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THE EVALUATION OF HIGH HYDROSTATIC PRESSURE EFFECTS ON BOVINE BLOOD CONSTITUENTS AND THE MICROBIAL SURVIVAL

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ABSTRACT

THE EVALUATION OF HIGH HYDROSTATIC PRESSURE EFFECTS ON BOVINE BLOOD CONSTITUENTS AND THE MICROBIAL SURVIVAL

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The main objective of this study was to investigate the effects of high hydrostatic pressure on the stability of blood constituents for the purpose of an effective reduction of viral and bacterial count. The effect of HHP treatment on the several blood constituents were analyzed at different HHP levels at 25 ^{0}C for 5 minutes. The bovine blood as the model material was separated into two major parts; namely, serum and blood cells by centrifugation. Erythrocytes were found to be mostly stable up to 220 MPa pressure treatment displaying only surface modifications, but the cells lose their morphology at 350 MPa. White Blood Cells and platelets were found to be more sensitive, being degraded at around 110 MPa pressures putting an upper limit for the HHP treatment for the whole blood. But serum components and parameters studied showed much higher stability up to 220 MPa pressure level.

The HHP treated blood cells were analyzed by FTIR spectroscopic technique and found to be stable in the macromolecular level. HHP treated proteins

display only minimal changes in their secondary structures shown by the artificial neural network and curve fitting studies. Changes in the lipid bands indicated the changes in the membranes of the blood cells.

In the microbiologic part of the study, *Listeria innocua* was found to be more stable than Bovine Herpes Virus type 1 as the model bacterium and virus respectively and their inactivation levels were compared with that of blood constituents.

Key Words: Pressure, Blood, FTIR, Virus, Bacteria.

ÖZ

YÜKSEK HİDROSTATİK BASINCIN SIĞIR KAN BİLEŞENLERİ VE MİKROBİYAL YAŞAM ÜZERİNE ETKİLERİNİN DEĞERLENDİRİLMESİ

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Bu çalışmanın esas amacı viral ve bakteriyel etkenlerin etkin olarak yok edilmesi amacıyla yüksek hidrostatik basıncın kan bileşenleri üzerine olan etkilerinin incelenmesiydi. 25 ^{0}C de beş dakikalık HHP uygulamasının çeşitli kan bileşenleri üzerine olan etkisi araştırıldı. Model materyal olarak alınan sığır kanı serum ve kan hücreleri olarak iki kısma ayrıldı. Eritrositler 220 MPa basınca kadar genel olarak dayanıklı bulundu fakat yüzeylerinde değişiklikler gözlendi fakat morfolojilerini 350 MPa tamamen kaybettiler. Plateletler ve beyaz kan hücreleri ise daha duyarlı bulundular ve 110 MPa civarında bozulmaya başladılar buda tam kanda yüksek basınç uygulamasının üst sınırını oluşturdu. Fakat çalışılan serum bileşenleri ve parametreleri 220 MPa 'a kadar çok daha fazla bir dayanıklılık gösterdiler.

Basınç uygulanmış kan hücreleri FTIR spektroskopisi metoduyla da incelendi ve makro moleküler düzeyde dayanıklı bulundu. Yapay sinir ağları ve

eğri uydurma metotlarıyla yapılan çalışmalarda basınç uygulanmış proteinlerin ikincil yapılarında sadece sınırlı değişiklikler gözlendi. Lipit bantlarında gözlemlenen değişiklikler kan hücrelerinin membranlarında gözlenen değişiklikleri gösterdi.

Çalışmanın mikrobiyolojik kısmında ise, model bakteri seçilen *Listeria innocua*'nın ve model virüs seçilen Sığır Herpes Virüsü tip 1'den daha dayanıklı olduğu görüldü ve inaktivasyon düzeyleri kan bileşenlerininkilerle karşılaştırıldı.

Anahtar Kelimeler: Basınç, Kan, FTIR, Virüs, Bakteri.

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CHAPTER 1

INTRODUCTION

1.1 Hydrostatic Pressure

1.1.1 What is Hydrostatic Pressure?

Pressure is simply defined as the force acting on a unit area. The standard unit for pressure is the Pascal, which is a Newton per square meter. If a motion of fluid is considered, the pressure caused by the kinetic motions of fluid particles is called hydrodynamic pressure. When a static fluid is considered, the pressure caused by the weight of fluid head is called hydrostatic pressure. Throughout this research investigation the pressure mentioned is hydrostatic pressure not produced by a fluid head on top, but a hydrostatic pressure produced by the compressibility properties of the liquid pressed in a closed system. In addition to above definitions, pressure is a state-variable defining the physical state of a system. When used in a chemical or biological manufacturing process pressure is often an important process variable to be controlled.

The main effect of pressure is to change the system volume. In compressible fluids such as gases the effect is large, for liquids and solids the effect is minimal. The reduction in the volume of a system causes a decrease in the intermolecular distances. When the volume is changed by the applied pressure, the work is done and the system energy increases.

There are two principles that express the effect of high pressure (Smelt, 1998). The first one is the le Chatelier principle which states that any phenomenon such as chemical reaction and phase change accompanied by a decrease in volume will be enhanced by increased pressure. The second one is isostatic pressure property stating that pressure is instantaneously and uniformly transmitted

independent of the size and the geometry of the material pressure is applied (Knorr, 1993).

1.1.2 The Use of High Hydrostatic Pressure in Biostructures

1.1.2.1 Food Science

High pressure may be used advantageously in many areas of biotechnology. One such area where industrial applications already exist is that of food processing (Tedford *et al*, 1999). For example, pressures up to 4 to 9 kbar (400-900 MPa) inactivate bacteria and irreversibly denaturate enzymes there by increasing shelf life while leaving vitamins and other nutrients unaffected, as opposed to thermal treatments. Pressures up to 4 to 5 kbar have been found to improve protein stability at high temperature and to affect both enzyme activity and stability (Hayakava *et al*, 1992).

1.1.2.2 Cancer Research

High hydrostatic pressure has also been used to develop cancer vaccines to combat this deadly disease of the century. Goldman *et al* (2000) developed a method in which tumor cells are exposed to high hydrostatic pressures in the presence of a biologically compatible crosslinker (CL - 2' – 3' – adenosine dialdehyde). The new topology acquired by the surface proteins of the modified cells found to stimulate the immune system after the high pressure processing. They also found that subsequent reduction of surface protein disulfides with N-acetyl–L–cysteine (NAC) could augment the immunogenic potential of PLC-modified tumor cells. The cells were exposed to pressures of up to 1,200 atmospheres for 15 minutes. The cells were washed and resuspended in cold sterile buffer and irradiated for further use. The mice were induced to develop lung metastases. After 7 days of incubation with the pressure treated cells the lung weights were measured and compared with the control ones. The weights of the mice treated with the cancerous cells treated with PCL-NAC and hydrostatic

pressure produced normal lung weights indicating immune response eliciting properties of the pressure applied tumor cells.

Ramakrishna *et al* (1993) used high pressure as a method of increasing the projection of MHC and tumor antigens in murine B16-BL6 melanoma cells evidenced by flow-cytometric analysis. The amount of pressure applied was the same as above.

In the following paper by the same research group, Eisenthal *et al* (1993) showed that in mice that were immunized with modified B16-BL6 cells; significant cell mediated immunity was observed whereas immunization with unmodified cells had only a marginal effect when compared to the results in controls with sham-immunized mice.

1.1.2.3 Membrane Science

The application of high pressure to membranes is an interesting research area (Kornblat *et al*, 2002). The application of high pressure to phosphotidyl choline multiplayer membranes causes (a) a linear increase in the melting temperature of bilayers, T_c, along with an interdigitation of acly chains from apposing leaflets (Braganza *et al*, 1986) (b) a tilting of the head groups (Peng *et al*, 1992), and (c) increases in the bilayer thickness and changes in the phase transition behaviors (Bonev *et al*, 1995 and 1996).

1.1.2.4 Protein Studies

The study of high pressure on proteins has received considerable attention in recent years. The perturbation of protein structure due to this parameter has been under investigation by various methods: most of the information relating to the thermodynamics and kinetics of protein stability has been revealed by the use of different spectroscopic methods (Cavaille *et al*, 1995). Nuclear magnetic resonance

spectroscopy has already been successfully applied to the study of some elementary movements on proteins (Samarasinghe *et al*, 1992).

The stability of an enzyme is related to both its conformational changes and its activity decrease after temperature or high pressure treatments (Mozhaev *et al*, 1996). With regard to pressure stability, the behavior of proteins is governed by Le Chatelier´s principle. Depending on the volume changes measured for the major interactions in proteins, electrostatic and hydrophobic interactions are the main targets of pressure, whereas hydrogen bonds are almost insensitive (Mozhaev *et al*, 1994). Covalent bond angles and distances are invariant in the biological pressure range (Weber *et al*, 1983). Hence, moderate pressures favor dissociation of oligomeric proteins, significant tertiary structure changes are observed beyond 200 MPa, whereas irreversible unfolding of small and monomeric proteins occurs at higher pressures (400-800 MPa). Generally speaking, low pressures induce reversible changes whilst pressures higher than about 500 MPa an induce denaturation which is in most cases irreversible, essentially due to chemical modifications or aggregation (Heremans *et al*, 1993 and 1998).

The occurrence of life on this planet seems to be restricted to narrow ranges of temperature and pressure. Laboratory experiments strongly suggest that the stability of proteins is dictated by these parameters (Smeller *et al*, 1999). In this respect it is interesting to note that the antagonistic effect of pressure on temperature induced phenomena that is characteristic for proteins, has also been observed in bacteria. Diagrams that show the effect of temperature and pressure on the viability of bacteria from the deep sea show a strong resemblance to those observed for the reversible and irreversible denaturation of proteins. It was found that the temperature of maximum denaturation for growth increases with increasing pressure up to about 100 MPa. This effect does not continue at higher pressures (Michels *et al*, 1997 and Hei *et al*, 1994). However, the phenomena of pressure stabilization of proteins can not be generalized (Konisky *et al*, 1995).

To review the effect of pressure on the protein structure and function it is instructive to organize the discussion considering four different levels of structural hierarchy: The primary structure describes the order of the covalently linked amino acids. This level is not affected by pressure (Heremans, 1993). The pressure-induced breaking of a covalent bond in a protein has never been observed in the range of a few GPa. The secondary structure describes the way in which the polypeptide chain forms either intra-molecular (to form a α- helix) or intermolecular (to form a β-sheet) hydrogen bonds. Pressure may have an effect on these structures and their transformations. This has important consequences for pressure induced conformational transitions and for pressure-induced denaturation of proteins in general. The tertiary structure describes how the secondary structure domains fold into a three-dimensional shape as a consequence of the non-covalent interactions of the amino acid side chains. Pressure does have profound effects on this structural level but with experimental methods they cannot easily be distinguished from the changes that place on the secondary structures (Heremans, 1993). The quaternary structure of proteins is formed by the interaction of non-covalent bonds between protein subunits under high pressure has received considerable attention recently (Weber, 1993).

As a general rule, pressure induces reversible effects on proteins below 100-200 MPa. These include protein compressibility, the binding of small molecules, changes in conformation and changes in the interaction of the subunits in multimeric proteins. Irreversible effects may include complete inactivation of enzymes and denaturation of proteins. The simplest possible scheme for such a reaction may be represented as follows (Heremans, 1993):

$$E + S \Leftrightarrow ES \Leftrightarrow ES^* \Rightarrow E + P$$

$$nE \Leftrightarrow E_n \quad \text{Subunit interactions}$$

$$E \Leftrightarrow E^* \quad \text{Conformational changes}$$

High pressure experiments on proteins provide opportunity to separate the effects of density and temperature (Heremens *et al*, 1998). High pressure is unique in that its perturbation of macromolecular structures in solution depends solely on the volume change of the process. However in the case of structural perturbations caused by high temperature and the presence of denaturing agents, these effects depend on many factors. An increase in temperature produces simultaneous changes in both the total energy, the volume, and temperature, whereas the effects of a denaturant depend on its binding properties (Silva *et al*, 1996).

1.1.2.5 Bacteriology

High hydrostatic pressure has been proposed as an alternative technique to thermal processing to destroy food-borne pathogens since it can inactivate microorganisms without altering the flavor and nutrient content of foods by Hoover *et al* (1989). Bacteria are observed to be injured or killed by hydrostatic pressure. These effects are dependent on the pressure level, temperature, application time and other environmental conditions such as composition of the medium.

