EVALUATION OF HAEMATOLOGICAL AND CYTOKINE RELEASE FROM CRYOPRESERVED BLOOD IN RESPONSE TO LIPOPOLYSACCHARIDE

A DISSERTATION SUBMITTED

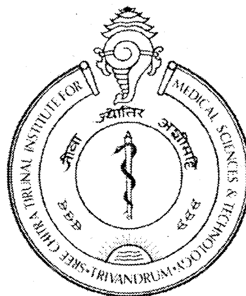
BY

MEGHA K B

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY



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DECLARATION

I, Megha K B, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Evaluation of haematological and cytokine release from cryopreserved blood in response to lipopolysaccharide**" under the direct supervision of **Dr. PV. Mohanan, Scientist In Charge, Division of Toxicology,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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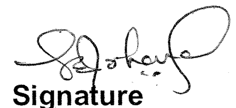
CERTIFICATE

This is to certify that the dissertation entitled "**Evaluation of haematological and cytokine release from cryopreserved blood in response to lipopolysaccharide**" submitted by **Megha K B** in partial fulfillment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Division of Toxicology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram-695011.



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The Dissertation

Entitled

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Submitted

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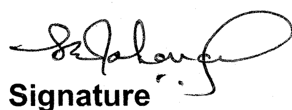

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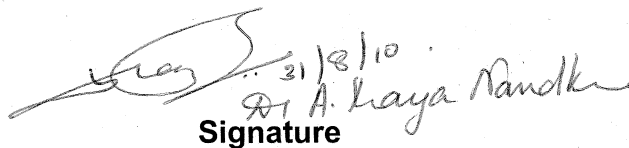

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Megha K B

List of abbreviations

AVP- Arginine vasopressin

BET- Bacterial endotoxin test

BMT- Biomedical technology

BSA- Bovine serum albumin

COX-2- Cyclooxygenase- 2

DMSO- Dimethyl sulphoxide

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme linked immunosorbant assay

EU- Endotoxin unit

h- Hours

IFN- Interferon

IL-1- Interleukin-1

IL-1β- Interleukin- 1β

IL-6- Interleukin- 6

IL-8- Interleukin-8

IL-12- Interleukin-12

IPT- *In vitro* pyrogen test

IV- Intra venous

LAL- Limulus Amoebocyte Lysate

LPS- Lipopolysaccharide

LTA- Lipoteichoic acid

MIP- 1β- Macrophage inflammatory protein- 1β

nm- Nanometre

NK- Natural killer

OVLT- Organum vasculosm of the lamina terminalis

PBS- Phosphate buffer saline

PGE2 – Prostaglandin E2

RBC- Red blood cell

SCTIMST- Sree Chitra Thirunal Institute for Medical Science and Technology

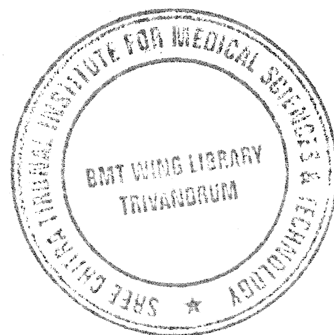
S- Seconds

TLR4- Toll like receptor 4

TMB- 3,3',5,5'- Tetra methyl benzidine

TNF-α- Tumor necrosis factor- α

WBC- White blood cell



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SYNOPSIS

Pyrogenic contaminants are major threat to various biological and medical products. These pyrogenic substances can initiate fever response by increasing the body temperature. Cytokines play an important role in the onset of fever and the major cytokines involved are IL-1β, IL-6, IL-8, TNF-α, MIP-1β etc. IL-1β is a prototypic proinflammatory cytokine which is released during the pathogenesis of fever. In human body IL-1β release leads to an increase in set point of body temperature and is one of the first responses to immune stimulating components. Lipopolysaccharides (LPS) from the cell wall of Gram negative bacteria are found to be a potent pyrogen, which creates surface contamination of various medical and biological products. Pro-inflammatory cytokine IL-1β binds to the receptor on the blood side of the organum vasculosum laminae terminalis, one of the circum ventricular organs of the brain and initiates the expression of the enzyme cyclooxygenase-2 (COX-2). This converts arachidonic acid to prostaglandin E2 (PGE2), which mediates an increase in temperature.