Alpas and Bozoğlu (2003) studied the pressure resistance of several *Listeria monocytogenes* strains at various temperatures at 250 and 350 MPa in different fruit juices. Among the Listeria strains studied *L. monocytogenes* CA was found to be the most pressure resistant strain and increasing pressurization from 250 to 350 MPa at 30 ^{0}C had an additional three to four log cycle reduction in viability, still leaving viable cells after 5 minutes indicating the time dependent or kinetic nature of pressure destruction of microorganisms. It was also evident that high pressure processing sensitizes the bacteria to acid conditions found in fruit juices indicating the importance of acidity on the pressure application. For successful commercial batch applications the pressure level is chosen to be less than 350 MPa. To keep the capital equipment costs down pressures should be no more than this. To achieve realistic levels of throughput batch processing times should be 5 min or less (Jordan *et al*, 2001). In another study (Alpas *et al*, 1999), it was shown that some strains of food-borne pathogens could be more resistant to

hydrostatic pressure treatment than others. But this difference holds only for lower temperature values such as 25 °C, whilst at elevated temperature levels such as 50 ^{0}C, this difference disappears. For a biologic fluid such as blood, high temperature values can not be achieved due to its high sensitivity coming from its constituent cells and other dissolved components such as proteins, hormones etc.

Alpas *et al* (1998) studied the viability of *Listeria innucua* for different pressure-time-temperature applications in peptone solution. They reached a 7 log decrease in the microbial load at 344.7 MPa, 50 °C and 9.1 minute. They also suggested that the incorporation of an antibacterial agent such as a bacteriocin could decrease the viability of gram negative and positive bacteria in foods under high pressure.

Alpas and Bozoğlu (2000) studied the combined effect of high hydrostatic pressure and bacteriocins on the inactivation of food borne pathogens in milk and orange juice. Their results revealed that when pressure treatment was used in combination with a bacteriocin-based biopreservative, more than 8 log-cycle reductions in cell population was achieved for the resistant strains of *Staphylococcus aureus* and *Listeria monocytogenes.*

Spilimbergo *et al* (2002) addressed the issue of high pressure application to foodstuff for bacterial sterilization purposes with the additional application of CO_2 as the super critical fluid agent. The application of CO_2 reduced the applied pressure level significantly from the levels of several thousand bars to more moderate levels of a few hundred bars. Bacterial spores are very compact and therefore stabile structures against several environmental conditions such as temperature and pressure. They obtained complete spore inactivation with employing different combinations of fast cycle-type treatment (30 cycles per hour), temperature (35 °C), and treatment time (30 minutes) for the pressure change levels of 80 bars. They hypothesized that the rate of bacterial inactivation may be governed by CO_2 dissolution inside the cell, which causes a decrease in the intracellular pH decrease and a significant modification of the cell membrane

permeability. They also concluded that the treatment with supercritical CO_2 could become an alternative to high pressure application in the field of microbial inactivation of food.

In the study of the effects of high pressure on the resistances of bacterial strains from *S. aureus* and *E. coli*, Alpas *et al* (2003), found that ribosomal denaturation appeared to be a major factor in cell death by both thermal and high pressure treatments using Differential Scanning Calorimetry. The level of the pressure was held to be 345 MPa for 10 min at 35 °C. The analysis of the calorimetric data for control samples and for the pressure applied samples showed that the sensitivities of bacteria against different physical stresses can be different. They also found that the apparent enthalpy and viability values decrease as a function of pressure although the reduction depends on the strain.

Niven *et al* (1999) also demonstrated that cell death due to elevated pressures may also be related to irreversible ribosome damage. They observed a correlation between loss of cell viability and decrease in ribosome-associated enthalpy in *E. coli* cells subjected to pressures of 50-250 MPa for 20 minutes. In the pressure-treated cells, the thermogram peak temperatures decreased, suggesting that the remaining ribosomes had adopted a less stable conformation.

Kaletunç *et al* (2003) evaluated the structural changes induced by high hydrostatic pressure of 250-500 MPa in *Leuconostoc mesenteroides* cells using DSC, TEM, SEM and viability measurements. High pressure treatments caused significant changes on the external surfaces and internal structures of cells. Dechaining and blister formation on the surface of the cells increased with pressure as observed for SEM micrographs. TEM results showed that, cytoplasmic components of the cells were also affected from high pressure with dense-compacted regions in the cytoplasm. DSC studies showed increasing denaturation of ribosomes related to the number of viable cells.

1.1.2.6 Virology

The presence of infectious virus particles in the blood is called viremia. These virus particles may be free in the blood or contained within infected cells such as lymphocytes. There are two different types of viremia namely active viremia and passive viremia. Active viremia requires virus replication, while passive viremia results when virus particles are introduced into the blood without viral replication at the site of entry (Flint *et al*, 2000). Related examples are given in the Table 1.1. Viruses could also cause hematogenous spread by escaping from local defenses to produce a disseminated infection by entering the blood stream. They may enter the blood directly through capillaries, by replicating in endothelial cells, or through inoculation by a vector bite. A virus spends 1 to 60 minutes in the blood. When they enter the blood they have access to almost every tissue in the body.

Although many viremias are of short duration, some viral infections are present in the blood for long times. For example, hepatitis B and C viruses or lymphocytic choriomeningitis virus cause viremias that last for years (Flint *et al*, 2000).

Viremias have diagnostic values and can be used to monitor the diseases, but this causes certain problems. Some of the diseases such as hepatitis and AIDS can be spread by contaminated blood and blood products. For some viruses it is difficult to know the number of infectious viral particles in the blood but the presence of certain markers in the blood allows people to estimate the virus titer via certain quantitative tests such as ELISA and in vitro nucleic acid amplification tests (Flint *et al*, 2000).

For reliable quantification of viral titers sensitive tests are required. Most animal viruses can be titrated on tissue-culture plates containing a monolayer of susceptible cells. In these assays each infectious particle induces an area or focus of cell-killing or transformation. Direct proportionality between virus particle count

and number of foci of infection is generally accepted for the bacterial and animal viruses (Flint *et al*, 2000).

Table 1.1 Some of the human pathogenic viruses invading blood (Flint *et al*, 2000).

Cell Type	Virus	Duration of viremia
Monocyte/macrophage	Dengue virus	Acute
	Rubella virus	Persistent
	Measles virus	Acute
	Lymphocytic chroriomeningitis virus	Persistent
	Human immunodeficiency virus	Persistent
	Cytomegalovirus	Acute
	Mousepox virus	Acute
B lymphocytes	Murine leukemia virus	Persistent
	Epstein-Barr virus	Persistent
T lymphocytes	Human immunodeficiency virus	Persistent
	Human T-cell leukemia virus type 1	Persistent
	Human herpes viruses 6 and 7	Acute
Erythrocytes	Colorado tick fever virus	Acute
Neutrophils	Influenza virus	Acute
Free in plasma	Polio virus	Acute
	Yellow fever virus	Acute
	Hepatitis B virus	Persistent

One of the pioneering studies on the use of hydrostatic pressure in the field of virology is that of Silva (1993). In the referred report, the studies of pressure on two different viruses, namely vesicular stomatitis virus and rotavirus were detailed. It was reported that 2.6 kbar pressure for 12 hours was enough to decrease the infectivity of vesicular stomatitis virus by a factor of 10^4, and 2.5 kbar pressure for 30 minutes was enough to reduce the infectivity of rotavirus by a factor of 10^5. It was noted that the inactive virus particles were as immunogenic as native counter parts indicating the possible use of high pressure for the production of pascalized vaccines. From the studies with protein dimers and tetramers, he concluded that pressure decreased the ability of monomers to aggregate. Therefore a conformational drift might cause the irreversible loss of aggregate formation. This referred study also paved way to understand protein-protein and protein-nucleic acid interactions with the use of high pressure.

The use of hydrostatic pressure makes it possible to control the dissociation of viruses, oligomeric proteins, and other subcellular assemblies (Silva, 1993). For many oligomers, there is a substantial hysteresis between the compression and decompression dissociation curves, suggesting the formation of a defective reassociated form that relaxes slowly to the native conformation. For multisubunit protein systems such as viruses, reassembly becomes even more difficult and, in some cases, stable but defective assemblages are formed. All of these effects can be explained as the result of a conformational change that occurs following dissociation. After decompression, the dissociated species get trapped in metastable conformations that may never recover the original form. The degree of reversibility of pressure effects varies in different viruses. In several viruses, non-infective particles are formed after a cycle of compression and decompression. These results demonstrate the potential of utilization of hydrostatic pressure to prepare non-infectious whole virus particles that are highly immunogenic for production of new anti-virus vaccines and even against cancer. The effect of high hydrostatic pressure on the bacteria and viruses is covered in the referred study separately.

Da Poian *et al* (1994) studied the effect of pressure on a plant virus, Cowpea Mosaic Virus on virus assembly using a nondenaturing concentration of urea at 2.5 kbar taking the advantage of fluorescence spectra as a measure of protein denaturation. As a RNA virus, capsid dissociation was observed to be reversible in the presence of RNA but irreversible without RNA indicating the stabilizing effect of ribonucleic acid molecule on the conformation of the virus particle.

Silva *et al* (1992) studied the effects of high pressure on a membrane enveloped virus (vesicular stomatitis virus) and observed the reduction of infectivity by a factor of 10^4 at 260 MPa for 12 hours of application. In addition the antibodies produced against the pressure applied virus were as active as that against the wild virus according to their virus neutralization results. Under the electron microscope they observed a bulge on the capsid after the pressure application and the membrane was partially deformed.

The effects of high hydrostatic pressure on herpes simplex virus type-1 and human cytomegalovirus were also studied by Nakagami *et al* (1992). They found that the pressure more then 300 MPa for 10 minutes of application at 25 ^{0}C inactivated the viruses and at higher pressures the inactivation level was increased. Their electron micrographs showed that the envelope of the viruses were damaged preventing the entry of the viruses to the cells.

Similarly, Jurkiewics *et al* (1995) studied on a HIV model virus, Simian immunodeficiency virus with 150 and 250 MPa pressures and found that at 150 MPa pressure after 10 hours virus titer decreased by 5 logs. They also concluded that the method could be used as a method to prepare inactive virus vaccines and they also concluded that virus inactivation was due to membrane and capsid deformation under high pressure.

Inactivation of bacteriophages is also possible under high pressure as studied by Moroni *et al* (2002). They subjected the lactococcal phages to

dynamical high pressure with several cycles and found that inactivation was proportional to both pressure level and the number of cycles. In buffer, a maximum inactivation of 5 log cycles was obtained with 200 MPa after 5 passes. Therefore, dynamical high pressure application holds promise for significant inactivation of lactococcal bacteriophages.

Bradley *et al* (2000) studied the inactivation of lambda phage in human plasma using pressure cycling technology at near zero (^{0}C) temperatures. The virus titer was decreased by 4-7 log after 275 MPa pressure application for 7.5 minutes of application at near zero temperatures. Interestingly they observed only a slight virus inactivation after the pressure application below -40 ^{0}C and 28 ^{0}C. They concluded that pressure could be an effective means of virus inactivation in plasma when applied in cycles at near zero temperatures.

1.1.2.7 Blood Functionality

Although high hydrostatic pressure was used advantageously in various scientific and industrial fields, its use in the blood-related fields has been limited. Pares *et al* (2000) studied the application of high pressure on porcine blood plasma as an additive both in feed and food industries. They found slight changes in the protein structures as evidenced by solubility and calorimetric analyses. But the water-holding capacity and hardness of the gels from pressurized plasma were similar to those of the control ones. They did not observe any differences between the microstructure of the pressurized ones and the control ones. Anyhow the appearance of the pressurized plasma was always different from the untreated ones.

Bradley *et al* (2000) studied the effects of pressure cycling technology on some of the proteins in human plasma at near-zero (0 ^{0}C) temperatures. They found that the activities of plasma proteins alkaline phosphatase and total amylase did not change significantly and the activities of IgG, IgM and factor X remained within the range of 104, 89 and 80 percent of the control values respectively.

1.2 Blood and Its Constituents

Blood is a liquid tissue which also contains several types of cells and cell fragments. It also contains many non-cellular components (Madigan *et al*, 2000). Changes in the blood components and properties reflect changes in the body metabolism including disease states. Since blood can be easily obtained from patients safely and easily, it is a very valuable source of material for clinical procedures. Blood is pumped by the hearth through a network of arteries and capillaries to various parts of the body and is returned through the veins. Blood also provides a means of contact between different parts of the body in a short time span. It is the most important mediator of the metabolism of the whole body. For the body to function properly, each specialized tissue cells should work in a physically and chemically stable environment. The transportation of the foods taken from outside to the tissues is carried out by the blood. Similarly, oxygen from lungs is carried in the blood to the cells of the body. Likewise, carbon dioxide and other chemicals from metabolic activities of the body were discarded via blood. Hormones and other signal molecules produced by several tissues are exchanged through blood. The body temperature is maintained in an acceptable level with the functioning of the blood. Water level, pH and osmotic pressure are equilibrated with the proper functioning of the blood. Moreover, the defense of the body against invading microorganisms and other adverse conditions is realized through blood and its components. All the white blood cell types participate in the defense of the body (Madigan *et al*, 2000).

1.2.1 Cells of Blood

Blood is composed of plasma and the cells floating in it basically. The basic cell types that are found in the blood are red blood cells, white blood cells and platelets. Normally, these cells stand in the blood in the form of a suspension (Yılmaz, 1984). When the cells are removed from blood, the remaining fluid is called plasma (Madigan *et al*, 2000). Plasma is a straw-colored liquid in which the blood cells are suspended. Fibrinogen is an important protein which undergoes a

set of reactions during the formation of a fibrin clot. If any anticoagulant such as EDTA or heparin is not added into the blood it forms a clot rapidly. The remaining fluid is called serum. Serum contains all the non-cellular components of plasma except for fibrin and it is widely used in biochemical and immunological investigations.

There are three main types of cells in the blood namely erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets). They all are produced in the bone marrow and stem from multi potent stem cell. The production of the cells of the blood is under the tight control of certain hormones and/or cytokines. If a sample of is taken and treated with an agent to prevent clotting and centrifuged, the red cells settle to the bottom, the white cells settle on top of them along with platelets forming a white layer (Madigan *et al*, 2000).

1.2.1.1 Red Blood Cells (Erythrocytes)

The most numerous cells in human blood and that of other mammals are erythrocytes, outnumbering leukocytes by roughly a factor of 1000. They are nonnucleated cells that function to carry oxygen from the lungs to the tissues. In other vertebrates they have nucleus. They carry out the transport of oxygen and carbon dioxide between the lungs and tissues. The protein hemoglobin does the job of carrying oxygen and part of the carbondioxide produced as a product of active metabolism. The heme molecule in hemoglobin gives the red color of blood. Erythrocytes constantly develop from stem cells. They are flexible cells so that they fit through tiny capillaries. The blood types of humans are due to the glycoprotein antigens on the surface of their erythrocytes. In human and mammals they have the shape of biconcave lens which increases the surface to volume ratio. They have a mean life period of 120 days, after which they are retained by the spleen and phagocyted by macrophages (Madigan *et al*, 2000).

1.2.1.2 White Blood Cells (Leukocytes)

White blood cells, or, leukocytes, are involved in antibody production and cell-mediated immunity. They have a rather short life cycle, living from a few days to a few weeks. Their number is much less that that of erythrocytes. Their number increases in the case of infection drastically. They have nuclei. Each class of white blood cells has distinct roles in the defense of body against the invading pathogens. Like all blood cells they are produced in the bone marrow. Under the influence of certain cytokines stem cells differentiate into different types of white blood cells. There are basically three types of white blood cells: lymphocytes and monocytes with clear cytoplasm, and three types of granulocytes with granules in their cytoplasm (Madigan *et al*, 2000). Monocytes are found in the blood and lymph (Pinchuk, 2002). They transform into macrophages after they move from the blood into tissues. Macrophages are large, phagocytic cells that engulf foreign materials (antigens), pathogens and also dead cell of the body. Lymphocytes are one of the main components of adaptive immunity. Most of them are in a resting state in the blood. But get larger when stimulated. There are three types of lymphocytes: T and B cells and natural killer cells. When the B cells are activated they grow quickly through clonal selection and become plasma cells which produce antibodies (humoral immunity). Granulocytes are the white blood cells that participate in non-specific defense mechanisms but also in specific cell responses when stimulated by cytokines (Pinchuk, 2002). Their characteristic feature is the presence of large granules in their cytoplasm (Kılıçturgay, 2003).

1.2.1.3 Platelets (Thrombocytes)

Platelets are small cell-like constituents that lack a nucleus and play an important role in preventing leakage of blood from damaged blood vessels (Kılıçturgay, 2003). Platelets clump together to form a temporary plug in a damaged vessel until a permanent clot forms through the action of various clotting agents, some of which are released from the platelets themselves. They are tiny plate-shaped bags containing chemicals for proper blood clotting such as serotonin,

epinefrin etc. They are produced from megakaryocytes. Each megakaryocyte produces about 1000-7000 platelets. When stained with Giemsa, they appear as purple aggregates. They are not real cells. They do not have a nucleus.

1.2.2 Physicochemical Properties of Blood

In mammals the composition of the blood is similar essentially. Human blood contains a high amount of water (approximately 83 %). Salts take up about 1 percent of the plasma. Proteins make up 6-8% of the blood plasma. They are almost equally divided between serum albumin and a great variety of serum globulins. Plasma proteins can be separated through electrophoresis. Plasma proteins also provide the blood its viscosity which is necessary in terms of the fluid properties of the blood while flowing through the veins (Yılmaz, 1984).

In general, blood is a sticky, viscous and red colored fluid. Its smell depends upon the origin of the organism. It tastes a bit salty due to high concentration of NaCl in it. In the body it has temperature slightly higher than that of the body depending of the tissue position. It has a melting point of about – 0.50 ^{0}C again depending on the origin of the animal. It has a specific weight of around 1.050 g/ml, and a pH around 7.35- 7.50. The viscosity of the blood depends on the temperature and composition and it is between 3.5-5.5 centipoises (Yılmaz 1984).

Changes in blood properties and constituents are sensitive reflections of body changes, including disease states. Because blood can be easily and safely obtained from patients, it is a valuable source of material for clinical analytical procedures, including most immune response assays.

1.3 Use of Fourier Transform Infrared Spectroscopy in the Blood Analyses

Since Fourier Transform Infrared Spectroscopy was used for the biophysical analysis of the cells of blood it is appropriate to include the previous

examples of scientific reports here. Gotshal *et al* (1997) proposed FTIR as a new method for blood diagnostics. According to their results FTIR along with artificial neural network analysis can be used instead of classical blood chemical-enzymatic analysis. They predicted serum protein, cholesterol and uric acid concentrations successfully. Gattoni *et al* (2003) studied the effect of isoflurane on erythrocytes using ATR-FTIR. They found significant changes in the structural and dynamic properties of phospholipids. These studies suggests that FTIR as a sensitive method and it can be employed in the analysis of blood and blood components under a variety of conditions with success. Liu *et al* (2002) used the technique to analyze diabetic platelets from human blood and to compare them with the control ones. They observed differences in the protein and membrane lipid components and their properties. Shen *et al* (2003) used the FTIR technique to determine the glucose concentrations in the blood plasma.

1.4 Advantages of Fourier Transform Infrared Spectroscopy

▪ FTIR spectroscopy is a rapid, sensitive and an inexpensive technique, which is easy to perform (Manoharan *et al*, 1993, Ci *et al*, 1999).

▪ FTIR spectroscopy can be used to analyze any material and is not limited to the physical state of the sample. Samples may be solutions, viscous liquids, suspensions, inhomogeneous solids or powders (Colthup *et al*, 1975).

▪ Digital subtraction (that is, point-by-point subtraction of the separate spectra by a computer) can be used to produce good difference spectra. This method has great advantages in obtaining infrared spectra in aqueous solutions (Campbell, 1984).

▪ The FT-IR technique yields high quality infrared spectra from sample amounts as low as few micrograms (Dighton *et al,* 2001).

▪ It is a non-destructive technique (Melin *et al*, 2000, Severcan and Haris, 1999, Çakmak *et al.*, 2003).

▪ Since a computer is already used to obtain the Fourier transform, it is easy to perform many scans to improve the signal-to-noise ratio (noise adds up as the square root of the number of scans, whereas signal adds linearly). It has dramatically improved signal to noise ratio by the averaging of numbers of scans per sample (Beaten *et al*, 1998).

▪ FTIR system provides opportunities to the user many digital manipulations of data, permanent data storage and quantitative calculations (Ci *et al*, 1999; Yano *et al*, 1996).

1.5 Molecular Biology of Bovine Herpes Virus-1 as the Model Virus

The model virus that has been used throughout this research study is bovine herpes virus type 1 (BHV-1). BHV-1 is a member of the family *herpesviridae.* Herpesviruses have been found in various organisms such as insects, reptiles, amphibians, and mullusks as well as in virtually every species of bird and mammal that has been investigated (Murphy *et al*, 1999). Also in cell cultures such as on MDBK cell monolayers, the virus forms cytopathic effect which allows researchers to quantitate the infective virus particles. Actually, cytopathic effect is the general name for visual effects of viral replication and growth that can be observed under light microscopy (Mahy *et al*, 1996). Such effects include thick-enlarged cells, dark color formation in the cytoplasm, large vacuoles filled with virus particles in the case of BHV-1.

Herpesvirus particles are generally unstable outside of the body (Murphy *et al*, 1999). The transmissions of the diseases they cause occur through close contact in particular mucosal contact. In addition to animals they also infect humans such as well known Herpes simplex virus causing cold sore, Human herpes virus-2 causing genital herpes disease, Cytomegalovirus (Human herpes virus 5), Epstein-Barr virus (Human herpes virus 4) each causing an illness. All herpes viruses can survive from one generation to the next via persistent, often latent infections, from

which virus is periodically reactivated and shed. BHV-1 is the causative agent of the respiratory disease called Infectious Bovine Rhinotracheitis (IBR) in cattle.

There are four subfamilies of the family *Herpesviridae*: *Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae*, and an unnamed subfamily comprising the channel catfish herpesvirus-like viruses. In general alphaherpes viruses grow rapidly, lyse infected cells, and establish latent infections primarily in sensory ganglia and have a broad host range. Other subfamilies have relatively narrow host range and slow growth rate. BHV-1 falls into the class of *α-herpesvirinae* subfamily of the family *herpesviridae* (Murphy *et al*, 1999).

BHV-1 has typical herpes virus morphology (Studdert, 1999). The virions are enveloped and about 150 nm in diameter. The double-stranded DNA genome is wrapped around a spool-like core. The DNA has a size of 140 kb and 72 % G+C in terms of moles. There is an icosahedral nucleocapsid 100 nm in diameter composed of 162 hollow capsomers: 150 hexamers and 12 pentamers. The nucleocapsid is surrounded by a layer of globular material called the tegument that is enclosed by a typical bilayer lipoprotein envelope within which are embedded 12 glycoproteins some of which appear as projecting spikes in negatively stained electron micrographs. There are up to 76 open reading frames (genes) coding for a corresponding number of individual proteins (Studdert, 1999) and about 40 of these proteins are structural, i.e. associated with the virion, whereas the rest are nonstructural being only found in infected cells.

Virus replication takes place in the nucleus of cells and results in the production of large intra-nuclear, eosinophilic inclusion bodies (Studdert, 1999). As in all herpes viruses, the attachment of the virus occurs via the binding of virion glycoprotein peplomers to host cell receptors, one of which is heparin sulfate proteoglycan. Then nucleocapsid enters the cytoplasm either by fusion of the virion envelope to the cell membrane or by endophagocytosis. Finally, the DNA-protein complex becomes naked by avoiding the nucleocapsid and enters the nucleus and ends the host cell macromolecular synthesis (Murphy *et al*, 1999).

The replication cycle of BHV-1 like all herpes viruses goes through cascades. First, tegument proteins along with cellular regulatory proteins initiate transcription and translation of some of the viral proteins belonging to the immediate early proteins (Jones, 2003). IE proteins activate E gene expression, and viral DNA replication ensues. L gene expression is also activated by an immediate early gene product, culminating in virion assembly and release. IE genes are essential for virus growth because they regulate viral gene expression. Although there are some differences in the IE gene expression in HSV-1 and BHV-1 productive infection, the same general cascade of viral gene expression occurs. After the viral DNA is replicated in the host cell nucleus it is placed into the newly formed capsid. Following this step, the nucleocapsid associates with the inner layer of the nuclear membrane and leaves it by budding. Matured virus particles gather in the vacuoles and are released by exocytosis or cytolysis (Murphy *et al*, 1999).

Another interesting feature of all herpes viruses is their ability to induce latency in their respective host cells. Latent infection is a state of persistent infection in which no symptoms are observed and no infectious virus is produced (Levy *et al*, 1994). In general, most transcriptional and translational processes are blocked and a limited portion of viral genome is allowed to be expressed. This form of viral growth can be seen in some viral families such as herpesviruses, retroviruses, and papovaviruses etc. The LR gene products are the only abundantly detected transcripts in latently infected neurons (Jones, 2003). It also inhibits apoptosis in the infected cells. Latency is the principal strategy used by the virus to evade immune defenses and for persisting a life-long time period in the host. HSV infects epithelial cells in the mucosa or skin, then enters peripheral nerve ends and travels intraaxonally to the sensory ganglia. In neurons it can establish latency and reactivate as a result of stress or immunosuppression from time to time. Throughout the primary infection, a strong immune response is elicited by the host including both neutralizing antibodies and an antiviral CD4 and CD8 T cell response. In contrast, during the latent period no immune response detects the HSV existence in neuronal cells (Nash, 2000).