The assays used for checking pyrogenicity are *in vivo* rabbit pyrogen test and Limulus Amoebocyte Lysate (LAL) assay. In the conventional rabbit pyrogen assay animals are injected with a sample (pharmaceuticals, IV fluids, extract of medical device etc.,), followed by the measurement of rectal temperature. LAL assay measures the coagulation of the amoebocytes of the horseshoe crab initiated by the cell wall components of Gram negative bacteria (LPS). Both the assays have an inherent problem with respect to the differences in sensitivity towards endotoxin due to species variation (animal to human). They are also non-quantitative and costly. In order to overcome this situation Hartung *et al.,* developed an *in vitro* human whole blood assay for pyrogenicity. The basic principle of this assay is to measure the cytokine production, especially IL-1β following challenge with endotoxins in human monocytes. Similarly, SCTIMST developed an *in vitro* ELISA method for the evaluation of IL-1β as marker for

pyrogenicity. The method involves the incubation of the sample with human whole blood followed by an enzyme immunoassay for the measurement of IL-1β. The main problem of the *in vitro* human whole blood assay is the lack of continuous availability of healthy, non-allergic, pathogen free human whole blood. Hence, an attempt was made in the present study to develop a cryopreserved blood system for the evaluation of pyrogenicity using the *in vitro* ELISA method developed by SCTIMST.

The present study is mainly focused on the evaluation of haematological parameters and IL-1β release from fresh-pooled, cryopreserved single and pooled blood from multiple donors. The IL-1β release was induced using lipopolysaccharide which is a strong endotoxin from the Gram negative bacteria.

The methodology includes the collection of blood from various donors and checking its complete haematological parameters using automated haematology analyzer and differential count using Leishman's staining. This is to check the alternations in the number of cells and cellular morphology of WBCs, which directly affects the cytokine release (IL-1β). The cryopreservation procedure was done by diluting the blood sample with 10% DMSO in phosphate buffer (pH 6.8). The diluted blood was then snapfreezed using liquid nitrogen (by dipping the tubes in the cylinder for 1 minute) and stored at -80°C. The cytokine release after challenge with LPS was studied in fresh-pooled blood from multiple donors, cryopreserved blood from single donor (Individual A, B and C) for 1, 10 and 25 days and pooled cryopreserved blood from multiple donors for 1, 10, 25, 45 and 75 days after storage. The IL-1β level was detected by ELISA.

From the present study it was observed that there was no abnormality in the haematological parameters while comparing with fresh blood. In cryopreserved blood from single donors, Individual A showed the maximum release of IL-1β

when induced with 5EU of LPS at 10h, 6h, between 9h and 10h, when assayed with cryopreserved blood stored at 1, 10 and 25 days respectively. When treated with 0.5 EU of LPS, the maximum release was found at 12h (1 day), 10h (10 days) and 11h (25 days after storage). In Individual B the maximum release of IL-1β (0.5EU) was found at 9h, 9h and 10h when used with cryopreserved blood stored for 1, 10 and 25 days. Similarly the maximum IL-1β release was observed at 5h on storage for (1, 10 days) and 6h (25 days after storage) when treated with 5EU of LPS. The maximum release of IL-1β (5EU LPS) was at 10h, 6h and 5h in cryopreserved blood of Individual C stored at 1, 10 and 25 days, whereas the maximum IL-1β release was observed at 17h (1 and 10 days) and 9h (25 days after storage) when treated with 0.5EU of LPS. In fresh-pooled blood the maximum release of IL-1β was observed at 2h when treated with 5EU (LPS) and at 5h when the concentration of LPS was 0.5EU.

In 1 day stored pooled cryopreserved blood, the maximum release of IL-1β was at 6h and 12h when treated with 5EU and 0.5EU (LPS) respectively. When 10 days stored cryopreserved pooled blood was used to detect the IL-1β release, the maximum peak was observed at 6h (5EU LPS) and 9h (0.5EU). The cryopreserved pooled blood at 25th day showed a maximum release of IL-1β at 5h and 10h after treated with 5EU and 0.5 EU of LPS. The 45 days stored pooled blood treated with 0.5 and 5EU of LPS induced a maximum level of IL-1β at 12h and 7h respectively. Treatment with 5 and 0.5EU of LPS with 75days stored pooled cryopreserved blood induced a maximum release of IL-1β at 9h and 15h of reaction.

Based on the results obtained, it can be concluded that the cryopreserved blood especially from multiple donors (screened and pooled) can be used as an alternative to the existing fresh human whole blood after further validation. This will definitely help to provide a continuous supply of non-allergic and pathogen

free blood, which will be readily available to the various segments of health care industries for evaluating the *in vitro* pyrogenicity using ELISA method.