1.6 The Aim of the Study

The main aim of this research investigation is that whether blood and blood products could be sterilized using high hydrostatic pressure without giving damage to the components of blood. The variables in such a process may be pressure and its application time, temperature, pressure increase and release speeds. As being a merely physical effect, pressure holds promise to be used in this important scientific and economic field, since high hydrostatic pressure has been successfully used in the field of food manufacturing commercially. Sterilization using high hydrostatic pressure as being a non-thermal process could be used on blood as a heat sensitive material. This is an important study, because if a considerably high reduction in the number of pathogens could be achieved using high hydrostatic pressure without giving too much harm to the blood or blood products it should be considered a success especially today in the age of HIV or hepatitis or other bacteriemic/viremic diseases severely injuring people's health and many country's economic status.

Firstly, in this thesis study the effect of high hydrostatic pressure on various blood components will be determined employing several biochemical and biophysical methods such as flow cytometry, enzyme and chemical assays, electrophoresis, light microscopy, atomic force microscopy and Fourier Transform Infrared Spectroscopy. Later the potential of sterilization of blood with high pressure will be considered for the two model microorganisms namely *Listeria innocua* and Bovine Herpes Virus-1. Finally, a conclusion will be reached as to whether high hydrostatic pressure can be used in the sterilization of the blood without giving too much destruction to the material according to the results obtained in the first two sections.

CHAPTER 2

MATERIALS AND METHODS

2.1 High Hydrostatic Pressurization Unit

The high pressure experiments were carried out in a high pressure cell shown in the Figure A.1 in the Appendix section which is situated in the Ultra High Pressure Laboratory in the Food Engineering Department. The system was designed to have a pressure chamber, a pressure generating unit and a control unit basically. The pressure generated by an electric motor system is transduced to the pressure chamber via hydrolic connectors containing grease and finally to a cylidric unit. Following the movement of the cylinder the pressure is generated in the pressure cell. The fluid used as the isostatic pressure transducing agent is water. The system has also a control unit to monitor and control the pressure, cells temperature and pressure level.

Pressurization times reported in this study did not include the pressure increase and release times. The unit is capable of operating up to 350 MPa pressure between 25 to 95 ^{0}C.

2.2 Biochemical and Biophysical Techniques Used in the Blood Analyses throughout This Study

The blood used in this research is post mortem bovine blood. The blood sample was taken into 100 ml glass bottle containing 1.2 mg/ml of anhydrous salt of Ethylene Diamine Tetra Acetic Acid (EDTA). The whole blood analyses and the analyses for the cells of blood were carried out with the blood samples containing EDTA but when the serum was of interest the whole blood taken was left to stand in the bottle without EDTA. When necessary the whole blood was spun at a maximum of 2000 rpm to prevent cell disintegration.

2.2.1 Flow Cytometry

The whole blood analysis was carried out with hematology flow cytometry system the MAXM (Beckman Coulter, USA) with an autoloader device. The device provides sensitive, fast and walk-away operations and with the computer connection the system allows rapid diagnostic detection. The system is calibrated with the reference blood on a daily basis. The device can analyze 24 different parameters simultaneously. The device carries out the cell number and cell volume analyses according to the Coulter principle (Coulter MAXM Analyzer Reference, 1995).

2.2.2 Electrophoresis of Serum Proteins

The HYDRAGEL 7 PROTEIN(E) (Sebia) gel system was used for the purpose of elucidating effects of high pressure on the serum proteins. The system is used for separation of human serum proteins in human serum by electrophoresis on alkaline buffered (pH 9.2) agarose gels (sebia user manual). The protein kits are used in conjunction with the semi-automated HYDRASYS instrument. The separated proteins are stained with amidoblack. The electrophoregrams provide a visual opportunity to evaluate protein pattern abnormalities. Densitometry provides accurate relative quantification of individual protein zones.

The samples were applied to the system after the gel was situated into the HYDRASIS instrument. The sample volume applied was 10 μl for each well. Sample application, electrophoretic migration, drying, staining, destaining and final drying were applied as instructed in the manual.

2.2.3 Light Microscopy

Light microscopy experiments were designed to investigate the morphological changes of the cells of blood under pressure. The whole blood

samples were subjected to 55, 154, 220 and 350 MPa pressures for 5 minutes at 25 ^{0}C unless otherwise stated. After the pressure application the samples were stained with the Wright Solution. 10 micro liters of the control and HHP treated blood samples were dropped onto the glass slides and the blood was spread on the glass with another glass slide gently. After the samples were dried thoroughly, the Wright Solution was applied on top for five minutes. The dye was washed off with distilled water followed by another five-minute waiting step. Then the slides were washed thoroughly with distilled water. The slides were dried completely. The images were viewed under the inverted light microscope and the digital pictures were taken and copied to the computer memory and evaluated.

2.2.4 Atomic Force Microscopy

Atomic Force Microscopy was used to see the effect of HHP on the outher structure of erythrocytes. For this purpose MMAFM-2/1700EXL model instrument within the contact mode was used.

The whole blood samples were fixed on the glass slide surfaces and allowed to dry. Then, the glass slides were cut into appropriate sizes to fit into the device holder and the samples were analyzed.

2.2.5 Biochemical Analysis of Serum Components

The components of the serum selected are: glucose, albumin, iron binding capacity, cholesterol, alanine aminotransferase activity (ALT), aspartate aminotransferase activity (AST), total immunoglobulin E (IgE) concentration and iron ion concentration.

All of the biochemical measurements mentioned below were carried out using Synchron Clinical System CX9 PRO (Beckman Coulter). The system allows automatic and fast measurement of a wide range of chemicals. A minute amount of serum material is enough to carry out most of the tests in the menu.

2.2.5.1 Glucose

GLU reagent is used to measure the glucose concentration by a timed endpoint method. In the reaction, hexokinase (HK) catalyses the transfer of a phosphate group from adenosine triphosphate (ATP) to glucose to form adenosine diphosphate (ADP) and glucose-6-phosphate. The glucose-6-phosphate is then oxidized to 6-phosphogluconate with the concomitant reduction of β-nicotinamide adenine dinucleotide (NAD) to reduced β-nicotinamide adenine dinucleotide (NADH) by the catalytic action of glucose-6-phosphate dehydrogenase (G6PDPH).

The system automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 100 parts reagent. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is directly proportional to the concentration of glucose in the sample and is used by the system to calculate and express glucose concentration (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389746 AA, November 2003).

2.2.5.2 Cholesterol

CHOL reagent is used to measure cholesterol concentration by a timed-endpoint method. In the reaction, cholesterol esterase hydrolyzes cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a colored quinoneimine product (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389726 AA, November 2003).

The system automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 100 parts reagent. The system monitors the change in absorbance at 520 nanometers. This change in

absorbance is directly proportional to the concentration of cholesterol in the sample and is used by the system to calculate and express cholesterol concentration.

2.2.5.3 Albumin

ALB reagent is used to measure albumin concentration by a timed endpoint method. In the reaction, albumin combines with bromocresol purple (BCP) to form a colored product. The system automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 100 parts reagent. The System monitors the change in absorbance at 600 nanometers. This change in absorbance is directly proportional to the concentration of albumin in the sample and is used by the system to calculate and express albumin concentration (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389705 AA, November 2003).

2.2.5.4 Iron

FE reagent is used to measure the iron concentration by a timed-endpoint method. In the reaction, iron is released from transferrin by acetic acid and is reduced to the ferrous state by hydroxylamine and thioglycolate. The ferrous ion is immediately complexed with the FerroZine Iron Reagent.

The system automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio used is one part sample to 8 parts reagent. The system monitors the change in absorbance at 560 nanometers. This change in absorbance is directly proportional to the concentration of iron in the sample and is used by the system to calculate and express the iron concentration. (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389743 AA, November 2003).

2.2.5.6 Total Iron-binding Capacity

Transferrin in serum or heparinized plasma is completely saturated by adding excess ferric ion in the form of ferric chloride. Any iron not bound to transferrin is absorbed by aluminum oxide in the column. Iron-bound transferrin in the supernatant is measured by the IBCT reagent.

IBCT reagent is used to measure the iron concentration by a timed-endpoint method. In the reaction, iron is released from transferrin by acetic acid and is reduced to the ferrous state by hydroxylamine and thioglycolate. The ferrous ion is immediately complexed with the FerroZine Iron Reagent. The system automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio used is one part sample to 8 parts reagent. The system monitors the change in absorbance at 560 nanometers. This change in absorbance is directly proportional to the concentration of iron bound to transferrin in the sample and is used by the system to calculate and express the total iron-binding capacity (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389754 AA, November 2003).

2.2.5.7 Alanine Aminotransferase Activity (ALT)

ALT reagent is used to measure analyte activity by a kinetic rate method. In the reaction, alanine aminotransferase catalyzes the reversible transamination of L-alanine and alpha-ketoglutarate to pyruvate and L-glutamate. The pyruvate is then reduced to lactate in the presence of lactate dehydrogenase (LDH) with the concurrent oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) to β-nicotinamide adenine dinucleotide (NAD).

The system automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 11 parts reagent. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is directly proportional to the activity of ALT in the sample and is used

by the system to calculate and express the ALT activity (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389708 AA, November 2003).

2.2.5.8 Aspartate Aminotransferase Activity (AST)

AST reagent is used to measure aspartate aminotransferase activity by an enzymatic rate method. In the reaction aspartate aminotransferase catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase (MDH) with the concurrent oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) to β-nicotinamide adenine dinucleotide (NAD).

The system automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 11 parts reagent. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is directly proportional to the activity of AST in the sample and is used by the system to calculate and express the AST activity (Beckman Coulter Inc, Synchron CX Systems Chemistry Information Sheet 389714 AA, November 2003).

2.2.5.9 Immunoglobulin E (IgE) Concentration

The device used in the detection of IgE concentration is BN100 system (Dade Behring, USA). After the deployment of about 200 micro liter plasma sample the system carries out the operations automatically and the results are displayed on a computer screen with a printer output facility attached.

The principle of the method is based on the technique immunonephelometry. Within the system polystyrene particles coated with antibodies specific to human IgE are aggregated when mixed with samples containing human IgE. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of IgE in the sample. Then the

result is computed after comparing the intensity with a known reference one (Dade Behring prescript for N Latex IgE mono, January 2002).

2.2.5.10 Immunoglobulin Titer (IgG)

In the micro neutralization experiments Madin-Darby Bovine Kidney (MDBK) cells sensitive to BHV-1 were used. In a 96-well plate each well was filled with 50 μl serum diluted 1/2 with Dulbecco's Minimum Essential Medium (DMEM). The virus at a certain titer (100 $TCID_{50}$)
was added on top of the serum samples and mixture was allowed to sit in the CO_2 incubator for 60 minutes. Following this 50 μl MDBK cells were applied on top of the mixture and put back into the CO_2 incubator for two consecutive days. Any possible cytopathic effect formation was observed under the light microscope during this time. For virus control samples, four wells were filled with 100 μl virus and 50 μl MDBK cells and for the cell control four wells were filled with 150 μl MDBK cells. Every sample was tested in parallel.

2.2.6 Fourier Transform Infrared Spectroscopy (FTIR) to Monitor Molecular Changes

2.2.6.1 Instrumentation

General structure of an FTIR device can be seen in the Figure 2.1.

Figure 2.1 General Structure of an FTIR device.

2.2.6.2.1 The Source

The light source for an FTIR spectrometer is a heated filament generally. The heated rod emits a continuous infrared spectrum. Infrared emission sources are of two kinds: Globar which is made up of silicon carbide, and Nernst Filament which is a mixture of the oxides of zirconium, yttrium and erbium for the mid-infrared region. The source should produce intense light in the targeted range (Stuart, 1997).

2.2.6.2.2 The Interferometer

The central apparatus of FTIR instruments is the Michelson interferometer, which causes the incident beam from the source to split into two equal beams at a beam splitter. This is followed by the recombination of these two beams at the beam splitter, where they undergo constructive and destructive interference with each other and their transmittance to the sample. This yields an interferogram (Stuart, 1997). The beam from the interferometer with a 90^0 angle is called the transmitted beam and is detected by the detector.

2.2.6.2.3 The Detector

A detector consists of a thermocouple basically. It should be sensitive enough to detect the radiation arriving from the sample (Stuart, 1997). Pyroelectric devices containing deuterium tryglycine sulphate are used generally.

The signal coming from the detector is amplified in an amplifier which contains a filter to eliminate high frequency contributions. The data are converted to a digital form by an Analog/Digital converter and sent to the computer for Fourier Transform of the spectra is taken.

2.2.6.2 Pretreatment of Blood Cells Prior to the FTIR Study

The whole blood was centrifuged to separate the two main parts of the blood, namely the cells of the blood and the plasma part. For this purpose, the blood was spun at 3000 rpm for 10 minutes at the room temperature. The blood fractionated into three main components: the erythrocytes, the white layer comprising the white blood cells and platelets and the plasma. The plasma part was gently taken into a different tube for further analysis without disturbing the white layer and then vortexed briefly to mix the cells. The cells of the blood part separated and analyzed.

2.2.6.3 Spectrum Accumulation and Data Processing

The spectral analysis was carried out by using a Perkin-Elmer spectrometer equipped with MIR TGS detector (Spectrum One Instrument, Perkin Elmer). 4 microliter of blood cell samples were placed onto clean ZnSc window without spacers. This amount of blood samples were optimized to obtain nice peaks with minimum scattering coming from water peaks. Each recording was carried out at 4 cm^{-1} resolution with 50 scan number. Before each set of experiments the background of the ZnSc window was taken along with the background absorption coming from water and carbon dioxide and the background spectrum was subtracted from the spectra of the samples automatically. Spectrum One (Perkin-Elmer) software was used for all of the data manipulations except for curve-fitting. ZnSc was obtained from Merck (Merck, Darmstadt, Germany).

After smoothing with a width of 13 in the Perkin-Elmer Spectrum software the smoothed spectra were taken to Win Bomem Easy software (Galactic Industries Corporation). The smoothed spectra were baselined first and then the curve-fitting was carried out in the amide I region of the spectrum with Gaussian-Lorentzian components using Grams/32. The baseline was always linear.

The spectrum of each pressure experiment was subtracted from the spectrum of water and the wavenumber values of all functional groups were recorded. The band positions were measured according to the center of weight.

Fourier Transformation is a mathematical tool which is used to quantitate the analysis of many physical processes. A spectrum in the time domain can be converted to a spectrum in the frequency domain. The presentation of the data is very simplified as a result.

2.2.6.4 Artificial Neural Network Analysis of Amide I Band

The amide-I band of the control and pressure treated samples were analyzed by Mete Severcan (Middle East Technical University, Electrical and Electronic Engineering Department) through the software developed by Severcan *et al* (2004). The software provides the predictions of the secondary structure content of the component proteins in the sample analyzed.

2.2.6.5 Statistical Analysis

The differences between the control and pressure treated groups were calculated by means Mann-Whitney Test with the Minitab Statistical Software Release 13,0 program. The statistical results are expressed as means ± standard deviation (SD). Significance was accepted at $p < 0.05$.

2.3 Inactivation of Bacteria in Blood Using High Pressure

2.3.1 Bacterial Strain

For the high pressure bacterial inactivation study, a *Listeria innocua* strain was chosen as the model bacterium. The bacterium was obtained from Middle East Technical University, Food Engineering Department. It was grown in Nutrient Broth at 37 ^{0}C. Nutrient Broth inoculated with *Listeria innocua* was transferred to a fresh broth 4-5 times every week.

2.3.2 Growth Curve of *Listeria innocua*

Nutrient broth media was inoculated with 2% (v/v) inoculum in 50 ml Erlenmeyer flasks studied in parallel. Samples were taken at the designated time intervals to monitor the absorbance changes. The blank for absorbance measurements was non-inoculated Nutrient Broth medium kept at -4 ^{0}C except for

the measurement times. A growth curve was obtained from the data gathered for 35 hours.

2.3.3 Preparation of Cell (*Listeria innocua*) Serum Suspensions before High Pressure Treatment of the Cells and Enumeration of the Cells

Listeria innocua cells that were taken after 24 hours were diluted 1:7 in the sterile fetal bovine serum to simulate real blood environment. To ensure the sterility the serum was also filter sterilized using 0.2 μm filters prior to the inoculation with *Listeria innocua*. The samples were sealed in sterile cryovials. The bacterial suspensions then were subjected to the designated pressure levels. Following the pressure application, dilutions of the each suspension was prepared in sterile 0.7 % peptone solution for both the pressure applied cells suspensions and control cell suspensions. For the total bacterial count, 0.5 ml pressure applied suspensions were inoculated into Tryptic Soy Agar media from serially diluted samples with pour plate method. Each dilution was studied in parallel. The plates were incubated at 37 ^{0}C for two days and the numbers of the colonies were counted.

2.4 Inactivation of Viruses in Blood Using High Pressure

2.4.1 Virus Strain

For the high pressure virus inactivation study, Cooper strain of Bovine Herpesvirus type 1 (BHV-1) was chosen as the model virus. The virus was obtained from culture/virus collection of Ankara University, Faculty of Veterinary Medicine, Virology Department.

2.4.2 The Growth and Viral Infectivity Assay of BHV-1

The virus was grown on Madin-Darby Bovine Kidney cell line (MDBK) with routine procedure. MDBK cells maintained with Dulbecco's Minimum Essential Medium (DMEM) and fatal bovine serum were incubated in 24-well plate

in CO_2 incubator overnight. The following day, after the cell monolayer formed on the surface of the plate the liquid part was discarded and virus suspensions were dispensed in 10-fold dilutions in 0.2 ml volumes on the confluent cells in duplicates and left for the attachment of the virus particles to the MDBK cells for one hour at 37 ^{0}C in CO_2 incubator. Following the attachment of the virus to the MDBK cell membrane, the wells were overlaid with 1 milliliter of 1.6 % carboxymethylcellulose to provide a semi-solid environment. The wells were observed under the light microscope and the plaque formation was checked by daily microscopic examinations. The virus plaques were counted generally after two days following the inoculation. Cells were fixed with 10 % formaldehyde and stained with crystal violet (0.35 %) in ethanol. The plaque numbers were counted at the end of the experiment.

2.4.3 Preparation of the BHV-1 Suspensions in the Fetal Bovine Serum for HHP Studies

The effect of high pressure on the viral infectivity was investigated by the following experiments. An original virus suspension with a certain viral titer (3,525,000 per milliliter) in the fetal bovine serum was prepared to simulate real blood environment since fetal bovine serum is devoid of any maternal antibodies which can neutralize the viral particles. The virus suspensions were kept in ice bath to prevent the further viral loss. Several levels of pressure were applied on the virus suspensions which were assumed to have the same virus titer since they all were prepared from the original stock suspension. The pressure levels applied were as follows: control, 55, 110, 154, and 220 MPa. The duration of the pressure application was 5 minutes for all the pressure levels and the temperature of the samples were equilibrated to 25 ^{0}C just before the pressure application. For each pressure level duplicate experiments were carried out. The dilutions of the samples were prepared in DMEM.

CHAPTER 3

RESULTS

3.1 Pressure Stabilities of the Cells of Blood

Since the stability of blood cells is the most critical point in the HHP treatment, flow cytometry, light microscopy and atomic force microscopy experiments were carried out to enlighten it.

3.1.1. Flow Cytometry of HHP Treated Blood Cells

The stabilities of the blood cells were detected using flow cytometry which is the method of choice when counting and sorting the cells of blood. The stability of the red blood cells under high pressure application was studied for 5 minutes of pressure application at 25 ^{0}C. The values were drawn as follows: percent original cell numbers were included in one of the axis and the pressure level was included in the other for the comparison reasons. The pressure levels tried were 55, 110,154, and 220 MPa. The amount of error was given as the mean +/- standard deviation in the plots below. The results show the mean values of at least four different experiments.

The results of the flow cytometry experiments are given in the Figure 3.1. The results showed that erythrocytes are very stable blood cells for the pressure range studied. The increase in their number at 220 MPa samples does not mean their number was increased after the 220 MPa HHP treatment since their number increase is physically impossible. At 220 MPa due to the shape differences the flow cytometer can not count the erythrocyte number exactly possibly. Since the number of peaks in the flow cytometer gives the number of erythrocytes their number does not change with the applied pressure meaning they retain their integrity.

Figure 3.1 The effect of pressure on percent original Red Blood Cell number at 25 ^{0}C for 5 minutes of HHP treatment, RBC: Red Blood Cell Number.

The effect of high pressure on the mean red blood cell volume was also tested for the aforementioned pressure levels. The results of the experiments regarding mean erythrocyte volume versus pressure level is given in the Figure 3.2. The results clearly show that there is no effect of high hydrostatic pressure on the erythrocyte volume over the pressure range studied. In the working principle of the flow cytometer the impedance change gives the cell volume. In other words the bigger the cell, the more resistance it creates to electric current. The results suggest that the erythrocets retain their structure and shapes under the pressure range studied.

Figure 3.2 The effect of pressure on percent original Mean Red Blood Cell volume at 25 ^{0}C for 5 minutes of HHP treatment, MCV: Mean Cell Volume.

Possible pressure-dependent hemoglobin content changes were also monitored using the flow cytometry method. The results of the experiment are presented in the Figure 3.3. The results clearly show that the hemoglobin content of the whole blood does not change with the HHP treatment up to 220 MPa for 5 minutes. The hemoglobin content of erythrocytes still retains their structural integrity and functional potential as is shown by the no change in their dye binding capacities. In other words not only erythrocytes keep their structure, they also keep their functions as well.

Figure 3.3 The effect of pressure on percent original Hemoglobin Concentration at 25 ^{0}C for 5 minutes of HHP treatment, HGB: Hemoglobin Concentration.

The effect of high pressure on the platelet number was also measured in the experiments with the whole blood samples. The results of the experiments are shown in the Figure 3.4. The results clearly show that platelets are stable up until 110 MPa pressure, but they loose their structure after that point. Their number goes down as much as around 50 % of their original value and the destruction is certain at 220 MPa. When compared with the erythrocyte structure they turned out to be quite unstable pressure up to 154 MPa. Although both erythrocytes and platelets are membranous sacks why one of them remains stable and the other is not turns out to be an important question to be answered.

Figure 3.4 The effect of pressure on percent original Platelet number at 25 ^{0}C for 5 minutes of HHP treatment, PLT: Platelet Number.

The effect of high pressure on the platelet volume was also studied using the same technique. The results of the experiments are given in the Figure 3.5. The results are in accordance with the previous one. In the both cases both the platelet number and the mean platelet volume goes awry after 110 MPa HHP treatment. The mean platelet volume goes down at 154 MPa. This experiment shows the fragility of the platelet structure.

The differences between the erythrocyte and platelet membranes should be responsible from their stability behaviors. In addition, the instability of platelets puts an upper level for the HHP treatment for the whole blood which is around 110 MPa.

Figure 3.5. The effect of pressure on percent original Mean Platelet Volume at 25 ^{0}C for 5 minutes of HHP treatment, MPV: Mean Platelet Volume.

3.1.2. Light Microscopy Studies

Light microscopy experiments were carried out to see the effect of high pressure on the cells of blood explicitly. The blood smears were prepared and stained as described in the previous section. Following the staining procedure the digital pictures were taken and stored on the computer memory. The results are presented separately for each type of blood cells.

3.1.2.1 Erythrocytes

In order to see the morphologic changes after the HHP treatment the whole blood samples were visualized after the treatments. The results can be seen in the Figure 3.6.

Figure 3.6 The effect of pressure on Red Blood Cell morphology at 25 ^{0}C for 5 minutes of HHP treatment (a) Control, (b) 154 MPa, (c) 350 MPa, X 400, (d) 350 MPa, X 1000

As seen under the light microscope the overall shapes of erythrocytes remain stable for 154 MPa pressure for the designated time intervals. Only some

degree of small protrusions was observed. The same behavior was observed for 220 MPa samples also. But when the pressure was increased to 350 MPa the whole picture changes drastically. Erythrocytes were seemed to be fused into each other losing cell membranes and losing their spherical shapes completely.

3.1.2.2 Platelets

Although platelets are also important components of blood they are difficult to observe under the light microscope due to their small sizes. They should be observed using other more powerful microscopic facilities such as electron microscopy or atomic force microscopy.

3.1.2.3 White Blood Cells

The pressure applied white blood cells were images using invert light microscope to increase the contrast to see the cell surface properties well. Similar changes in the morphologies of white blood cells were observed depending on the level of the pressure applied. In the Figure 3.7 white blood cells are seen purple ones while the red blood cells stain red after the staining with the Wright Dye.

The results suggest that white blood cells are stable against 110 MPa HHP treatment for 5 minutes, but the cell morphology starts to show shape disorders at 132 MPa. Some white blood cells start to disintegrate at this point also. However, at 220 MPa pressure complete disintegration was observed.

Figure 3.7 The effect of pressure on percent original White Blood Cell morphology, at 25 ^{0}C for 5 minutes of HHP treatment, (a) Control, (b) 110 MPa, (c) 132 MPa, (d) 220 MPa, X400.