CHAPTER 1
INTRODUCTION

1.1. Background

The safety of biologicals/ biotechnological/ biomedical products mainly depends on the assessment of pyrogenicity and is an important safety parameter in quality control system. Pyrogens are heterogeneous group of fever inducing substances derived from Gram positive, Gram negative bacteria, fungi and viruses. They can provoke immune reaction as a part of the innate immune defense and is driven by monocytes, macrophages and neutrophilic granulocytes as well as the complement system. Pyrogens evoke the response by producing endogenous pyrogens such as prostaglandins and the proinflammatory cytokines such as Interleukin-1, Interleukin-6 and Tumor Necrosis Factor-α. The human system reacts effectively to highly conserved structures from different bacterial strains. The most potent stimuli are endotoxins from Gram negative bacteria. The lipopolysaccharides (LPS) of the outer cell membrane influence a cascade of defense mechanisms known as inflammation and fever. When cells of the immune system monocytes and macrophages present in blood come in contact with pyrogenic contaminations, they release mediators which transmit fever reaction within the organism.

Fever also known as pyrexia is a condition so common that all of us have experienced several times in our lives. It is defined as a regulated increase in internal body temperature to levels above normal and oral temperature greater than 99.5°F typically considered as fever. It is mainly triggered by stimulation of thermoregulatory neurons in the hypothalamus at the base of the brain. The brain commands the autonomic and behavioral effector mechanisms to increase the body heat loss, thereby inducing an active rise in temperature. The regulation of

fever is accomplished by the action of two types of endogenous cytokines namely pyrogens and antipyretics. Fever is the result of the communication between the peripheral immune system and the brain. The proinflammatory cytokine, Interleukin (IL-1β) binds to the receptor on the blood side of the organum vasculosum laminae terminalis, one of the circum ventricular organs of the brain and initiates the expression of the enzyme cyclooxygenase-2 (COX-2). This converts arachidonic acid to prostaglandin (PGE2), which mediates an increase in temperature.

The history of discovery of cytokines led the investigation into the triggers for the febrile response. Charles Dinarello and Steven Mizel independently cloned the IL-1 and found that it was same as the fever inducing factor produced by leukocytes known as leukocyte pyrogen. Cytokines are soluble proteins or glycoproteins produced by leukocytes and also by other cell types. They are involved in cell proliferation, differentiation and survival factors. Cytokines are of mainly two types; endogenous pyrogen which induces fever and includes IL-1, IL-6, IL-8, macrophage inflammatory protein-1β (MIP-1β) and interferon-γ. Endogenous antipyretics limit the fever, which include IL-10, arginine vasopressin (AVP), α-melanocyte stimulating hormone (α-MSH) and glucocorticoids. It is the sum of the relations of pyrogenic and antipyretics that is responsible for the elevation of temperature and duration of fever.

IL-1β is one of the main proinflammatory cytokine expressed by many cells including macrophage, NK cells, monocytes and neutrophils. IL-1β was chosen as a readout parameter because it is constantly released upon exposure to low concentration of pyrogenic stimulus and can be readily measured by ELISA.

The methods used for checking pyrogenicity are *in vivo* rabbit pyrogen test and Limulus Amoebocyte Lysate (LAL) assay. In the conventional rabbit pyrogen

assay animals are injected with a sample (pharmaceuticals, IV fluids, extract of medical device etc.,), followed by the measurement of rectal temperature. The assay has an inherent problem with respect to the differences in sensitivity of species (animal to human) towards endotoxin and currently requires 200,000 rabbits each year in Europe. After a recuperation period of 2-3 weeks, the animals can be used again for pyrogen assay; provided that the test substances cause no permanent changes in the immune system of rabbits.

Limulus Amoebocyte Lysate (LAL) assay measures the coagulation of the amoebocytes of the horseshoe crab initiated by cell wall components (LPS) of Gram negative bacteria. The assay cannot detect smaller quantity of LPS or the LPS equivalents from Gram-positive bacteria and fungi.

Human whole blood assay was developed by Hartung and Wendel which measures cytokine production i.e. IL-1β following challenge with pyrogens in human monocytes. It is more sensitive than the rabbit or LAL assays and has an advantage of being able to examine the strength of the reactive directly in human subject.

1.2. Review of literature

1.2.1. Pyrogens

The word "pyrogen" comes from the Greek word "pyros" meaning "fire". Pyrogens may be defined as any substance that causes fever. The pyrogens of concern to the pharmaceutical industry are bacterial endotoxin (lipopolysaccharides, LPS) from Gram-negative bacteria since it most frequently contaminates the parenteral medicinal products [Mascoli & Weary, 1979]. An immune response can be evoked by microbiological components such as LPS [Beutler & Rietschel, 2003], lipoteichoic acid (LTA) [Ginsburg, 2002], the LPS equivalent in Gram- positive bacteria, its cell wall which consists of peptidoglycan

[Wang *et al.*, 2003] , fungal spores [Tran *et al.*, 2003] and viral pathogens [Bowie & Haga, 2005]. It was noted that medical products which are sterile may be pyrogenic in many situations [Daniels *et al.*, 2000; Haishima *et al.*, 2001; Nakagawa *et al.*, 2003].