3.1.3. Atomic Force Microscopy Results

Atomic Force Microscopy experiments were carried out to see any morphological changes on the red blood cells in more detail. The results of the experiments can be seen in the Figure 3.8.

a 50X50 μm

b 12X12 μm

c 50X50 μm

d 50X50 μm

e 12X12 μm

f 50X50 μm

g 50X50 μm

h 12X12 μm

i 50X50 μm

Figure 3.8 The effect of pressure on percent original Red Blood Cell morphology studied with Atomic Force Microscopy at 25 ^{0}C for 5 minutes of HHP treatment; (a), (b) and (c) Control; (d), (e) and (f) 132 MPa; (g), (h) and (i) 220 MPa.

After the first visual inspection, there seems to be no changes in the appearances of the erythrocytes after the 5 minute pressure application of 132 MPa pressure. But when the pressure level was raised to 220 MPa for the same duration the effect was more pronounced. The wrinkles and at the highest panel indicate the effect of pressure in more detail. At the second and third panel above indicates that cells do not remain as separate objects rather like being fused into each other after the pressure application which was not so clearly detectable in the light microscopy results.

3.2 Stability Analysis of Serum Components

Experiments were designed to enlighten the effect of pressure on the components of bovine plasma to compound the previous study on the cells of

blood. An analysis of pressure effects on some of the plasma components of blood was carried out. The components of plasma that were analyzed were selected largely arbitrarily. The analyzed components were glucose concentration, albumin concentration, iron binding capacity, iron concentration, triglyceride concentration, cholesterol concentration, alanine aminotransferase activity (ALT), aspartate aminotransferase activity (AST) and total immunoglobulin E (IgE) concentration and iron ion concentration. All the parameters were studied in parallel. Following these analyses, an electrophoresis study was carried out to investigate the effects of pressure on the serum proteins. In the Table A.1 in Appendices section, the results of the biochemical analyses can be seen for 132, 220 and 242 MPa pressure for 5 minutes at 25 ^{0}C.

The results indicate that there is no adverse effect of high pressure application on the glucose and cholesterol concentrations under the prescribed conditions. Since these molecules are small molecules, the pressure were not expected to be effective in the range studied. Iron is also en element which is not expected to be affected by pressure.

In the case of proteins, albumin seemed not be affected by high pressure application. ALT specific activity seemed to be affected only for 242 MPa pressure for only a limited amount which might be indicative of denaturation. AST specific activity looks increased slightly which can be seen in some enzymes for relatively low pressure levels.

In addition an important characteristic of blood, iron binding capacity essentially did not change for the pressure levels studied.

In addition to all these parameters total triglyceride concentrations were also analyzed, but unfortunately the concentrations could not be obtained due to the very high concentrations of the compound in the bovine plasma. The device working parameter ranges for triglyceride were adjusted according to human

plasma. It could have been studied by diluting the bovine plasma but the dilutions would not have simulated real bovine plasma environment under high pressure.

Another interesting point to recapitulate is the results obtained with Total IgE concentrations. The kit used to detect IgE concentrations were produced to detect human IgE concentrations employing monoclonal antibodies raised in mice against human IgE molecules. Since the kits contain antibodies specific to human IgE molecules, they were not supposed to react against bovine IgE molecules, but they did. This could be a consequence of a possible cross-reaction between bovine IgE molecules with antibodies raised against human IgE molecules. All the results are depicted in the Figure 3.9 for comparison reasons.

Figure 3.9 The effect of pressure on several blood parameters at 25 ^{0}C for 5 minutes of HHP treatment for 132, 220 and 242 MPa.

3.2.1 Virus Micro Neutralization Experiments

The effect of high pressure on the structure and function of immunoglobulin molecules, virus micro neutralization experiments were carried out. *In vivo* or *in vitro* blocking of infectious virus particles by its homologous antibodies is called virus neutralization. In other words in the process of neutralization virus losses its infectivity upon binding to antibodies specific to its surface epitopes. The test is based on the treatment of the virus suspension with antibodies for some time following this its test in antigen-antibody binding sensitive systems such as cell cultures. The test is so sensitive to detect small amount of antibodies and specific enough to detect serotypes of the same virus strain. Micro neutralization is so called "micro" due to small sample volumes it requires. Two bovine serum samples which were already known to have high antibody titers against Bovine herpes virus-1 were subjected to 154 MPa and 242 MPa pressures for 5 minutes at 25 ^{0}C. The results are given in the Table A.2 in the Appendices section and also the results are pictured in the Figure 3.10.

Figure 3.10 The effect of pressure on antibody titer at 25 ^{0}C for 5 minutes of HHP treatment for control, 154 and 242 MPa, blue bar denotes the serum number 1 and red bar denotes serum number 2.

3.3 Pressure Stability of Serum Proteins by Zonal Electrophoresis

The stability of the serum proteins were examined with zonal electrophoresis. The experiments in duplicate were carried out at 132, 220 and 242 MPa pressures for 5 minutes of pressure application. The electrophoresis results are given in the Figure 3.11.

Figure 3.11 The effect of pressure on protein components of bovine serum at 25 ^{0}C for 5 minutes of HHP treatment; Lanes (1) and (2) Control, (3) 132 MPa, (4) and (5) 220 MPa, (6) and (7) 242 MPa. Bands assigned: (a) Serum Albumin, (b) α-globulin, (c) β-globulin, (d) γ-globulin bands.

The bands on the electrophoresis gel were evaluated according to a densitometer and turned into areas accordingly. To save from the space the results of only one of the duplicate experiments were placed in here. The results were the same for all of the experiments as evidenced from the areas under the sectioned parts in the Figures 3.12.

Figure 3.12 The effect of pressure on protein components of bovine serum at 25 ^{0}C for 5 minutes of HHP treatment; Lanes (a) Control, (b) 132 MPa, (c) 220 MPa, (d) 242 MPa. Sections assigned in the panel a are Serum Albumin (1), α-globulin (2), β-globulin (3), γ-globulin (4) bands. The ordinate is absorbance in arbitrary units.

The areas under the curve were sectioned and calculated accordingly. The area under the curve corresponds to the concentration. The relative areas are given over a total of 100 in the Figure 3.13.

Figure 3.13 The effect of pressure on the relative areas of protein component band of bovine serum at 25 ^{0}C for 5 minutes of HHP treatment for different pressure levels; Sections assigned: (1) Serum Albumin, (2) α-globulin, (3) β-globulin, (4) γ-globulin bands.

The results suggest that the electrophoretic mobilities of the each group of serum proteins are not affected by the HHP treatment for the pressure range of up to 242 MPa for 5 minute of application. In addition, band areas did not change after the HHP treatment meaning that the component proteins in each group of sections did not lose their electrophoretic mobilities either. The results of the serum electrophoresis band areas are given in the Table A.3 in the Appendices section.

3.4 The Effect of High Pressure on Blood Cells Studied by Fourier Transform Infrared Spectroscopy

Since the blood contains a high amount of water which is a major disadvantage of FTIR technique the water spectrum was subtracted from the raw spectrum. The success of the subtraction process can be ascertained from the removal of the water band around 2100 cm^{-1}. The Figure 3.14 depicts the spectra belonging to (a) average of the blood cells spectrum (control), (b) double distilled water spectrum and (c) water subtracted blood cells.

Figure 3.14 FTIR spectrum of blood cells blood (blue), distilled water (red) and water subtracted blood cells (black).

3.4.1 Band Assignments of the Spectrum

The band assignment of blood cells were carried out after the water spectrum was subtracted from the raw spectrum and the peaks were numbered as shown in the Figure 3.15. The band assignments of the spectrum are given in the Table 3.1 as the wavenumbers of the all the functional groups.

Figure 3.15 FTIR spectrum of blood cells.

The wave number readings are shown in the Table A.4 in the Appendices section.

Table 3.1 General band assignments of FT-IR spectrum of blood cells.
a : Stuart,1997, b : Çakmak et al, 2003, c : Barth, 1999, d : Cannon et al, 2003, e : Lavoie et al, 2003, f : Sastry et al, 2001.

Band Number	Wavenumbers (cm^{-1})	Definition of the spectral assignment
1	2959	CH_3 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates and nucleic acids(a)
2	2936	CH_2 asymmetric stretch: mainly lipids, and little contribution from, proteins, carbohydrates and nucleic acids (a)
3	2872	CH_3 symmetric stretch: mainly proteins, with the little contribution from lipids, carbohydrates and nucleic acids (a)
4	2852	CH_2 symmetric stretch: lipids with little contribution from proteins , carbohydrates and nucleic acids (a)
5	1654	Protein Amide I (mainly C=O stretching) (a)
6	1546	Protein Amide II (N-H bending, C-N stretching) (a)
7	1468	CH_2 scissoring (lipids) (a)
8	1460	CH_3 asymmetric bending (lipids) (a)
9	1453	CH_2 bending (mainly lipids, with the little contribution from proteins) (b)
10	1399	Ca^{+2} chelation to Asparagine and Glutamine carboxilate groups (c)
11	1366	CH_2 Wagging (Cholesterol) (d)
12	1307	C-O ester bond, CH2 wagging (lipids) (e)
13	1300	Proteins Amide III (C-N stretching, N-H bending, C=O stretching, and O=C-N bending) (a)
14	1249	Phosphate bands (a)
15	1170	CO-O-C asymmetric stretching (lipids) (a)
16	1126	CH_3 rocking and CO stretching (a)
17	1105	DNA band (f)
18	1090	PO_2^- symmetric stretching (a)

The wavenumber values of the control and HHP treated sample values were tested whether they are statistically significantly different from the control values. They were found to be statistically insignificantly different from the control values.

3.4.2 The Amid I Protein Band of Blood Cells

Figure 3.16 shows the baseline corrected, normalized average FT-IR spectra region of control (red), 132 MPa (black) and 220 MPa (blue) HHP treated blood cells in the 1715-1600 cm^{-1}.

Figure 3.16 Amide I Region of blood cells, control (red), 132 MPa (black) and 220 MPa (blue) HHP Treated at 25 ^{0}C for 5 minutes.

Figure 3.17 shows the second derivative spectra of blood cells: control (blue), 132 MPa HHP treated (green), and 220 MPa HHP treated (red) blood cells. Second derivative approach is used to find the number of peaks in the major peak. Every minimum point in the second derivative spectrum corresponds to a maximum point in the absorbance spectrum. The gradual loss of the peak minimum at 1681 cm^{-1} with the increasing pressure level is noticeable in the spectra which corresponds to a partial loss in one of the turns and bends part of the secondary structure.

Figure 3.17 Second Derivative Spectra of control, 132 MPa and 220 MPa HHP Treated Blood Cells: Control (blue), 132 MPa (green), and 220 MPa (red) HHP treated sample at 25 ^{0}C for 5 minutes.

The secondary structural assignments of the second derivative spectra were carried out according to Stuart (1997) and are given in the Table 3.2.

Table 3.2 Secondary structure band assignments of amide I region.

Wavenumber (cm^{-1})	Secondary Structure Type
1690	Turns
1681	Turns
1656	α-helix
1648	Random Coil
1637	β-sheet
1630	β-sheet
1609	Tyrosine Side Chains

3.4.3 The Analysis of Protein Secondary Structure Elements with an Artificial Neural Network Algorithm Based on FTIR Data

An Artificial Neural Network algorithm was used to predict the relative amounts of several secondary structural elements of blood cells for different pressure levels as shown in the Table 3.3. The α-helix, β-sheet, and turn structures are a combination of different possible β-sheet structures, respectively.

Table 3.3 The Effect of HHP on the Secondary Structural Elements of Cells of Blood Studied by the Artificial Neural Network Algorithm Method.

	α-Helix	β-Sheet	Turns	Random Coil
Control	62.04	17.42	10.27	10.27
132 MPa	65.01	15.32	10.24	9.43
220 MPa	65.85	13.93	10.41	9.81

3.4.4 The Analysis of Protein Secondary Structure Using Curve Fitting Method Based on FTIR Data

The underlying bands of the amid I region were studied by the method of curve-fitting in the 1700 and 1600 cm^{-1} region. For this purpose a smoothing width of 13 is used to decrease the noise in the spectrum before curve-fitting procedure was applied. The method is used to calculate the areas of the component bands under the amid I band. The peak positions of the sub bands were obtained from the second derivative spectrum of the control spectrum after. The algorithm converged for a total of seven peaks when the areas of the sub bands equals the area of the amid I peak. Figure 3.18 shows curve fitting of the amide I region.

Figure 3.18 Curve fit results of the amide I region of the experimental curve (red), curve-fitting result (blue), ordinate is absorbance in arbitrary units.

The curve-fitting results are given in the Table 3.4. As seen from the Tables 3.4 and 3.5 the results obtained from The Artificial Neural Network and curve fitting methods are in agreement with each other.

Table 3.4 Curve-fitting results of the Amide I region of the control sample of blood cells.

Wavenumber (cm^{-1})	Secondary structure	Area under the sub band	Percent of the amide I band area
1690.42	Turns	0.22963	0.74
1679.47	Turns	1.44731	4.66
1667.98	Turns	1.34006	4.31
1655.83	α-helix	19.74480	63.65
1643.58	Random Coil	2.50948	8.09
1638.19	β sheet	1.45923	4.70
1627.31	β sheet	4.29235	13.84

Table 3.5 The correlation between the Artificial Neural Network and Curve Fitting Methods

Type of Secondary Structure	The Method and the Percent Secondary Structure Type	
	Curve Fitting	Artificial Neural Network
α-helix	63.650	62.040
β-sheet	18.536	17.420
Turns	8.104	10.270
Random Coil	9.710	10.270

The correlation between the two approaches is shown in the Figure 3.19.

Figure 3.19 Comparison of the artificial neural network and curve-fitting approaches in the determination of the secondary structure elements of the cells of blood study.

3.4.5 The Effect of HHP Treatment on Different Regions in the FTIR Spectrum of Blood Cells of Control, 132 MPa and 220 MPa Treated Samples

Since the FTIR technique provides useful information about the structure and function of the samples, the other spectral regions were also investigated. The spectra were interactively baselined first from the two arbitrarily selected points. Then a normalization step was carried out with a manuel baseline option at one of the baseline point used in the previous step so that the spectra are baselined at the same two points for precise comparison of the HHP treated and control samples.

Figure 3.20 shows FT-IR spectra of control (black), 132 MPa (blue) and 220 MPa (red) HHP treated blood cells.

Figure 3.20 Comparison of the 3010-2830 cm^{-1} region of the control (black), 132 MPa (blue) and 220 MPa (red) HHP treated cells of blood at 25 ^{0}C for 5 minutes. The spectra were normalized with respect to the 2958,63 cm^{-1} band.

The spectra of the control and HHP treated samples show almost no changes for this part of the spectrum. This result indicates that high pressure does not induce any variations in the lipid bands.

Figure 3.21 shows that the small changes in the band areas and positions, although being statistically insignificant, are observed in the 1480-1430 cm^{-1} region. The peaks in this region are representative of lipid functional groups whose

changes mainly arise from which indicates morphologic changes in membrane lipids after the HHP treatment.

Figure 3.21 Comparison of the 1481-1431 cm^{-1} region of the control (red), 132 MPa (black) and 220 MPa (blue) HHP Treated Cells of Blood at 25 ^{0}C for 5 minutes. The spectra were normalized with respect to the 1453,87 cm^{-1} band.

When the same analysis was carried out in the region of 1431 and 1361 cm^{-1} a similar behavior was observed. After the interactive baselining at the points of 1431.45, 1374.13 and 1361.55 the normalization within the same region yielded the spectra given in the Figure 3.22. Subtle changes are apparent between the control and HHP treated samples.

Figure 3.22 Comparison of the 1431-1361 cm^{-1} region of the control (red), 132 MPa (black) and 220 MPa (blue) HHP Treated Cells of Blood at 25 ^{0}C for 5 minutes. The spectra were normalized with respect to the 1399,04 cm^{-1} band.

The same analysis was applied between the points of 1361 and 1141 cm^{-1}. The bands at around 1307, 1300 and a shoulder around 1249 cm^{-1} remained same for control, 132 MPa and 220 MPa HHP treated samples as seen in the Figure 3.23. The band at 1170 cm^{-1} is informative of lipid structure and shows a decrease in its area as the pressure level increased.

Figure 3.23 Comparison of the 1361-1141 cm^{-1} region of the control (red), 132 MPa (black) and 220 MPa (blue) HHP Treated Cells of Blood at 25 ^{0}C for 5 minutes. The spectra were normalized with respect to the 1307.60 cm^{-1} band.

3.5 High Pressure Inactivation of Microorganisms

3.5.1. Pressure Inactivation of *Listeria innocua* as the Model Bacterium

Listeria innocua was chosen as the model bacteria for the high pressure inactivation studies in this research investigation. It is a gram positive, non pathogenic bacterium but holds many characteristics very similar to a relatively pressure-resistant pathogen *Listeria monocytogenes.* Since the highest bacterial resistance against pressure is developed in the early stationary phase, initially the growth curve of *Listeria innocua* was obtained at 37 ^{0}C in Nutrient Broth media.

The growth curve of *Listeria innocua* is given for 35 hours in the Figure A.2 in the Appendix Section. From the Figure A.2 the early stationary phase of *Listeria innocua* under the aforementioned conditions was estimated to be around 24 hours after the inoculation time.

The viability loss of *Listeria innocua* cells at 176 and 220 MPa at 25 ^{0}C for 40 minutes was studied. The colony numbers were noted for each plate given the Table A.51 and Table A.5.2 in the Appendices section. In the tables some panels were noted to be Too Numerous To Count due to very high number of colony numbers. The results can be seen comparatively in the Figure 3.24 also.

Figure 3.24 The effect of HHP treatment on the survival of *Listeria innocua* in fetal bovine serum at 25 ^{0}C for 40 minutes.

The results suggest that for 40 minutes of pressure application at the level of 220 MPa inactivates an important portion of the initial microbial load, whereas 176 MPa pressure level leaves most of the initial load unaffected. The application of 176 MPa pressure for 40 minutes inactivated the microorganism only 79.95 % of the original number, whereas the application of 220 MPa pressure for 40 minutes was found to be much more effective with 23.54 % of the original count.

3.5.2 High Pressure Inactivation of Viruses Contaminating the Blood

BHV-1 represents blood contaminating enveloped viruses here. BHV-1 has an envelope as many human pathogenic viruses. It allows researchers to detect the infective virus particles through a simple yet reliable plaque test. The virus plaques can be seen under the inverted light microscope as follows. The viral growth causes the cells to lyse and form foci. Each focus is considered to stem from an infective virus particle and two of them are visualized as shown in the Figure 3.25.

Figure 3.25 The virus plaques on MDBK cells stained with crystal violet.

To inactivate the virus completely the same experimental set up was applied to the virus suspension with the pressure level of 242 MPa but with different pressure application times and the complete viral inactivation was achieved as can be seen from the table below. The time periods that were applied were 5, 10, 20, and 40 minutes. In all of the well any plaque formation could not be observed which means complete inactivation of the virus with the high pressure application. The plaque numbers are given in the Table A.7 and Table A.8 in the Appendix section. Even at the highest dilutions tested, no sign of viral growth was observed. The results were plotted taking the averages of each sample as can be seen in the Figure 3.26. As the results suggest high pressure application inactivates the infective viral particles considerably depending on the level of pressure.

Figure 3.26 The effect of HHP treatment on the active virus titer in fetal bovine serum at 25 ^{0}C for 5 minutes.

CHAPTER 4

DISCUSSION

The first step in this research was a detailed stability analysis of whole blood and blood constituents upon HHP treatments. Since blood is a suspension of various cells in a solution phase called plasma, it was more appropriate to analyze each one in sequence. First, HHP treated and untreated cells of blood were analyzed using flow cytometry, light microscopy and atomic force microscopy techniques. Then in the second part of the study HHP treated and untreated serum components were analyzed by biochemical analysis techniques and microbiological methods. As the third part of the study FTIR analysis was performed to see the effect of pressure blood cells. In the last part the inactivation of a model bacterium and a model virus in blood was investigated.

The results of the flow cytometry study indicated the outstanding stability of red blood cells against HHP. Even at the pressure level attained (242 MPa) they remained around the same cell numbers and mean cell volumes. The small increase in the erythrocyte number at 220 MPa pressure should be regarded as the effect of morphological changes since it is physically impossible for the erythrocytes to increase in number. Small error bars Figure 3.1 strongly indicates that erythrocyte number is not different from that of control ones. Their overall morphological state after the pressure application showed small changes as small disturbances at their surfaces at 154 MPa pressure as seen by light microscopy, of the surface structure. They finally lost their characteristic shapes at 350 MPa and found to be fused each other completely (Fig.3.6). The Atomic Force Microscopy study showed small perturbations on the red blood cells where these changes that are not detectable under the light microscope. Pressure application seems to be rendering the erythrocyte membranes more flexible allowing them to be fused to each other as shown in the Fig 3.8. Similarly the hemoglobin content of the whole blood did not change in the pressure range studied as shown in the Figure 3.3.

The flow cytometry study indicated that platelets are not stable at the pressure levels of 154 MPa or beyond. Their number rapidly decreases at these elevated pressure levels. The results indicate that their cell membranes are more fragile to pressure effects. This instability results puts a higher limit on the pressure level which is somewhere between 110 and 132 MPa if the sterilization of the whole blood is considered. Their cell volumes showed a similar decrease to what after the same pressure level.

The pressure stability of white blood cells is similar to that of platelets indicated by the light microscopy results (Fig 3.7). From the pictures obtained under the light microscope it is possible to obtain the integrity data of the cell directly and also visually. According to the pictures in the previous chapter, the white blood cells start to loose their integrity around 132 MPa. But still some of the cells seem stable under pressure as judged from the pictures. At 220 MPa pressure they loose their structure completely. It is not possible from the light microscopy results whether the cell cells are live or not. But when they loose their overall structure it could be said that they are not live anymore.

The pressure stability of small molecules such as glucose and cholesterol and ions such as iron were found to be the same in the range of 0.1 – 242 MPa pressure. Iron binding capacity also did not change which indicates that molecular binding receptor is not altered upon pressure applied. The concentration of the protein Albumin did not change as it has given similar activity with the color forming chemicals.

The activities of the enzymes ALT and AST did not show important variations after the pressure applications indicating high stability of theses proteins against pressure. In the case of ALT a small decrease observed in the activity of the enzyme indicates denaturation behavior similar to the previous reports (Heremans *et al*, 1993 and 1998). In contrast with the AST case the activity of the enzyme slightly increases with the applied pressure depending on the pressure level. The increase of the enzymatic catalysis under and after the pressure

application was observed with different enzymes before, actually the catalytic activities of enzymes show complex pressure dependences (Grob *et al*, 1993 and Mozhaev *et al*, 1996). This indicates that pressure favors the active form of the enzyme by reducing its molecular volume which also implies that pressure exerts structure changing effects on the protein molecule enhancing or reducing their activities depending on the nature of their activities.

A similar behavior to the AST protein was observed with an important component of blood proteins namely immunoglobulin. IgE and IgG show full recovery after the pressure application. The quantitatively same results obtained for IgE indicated that the cross reaction between an antibody conjugate raised in mice against human IgE molecules and bovine IgE molecules could be used for such stability studies. Similarly the stability of IgG molecules evidenced in the virus neutralization experiments showed that the immunoglobulin fold found in the immunoglobulin structure is not affected by the high pressure application. These results also indicate that anti-parallel beta sheet structures are not adversely affected by the pressure application up to 242 MPa level.

Pressurized Serum proteins showed similar stability behavior after the gel electrophoresis indicating no changes of hydrodynamic radius or shapes. Since both the pressure applied and control samples all of the groups of proteins showed the same electrophoretic mobility it can be concluded that the overall structures of the serum proteins is not affected with the high pressure applied. The results with the serum electrophoresis support the results obtained in the functional studies above. The same results obtained with the area measurements obtained indicate that all of the serum protein groups do not lose their component proteins evidenced from the same shape and the area of the graphics. No change in the electrophoretic mobility indicates no changes in the overall molecular volumes or shapes in the technique of zonal electrophoresis.

The changes in the protein part of the blood cells were investigated in terms of changes in their secondary structure components using FTIR spectroscopic

technique. Both artificial neural network algorithm and curve fitting procedure were applied to the FTIR data. The results have indicated that HHP treatment has not changed the secondary structure properties of the cells of blood grossly. It is known that if there is a loss in α-helix or β-sheet structure and an increase in random coil structure, protein denaturates (Freifelder, 1982). In the current study no increase in the random coil structure was observed due to HHP treatment. This shows that the pressure used in the current study does not denaturate proteins. However a slight decrease in β-sheet structure and a slight increase in α-helix structure were observed. Previous studies showed that in some disease states there is a tendency from α-helix to β-sheets. It means that α-helix structure decreases but β-sheet structure increases (Kneipp *et al*, 2003). However in our case we observed opposite results: α-helix structure increased and β-sheet structure decreased. This may indicate that HHP treatment may stabilize protein structure.

The FTIR study clearly indicated that small changes in the lipid band positions and areas were observed although they were not statistically significant which showed that there are changes in the membranes of blood cells upon the HHP treatment.