Pyrogens can be classified as exogenous and endogenous in origin. Exogenous pyrogens include metabolic substances and cell wall components of microorganisms. Exogenous pyrogens induce fever through interacting with cytokines, which are referred to as endogenous pyrogens [Roth & De Souza, 2001] and also with Toll-like receptor belongs to the IL-1 receptor family [Dinarello, 2004]. Endogenous pyrogens are immune cytokines, which include Interleukin-1 (IL-1) family, Interferon α (IFN-α), Tumor Necrosis Factor (TNF), IL-6, GM-CSF and Macrophage inflammatory protein-1 (MIP) [Kluger, 1991]. The presence of pyrogens in the body initiates the secretion of pro-inflammatory cytokines and when high concentration of pyrogens enter into the blood stream it causes fever, septic shock or even death [Cohen, 2000; Hartung *et al.*, 2001].

1.2.2. Lipopolysaccharides

Bacterial endotoxin consists largely of lipopolysaccharides (LPS) from the cell wall of Gram negative bacteria. This LPS stimulates monocytes/macrophages via interaction with CD14 and Toll Like Receptor 4 (TLR4) [Beutler & Rietschel, 2003]. Lipid A have been identified as the biologically active components of LPS and when coupled to serum albumin it was shown to cause fever in rabbits [Dinarello, 2004]. Endotoxin is a very potent stimulus for a wide range of cells (leukocytes, platelets, endothelial cells, and epithelial cells) both *in vitro* and *in vivo*. Some cells such as leukocytes require minute amounts of LPS to be activated [Wright *et al.*, 1991] while others such as platelets need a larger concentration [Csako *et al.*, 1988]. LPS are an integral part of the outer cell membrane of Gram negative bacteria. Endotoxins consist of a lipid component

(Lipid A), a core oligosaccharide and a long heteropolysaccharide chain (the O-specific chain) (Figure 1. 1) representing the surface antigen (O-antigen). The O-antigen is generally composed of a sequence of identical oligosaccharides and is strain specific [Petsch & Anspach, 2000].

Lipid A is the most conserved part of endotoxin and is responsible for most of its biological activities. A single *E. coli* contains about 2 million LPS molecules per cell. Endotoxins are shed upon cell death (in large amount) but also during growth and division. They are highly heat-stable and hence are not destroyed under regular sterilizing conditions: a temperature of over 250°C (30 minutes) is necessary to inactivate endotoxins.

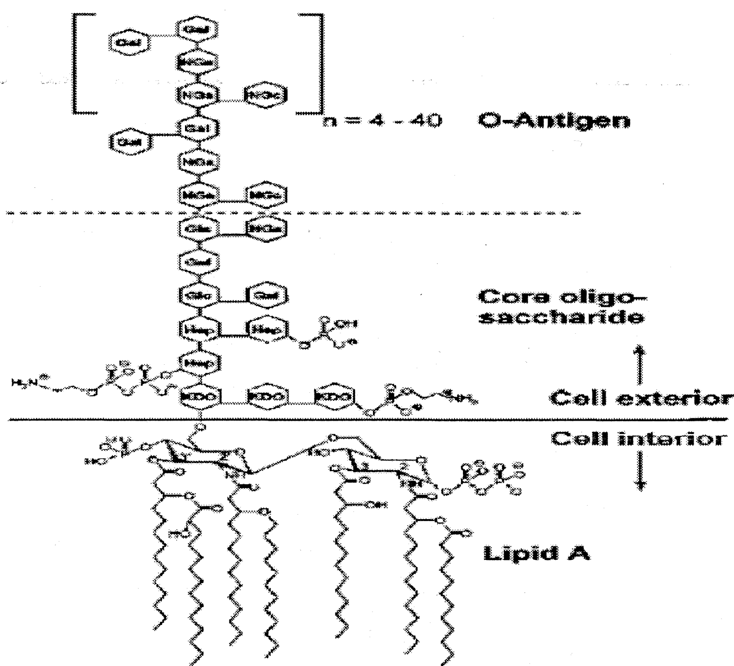


Figure 1.1: Chemical structure of endotoxin *E.coli* 0111:B4 (Adapted from Maud B Gorbet, 2005)

Endotoxins have a net negative charge in solutions. They can form aggregates (micelles or vesicles) with high stability depending on the solution characteristics

(pH, ions, surfactants etc.); thus the size of endotoxin ranges from 10–20 kDa monomers to over 1000 kDa vesicles. Due to their hydrophobicity, endotoxin adsorbs readily to hydrophobic materials and also binds to cationic materials through their phosphate groups. Endotoxins have very strong biological effects when entering the blood stream with symptoms ranging from fever and shivering to hypotension, adult respiratory distress syndrome, disseminated intravascular coagulation and endotoxin shock in human and animals. The potential to induce fever has led endotoxins to be referred as pyrogens. Most cases of sepsis (a systemic inflammatory response to a local infection) results from Gram negative bacteria and its pathophysiology initiated by LPS and stimulate the synthesis of inflammatory mediators such as the cytokines IL-1β, IL-6 and TNF-α. The critical level of endotoxin leading to sepsis is not clear since it depends on the virulence of the organism, the sites of infection, host response and genetic factors. Endotoxins have been reported to trigger the complement pathway, coagulation and kinin systems [Lynn & Golenbock, 1992].

1.2.3. Mechanism of fever

Fever is one of the most recurrent signs of many clinical disorders, particularly infections. When pathogenic microorganisms enter the circulation and stimulate the leucocytes to release endogenous mediators, it transmits signals to increase the thermostatic set point at the level of the preoptic area of the hypothalamus [Dinarello *et al.*, 1988; Saper & Breder, 1994]. These signals are further transmitted to the brain and thereby complex thermoregulatory mechanisms are triggered to increase the body temperature. The thermoregulatory mechanisms of the organism are intact and function as to maintain the body temperature in a cool environment at the onset of fever.

The increase in the body temperature during infection leads to several advantages, including improved bactericidal activities of neutrophils and

macrophages [Dinarello & Wolff, 1978] and inhibition of bacterial growth at high body temperatures [Duff, 1986; Saper & Breder, 1994]. The activated adaptive responses such as synthesis of acute phase proteins, iron sequestration, metabolic switch from glucose utilization (a good substrate for bacterial growth) to proteolysis and lipolysis, anorexia or somnolence [Saper & Breder, 1994].

Pathogenesis of fever indicates that pyrogenic substances are synthesized by leucocytes upon stimulation with bacterial cell wall components. These substances secreted by blood leucocytes are called endogenous pyrogens. Mononuclear cells are able to synthesize protein mediators called cytokines, some of which had potent 'endogenous pyrogen'- like properties. The most important cytokines involved in the pathogenesis of fever are IL-1β, TNF-α, IL-6 and IFNs.

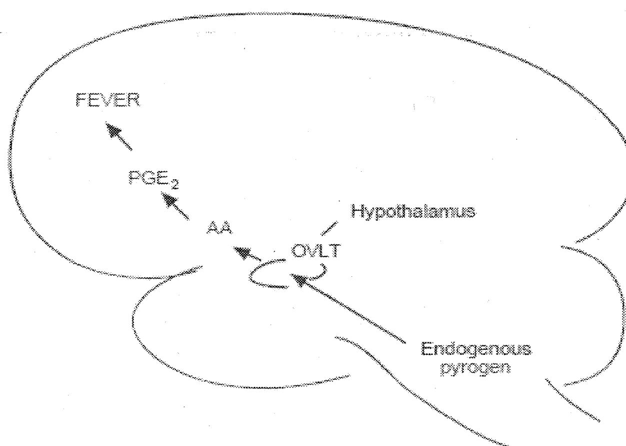


Figure 1.2: Induction of fever: the classical model. [Netea *et al.*, 1999].

In the classical model, exogenous pyrogens such as bacterial products stimulate monocytes /macrophages and T-lymphocytes to produce inflammatory cytokines [IL-1β, TNFα, IL-6, IFNs]. These circulating cytokines with endogenous pyrogenic properties reaches the central nervous system through fenestrated capillaries at the level of the organum vasculosum of the lamina terminalis (OVLT) [Katsuura

et al., 1990]. The cytokines may act either directly on nervous structures in the OVLT or alternatively on astrocytes, microglial cells and neurons at the site responding to cytokine production of prostaglandins especially of the E_2 class (PGE2). Passive diffusion of prostaglandins into the brain and/or stimulation of production of neurotransmitters by neurons with cell bodies in the vicinity of circum ventricular organs direct to the activation of the coordinated endocrine, autonomic and behavioural responses leading to fever [de Vries *et al.*, 1994; Dinarello *et al.*, 1988; Luheshi & Rothwell, 1996; Saper & Breder, 1994].