The pressure stability of the model bacterium was determined in the blood environment and high pressure application reduced the bacterial load down to its 80.00 % and 23.54 % of the control values at 176 and 220 MPa pressure application of 40 minute duration in fetal bovine serum environment respectively. The results are similar to the results obtained with the same organism in different buffer conditions in the literature (Alpas *et al*, 1998). The requirement of pressure above 220 MPa makes it difficult to use this pressure in order to inactivate bacterial cells in whole blood. Above 176 MPa pressure white blood cells and platelets are destroyed. However, the high pressure application is possible to eliminate the bacteria from serum or plasma components of blood without destroying serum or plasma components such as immunoglobulins and other serum proteins, small organic molecules.

The pressure stability behavior of the model virus was determined at different pressure levels in the blood environment. Interestingly, the pressure stability of the model virus, BHV-1, was found to be lower than that of the model bacteria, *Listeria innocua.* The pressure stability behavior of the virus opens up new possibilities for the application of HHP on blood and blood derivatives. The considerable reduction in the virus titer after the pressure application where no adverse effects of the application was seen on the blood components supports the basic hypothesis of the research investigation. Since the titer of the pathogenic viruses in the case of viremia is not high as the ones used in the research the elimination can be achieved with a greater success.

The higher pressure requirements for the similar herpes virus studies under pressure showed different results for the viral stability studies. The differences could stem from the effect of different pressure increase rates used in the relevant studies. The effect of the rate of pressure increase on the virus and bacterial pressure stability should be well characterized (Nakagami *et al*, 1992).

Overall results of the effect of the HHP treatment on blood constituents and survival of bacterial and viral agents are summarized in the Table 4.1. According to these results red blood cells are stable to HHP treatment up to 220 MPa. However, platelets and white blood cells appear to be degraded after 110 MPa HHP treatment. Serum proteins and small molecules kept their functions pressures up to 242 MPa. Although *L. innocua* was partially stable to HHP treatment up to 220 MPa an important portion of BHV-1 was inactivated above 110 MPa.

Table 4.1 The comparison of stability behavior of blood components versus model microorganisms studied. S: Stable, US: Unstable, RBC: Red Blood Cells, WBC: White Blood Cells.

Pressure Level (MPa)	RBC	Platelets	WBC	Serum Proteins	Small Organic Molecules	*L.innocua* Percent Survival	BHV-1 Percent Survival
55	S	S	S	S	S		94.33
110	S	S	S	S	S		68.79
132	S	-	-	S	S	-	-
154	S	US	-	S	S	-	28.37
176	S	US	-	S	S	80.00	-
220	S		US	S	S	23.54	0.47
242	S			S	S		0.00
350	US			-	S		

CHAPTER 5

RECOMMENDATIONS

In order to see the effect of high hydrostatic pressure on the blood cells, the function tests should be carried out which requires successful separation of erythrocytes, platelets and different white blood cells. An FTIR and DSC study could accompany this detailed stability study.

The stability of the different components of serum should be studied for a wider selection of proteins, enzymes, hormones etc.

Since the high pressure application causes minor changes in the secondary hence tertiary structures of proteins of blood cells possible consequences of these changes should be studied in detail. Such a study can be compounded by a two dimensional electrophoresis approach followed by a mass spectrometric study to identify affected proteins separately.

Relative stabilities of α-helices versus β-sheets under high pressure can be studied via molecular dynamics studies and spectroscopic methods.

The effect of high pressure on the components of blood should be studied in immunological terms to investigate the long term effects of the pressure applied blood to the body in a model organism.

Since FTIR spectroscopy is adversely affected by the presence of water in blood cells and serum a similar spectroscopic study can be carried out using Raman Spectroscopy. Because water peaks is weak in Raman spectroscopy blood can be studied with it. Raman spectroscopy can also provide additional information on the vibrational states on the molecular group level.

The aforementioned studies should be carried out with using human blood this time for the real purpose of this research investigation and for the comparison of the stability of bloods from different origins. In addition the effect of pressure on the blood groups and other antigenic properties should also be tested.

High hydrostatic pressure in cycles studies should be carried out to see the effect of the pressure in cycles on blood and on the inactivation kinetics of several model microorganisms and pathogens as well.

The same study should be carried out with human pathogens when possible. Since some human pathogen viruses such as hepatitis B and C viruses can not be grown on cell cultures appropriate model viruses can be studied in place of them. Bovine viral diarrhea virus can be studied as a model of hepatitis C virus and duck hepatits B virus could be a model for human hepatitis B virus. In addition the stabilities of DNA and RNA viruses could be ascertained using hydrostatic pressure as the destabilizing agent.

The effect of high pressure on the virus structure can be studied with FTIR and DSC. For such a detailed study the virus should be purified well. From such a study the effects of the pressure on the virus structure can be obtained similar to that of in bacteria. The effect of pressure on the one step growth curve of the virus and penetration kinetics could also be studied to increase the information on the subject.

Since herpes viruses also have a latent form of period in their host cells the effect of pressure on the latent form of the virus can be studied in ganglioside cells.

Since the model virus was completely inactivated with the high pressure application, the technique could be used for the production of vaccines against the disease. The advantageous of the high pressure application technique is in its nature. It is completely a physical technique which does not include any noxious chemicals to inactivate the virus or some additional chemicals to eliminate the adverse effects

caused by the virus inactivating chemicals. The immunological response of the animals against the killed virus vaccine should be studied.

The effect of hydrostatic pressure on the virus structure appears to be on the membrane part for the model virus used. Since the deformation of the membrane causes the loss of activity the deformation of the membrane could be studied using artificial membranes as models for real biological membranes. For such a study DSC and FTIR techniques would best suit.

The effect of high pressure on the other types of blood pathogens such as parasites, fungi etc should be studied as well.

The effect of hydrostatic pressure on the blood rheological properties may be important for its proper use after the application. In other words the changes in the blood cell number and shapes may be reflected in the rheological properties of blood namely whole blood viscosity and surface tension. The both are important for blood to carry out its function in body. Surface tension is important in the respiration process in the lungs and in the contact between blood and the blood vessels. Viscosity is the principle determinant in retaining its flow properties during the flow of blood. Such effects could also cause the blood pressure to change in a live organism. Possible rheological changes could also be studied using fundamental laws of fluid mechanics along with numerical computational simulation models in parallel to the experimental study.

CHAPTER 6

CONCLUSIONS

The high hydrostatic pressure application was found to be an effective way of eliminating pathogenic blood contaminating viruses from the whole blood at pressures up to 110 MPa and from the serum at pressures up to 242 MPa without destroying the cells of blood and serum components respectively. The elimination could be partial and dependent on the pathogen studied. Since some of the viral pathogens are slowly growing microorganisms their partial elimination in blood may prove very beneficial for the patient.

Bacteria are more resistant to high pressure application in the blood conditions. It seems impossible to eliminate an important portion of bacteria from blood without destroying the cells of blood within the pressure limits studied in this study. But in plasma or serum high pressure can be used safely to achieve important reductions in the blood contaminating pathogenic bacteria.

High pressure application causes minor changes in the secondary structures of some of the blood cell proteins. While the proportion of α-helices increases the proportion of β-sheets decreases slightly. This gives an advantage for using HHP application at those indicated pressures without affecting the functions of blood cell proteins.

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APPENDIX

Figure A.1. The High Hydrostatic Pressure Unit.

Figure A.2 The Growth Curve of *Listeria innocua* at 37 ^{0}C in Nutrient Broth

Table A.1. The effect of 132, 220 and 242 MPa pressure application on some of the bovine blood parameters at 25 ^{0}C for 5 minutes.

		Before Pressure Application		132 MPa		220 MPa		242 MPa	
Component	Units	Run 1	Run 2	1	2	1	2	1	2
Glucose	mg/dL	72	73	71	73	72	73	72	72
Albumin	g/dL	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Iron Binding Capacity	µg/mL	268	270	257	268	267	267	259	266
Cholesterol	mg/dL	93	94	90	93	90	93	91	91
ALT	IU/L	16	16	16	15	15	16	15	14
AST	IU/L	44	45	45	45	46	48	48	49
Total IgE	IU/mL	28.7	27.2	25.0	26.5	29.4	28.7	29.4	23.4
Iron	µg/dL	59	59	56	57	55	58	55	54

Table A.2. The antibody titers for the serum number 1 and 2, for the control, and after 154 and 242 MPa HHP treatments.

Serum No:	Antibody Titers		
	Control	154 MPa	242 MPa
I	48	24	48
II	24	32	32

Table A.3. Serum electrophoresis band areas.

	Sections on the Plot			
Experiments	1	2	3	4
Control,1	32.4	9.0	4.1	54.5
Control,2	32.3	8.6	4.3	54.8
132 MPa	32.4	8.7	3.8	55.1
220 MPa,1	33.5	8.3	4.3	53.9
220 MPa,2	33.1	8.3	4.2	54.4
242 MPa,1	32.6	8.6	4.3	54.5
242 MPa,2	32.2	8.7	4.6	54.5

Table A.4. Wave Number Readings for the Control, 132 and 220 MPa HHP Treated Blood Cells.

Wavenumbers (cm^{-1})	Control	132 MPa	220 MPa
2959	2959.46 +/-0.33	2959.46 +/- 0.48	2959.38 +/- 0.190
2936	2936.25 +/- 0.61	2936.45 +/- 1.17	2936.35 +/- 0.50
2872	2872.86 +/- 0.03	2872.87 +/- 0.28	2872.85 +/- 0.14
1654	1654.64 +/- 0.33	1654.93 +/- 0.08	1654.94 +/- 0.15
1546	1546.99 +/- 0.16	1547.20 +/- 0.12	1547.31 +/- 0.16
1468	1468.12 +/- 0.47	1467.75 +/- 0.20	1467.23 +/- 0.58
1460	1460.58 +/- 0.46	1460.03 +/- 0.50	1460.27 +/- 0.39
1453	1453.49 +/- 1.36	1452.94 +/- 0.49	1452.79 +/- 0.56
1399	1398.02 +/- 0.20	1398.02 +/- 0.20	1398.14 +/- 0.60
1366	1366.27 +/- 0.27	1366.08+/- 0.15	1365.93+/- 0.29
1307	1307.65 +/- 0.27	1307.74 +/- 0.17	1307.36 +/- 0.53
1300	1300.38 +/- 0.75	1300.69 +/- 0.660	1298.13 +/- 2.85
1249	1249.79 +/- 1.12	-	1248.07 +/- 1.67
1170	1169.97 +/- 0.52	1170.850 +/- 0.33	1170.98 +/- 0.74
1126	1126.07 +/- 0.01	1126.17 +/- 0.32	1126.83 +/- 0.67
1105	1105.86 +/- 0.50	1106.01 +/- 0.250	1106.32 +/- 0.83
1090	1090.82 +/- 0.54	1089.22 +/- 1.580	1091.16 +/- 1.12

Table A.5.1 Colony Numbers of *Listeria innocua* before and after 220 MPa HHP Treatment at 25 ^{0}C for 40 minutes for duplicate experiments.

Pressure Level: 220 MPa.

Dilution	Original Sample	I	II
10^{-4}	Too Numerous to Count	249; 290	Too Numerous to Count
10^{-5}	130; 127	38;31	32;20

Table A.5.2 Colony Numbers of *Listeria innocua* before and after 176 MPa HHP Treatment at 25 ^{0}C for 40 minutes for duplicate experiments.

Pressure Level: 176 MPa.

Dilution	Original Sample	I	II
10^{-5}	96;106	84;76	85;78

Table A.6. BHV-1 Infectivity Assay Results after HHP Treatments, for control,55, 110, 154, and 220 MPa HHP Treatments at 25 ^{0}C for 5 minutes,

TNTC = Too Numerous to Count.

	Control		55 MPa		110 MPa		154 MPa		220 MPa	
Dilutions	I	II	I	II	I	II	I	II	I	II
10^{-1}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	32	35
10^{-3}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	107	115	1	5
10^{-4}	80	61	70	63	45	52	17	23	0	0
10^{-5}	12	13	-	-	-	-	-	-	-	-
10^{-6}	0	1	-	-	-	-	-	-	-	-

Table A.7. BHV-1 Infectivity Assay Results after HHP Treatments, For 242 MPa HHP Treatment for control, 5, 10, 20, and 40 minutes at 25 ^{0}C.

TNTC = Too Numerous to Count.

	Control		5 Minutes		10 Minutes		20 Minutes		40 Minutes	
Dilutions	I	II	I	II	I	II	I	II	I	II
10^{-1}	TNTC	TNTC	0	0	0	0	0	0	0	0
10^{-2}	TNTC	TNTC	0	0	0	0	0	0	0	0
10^{-3}	TNTC	TNTC	0	0	0	0	0	0	0	0
10^{-4}	31	34	0	0	0	0	0	0	0	0
10^{-5}	3	5	-	-	-	-	-	-	-	-
10^{-6}	0	1	-	-	-	-	-	-	-	-

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