An alternative to the classical model is the local production of cytokines at the tissue level which plays a critical role in the pathogenesis of fever. Cytokines produced in the infected tissues may induce the release of secondary mediators with endogenous pyrogenic properties. Invasion of the host by microorganisms with concomitant exposure to exogenous pyrogens at the tissue level leads to activation of the monocytes. The activated monocytes migrate through the blood stream with no or very little production of cytokines and eventually adhere to endothelial cells in OVLT [de Vries *et al.*, 1994]. The synthesis and / or release of proinflammatory cytokines from either the mononuclear cells or activated cells take place from here.

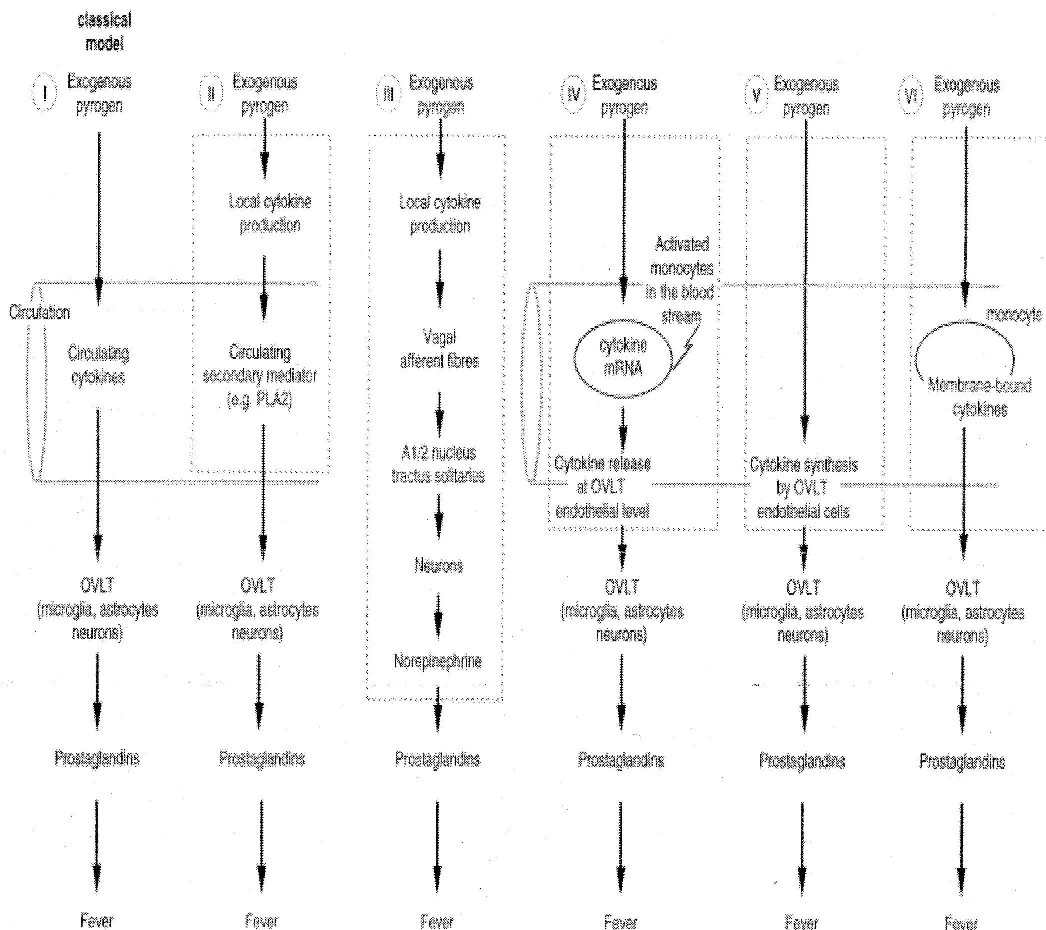


Figure 1.3: A multi pathway model for the pathogenesis of fever. [Netea *et al.*, 1999].

1.2.4. Cytokines

Cytokines are small non structural proteins with a molecular weight ranging from 10-30 KDa. They possess potent biological activities and are usually produced in disease states or following antigenic challenges rather than in health. They are essential for a functional immune system. Cytokines are classed broadly into proinflammatory cytokines and anti inflammatory cytokines. Pro-inflammatory cytokines include IL-1, TNF-α, IL-6, IL-12 and IL-18 where as anti inflammatory cytokines are IL-4, IL-10 and IL-13. Each cytokine is a product of a separate

gene and they activate the cells via their own highly specific surface receptor. Cytokine receptors are expressed on most cells, which accounts for their role in several biological and pathological processes. Cytokines are of endogenous pyrogens {IL-1, IL-6, IL-8, Macrophage inflammatory protein- 1β (MIP-1β) and Interferon-γ} and endogenous antipyretics {IL-10, arginine vasopressin (AVP), α- melanocyte – stimulating hormone (α- MSH) and glucocorticoids}. The sum of the interactions of pyrogenic and anti pyretic cytokines is responsible for the height and duration of a fever response. Cytokine interactions are dependent on a variety of factors like species, mode of infection and strength of the fever inducing stimulus. Cytokines show pleiotropism (they have multiple target cells and multiple actions), redundancy (different cytokines may share similar actions) and feed back mechanism (they can increase or decrease the level of their own or other cytokines).

The term interleukin was used to connote 'between leukocytes'. The interleukin-1 family of ligands consists of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1rn). IL-1β is thought to be primarily secreted form of IL-1, whereas IL-1α is thought to remain mainly cell associated [Auron *et al.*, 1987]. The IL-1rn is an inhibitory protein that binds to IL-1 receptors without including an intracellular signal and thus acting as a true antagonist of IL-1 inducible effects [Dripps *et al.*, 1991]. IL-10 is a protein product of T helper 2 subset cells that was originally described as a "Cytokine synthesis inhibitory factor". IL-10 inhibits the LPS induced production of many cytokines implicated in fever including IL-1β, IL-6 and TNF-α [Fiorentino *et al.*, 1991]. Pro-inflammatory cytokines communicate to the brain via a neural pathway involving activation of the vagal afferent by interleukin 1β in addition to blood borne routes. Pro-inflammatory cytokines IL-1α, IL-1β, TNF-α and IL-6 are released by peripheral immune cells in response to pathogenic challenge [Janeway & Medzhitov, 1999]. In the human body IL-1β release leads to an increase in the set point of the body temperature and is one of the first

responses to an immune stimulating compound [Dinarello, 2002]. Interleukin-1β was preferred as a readout parameter for the whole blood assay since it is consistently released in a concentration dependent manner on exposure to minimal pyrogenic stimulation and can be readily measured by ELISA [Daneshian *et al.*, 2009].

1.2.5. Assays for detecting pyrogenicity

Main assay for detecting pyrogenicity are the *in vivo* rabbit pyrogen and Bacterial Endotoxin Test (BET). *In vivo* rabbit pyrogen detects endotoxin and other pyrogens. The assay involves measuring the rise in body temperature following the intravenous injection of a sterile solution to be examined. The principle of the BET is the extracellular coagulation of the blood (haemolymph) of the horse shoe crab *Limulus polyphemus* [Levin & Bang, 1964]. The rabbit pyrogen assay is not suitable for sera, radiopharmaceuticals, chemotherapeutics, analgesics, cytokines, immunosuppressive agents etc., [Morath *et al.*, 2001]. The BET gives false positive results with glucan like structure and many herbal medicines and it does not reflect the potency of the LPS in humans [Dehus *et al.*, 2006]. Rabbit and the BET assay have a different sensitivity compared to humans towards different microbial components [Devleeschouwer *et al.*, 1985]. Both the assays have shortcomings like qualitative and semi quantitative, differences between the reaction of rabbits and human towards different pyrogen classes [Schindler *et al.*, 2004].

1.2.6. An *in vitro* approach towards pyrogenicity

The first pyrogen assay based on human whole blood stimulation by pyrogens was developed by Hartung *et al.* [Hartung *et al.*, 2001; Morath *et al.*, 2001] . The whole blood pyrogen assay has two stages for the detection of pyrogenic contamination. This involves incubation of the sample with human blood followed by an enzyme immunoassay for the measurement of IL-1β. As an alternative to

the *in vitro* pyrogen test [IPT] models the human endogenous fever response. It measures the release of the mediator IL-1β in whole blood assay upon incubation with the sample substances [Hartung & Wendel, 1995] . The pyrogen test developed by Hartung and Wendel is based on the principle that the sample to be tested is incubated with a small amount of blood taken from a healthy donor. Any pyrogenic activity independent of its chemical nature induces the formation of IL-1β, which can be determined by ELISA. IPT based on human whole blood cytokine release exploits the natural human reaction in response to pyrogens to detect contaminated ingestible drugs [Hartung & Wendel, 1995]. The whole blood test recognizes all known pyrogens that are relevant to humans and reflects their relative potency [Fennrich *et al.*, 1999]. The blood monocytes produce pro-inflammatory cytokines in response to any pyrogen present, in a concentration dependant manner and the IL-1β produced can be detected by enzyme linked immunosorbant assay (ELISA). Human whole blood containing monocytes was chosen as an accessible source of primary human immune cells. The advantage of using human whole blood instead of isolated cells such as blood monocytes is that all the immune cell types and serum components are present in their natural composition, and sources of contamination or pre activation of the cells are reduced as no isolation procedures are required [Daneshian *et al.*, 2009].

Based on the review of literature it was well documented that the pyrogenicity are assessed by Rabbit pyrogen and LAL assay. There was lot of lacunae in detecting the pryogencity of medical devices, blood derived and tissue engineered products. In order to achieve the above target the Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST) developed an *in vitro* ELISA method for detecting the pyrogenicity of compounds of any nature, which is based on human whole blood (Mohanan *et al.*, 2010).

The availability of healthy, non allergic whole blood at the laboratory conditions is little difficult. To overcome such situation an effort was made in the present study to develop a cryopreserved blood system for the evaluation of pyrogenicity using the above ELISA method developed by SCTIMST.

1.3. Hypo dissertation

Pyrogenecity testing includes rabbit pyrogen test and LAL assay. But these tests have a number of drawbacks like, it shows species variation, costly, has ethical issues etc. The collection of fresh human whole blood from healthy, non-allergic donors when necessary, is one of the difficulties faced when using human whole blood for pyrogenicity assay where IL-1β is the pyrogenicity marker. To overcome all these difficulties, an idea was put forward to evaluate cryopreserved blood system as a suitable candidate. Here an attempt has been made, to prepare cryopreserved blood from single donor and pooled blood (multiple donors) to evaluate the IL-1β release on induction with LPS. IL-1β was measured using the SCTIMST developed *in vitro* ELISA method.

1.4. Objective

The main objectives of the present study are

1. Evaluation of haematological parameters of the fresh, cryopreserved single donor and pooled blood from multiple donors.
2. Detection of IL-1β from fresh pooled blood, cryopreserved blood from single donor and multiple donors treated with lipopolysaccharides (LPS) using the SCTIMST developed *in vitro* ELISA method.

CHAPTER 2
MATERIALS AND METHODS

2.1. Materials

- Ethylenediaminetetraaceticacid [EDTA] extra pure
- RPMI 1640 (Himedia. India)
- Dimethylsulphoxide [DMSO, Biotechnology performance certified]
- Interleukin1-β human recombinant expressed in *E.coli*
- Endotoxin standards (Sigma, USA)
- Leishman's eosin methyleneblue solution for microscopy (Merck, India)
- Albumin fraction V from bovine serum for biochemistry (Merck, Germany)
- TMB plus liquid 1 component substrate (Amresco, India)

2.2. Equipments

- Heraeus Kelvitron T (Germany) hot air oven
- Fisher Scientific AB15+ (UK) pH meter
- Sanyo MCO- 18AIC (Japan) Carbondioxide incubator
- ESCO (Singapore) Air stream vertical laminar flow cabinet
- Olympus CH-2 (Japan) microscope
- Carl Zeiss- Axiostar (USA) microscope
- Asys Expert plus (Austria) ELISA plate reader (Nunc 96 well ELISA plate) with Digi Swift software
- Eppendorf centrifuge 5810 R (Germany)
- Sysmex K4500 automatic hematology analyzer (Japan)
- Operon Deep freezer -80°C (Korea)

2.3. Methods

2.3.1. Collection of blood

Fresh human whole blood collected from healthy and non allergic donor who has not taken any medication for at least 14 days prior to blood collection. Blood was drawn by venipuncture using sterile syringe and transferred into depyrogenised tubes containing EDTA.

2.3.2. Analysis of blood Parameters

The above collected blood was subjected to analyse the haematological parameters such as WBC, Haemoglobin, RBC and platelet count using an automatic hematology analyzer (SYSMEX K4500).

2.3.3. Evaluation of differential count of the blood using Leishman's staining

2.3.3.1. Preparation of smear

A drop of blood was placed on a clean grease free slide. A thin film of blood was made by spreading the drop of blood evenly across the slide using a smooth edged spreader.

2.3.3.2. Leishman's staining

The air dried smear was kept on a staining rack. 10 to 15 drops of Leishman's stain was poured over the smear. It was kept for 1 to 2 minutes for fixation. The stain was diluted with phosphate buffer (pH 6.8) and kept for 7 to 10 minutes for staining. The stained slides were then washed gently with tap water and dried. The stained slides were viewed under microscope to examine the quality of staining and then focused under oil immersion objective (100X). The differential count was done in a zigzag manner and percentage of cells was recorded.

1. Fresh human whole blood without dilution was immediately stored at 4°C, -21°C and -80°C. This blood was thawed after 24h, smeared on a clean glass slide and stained. The stained slide was subjected to WBCs count.
2. Fresh human whole blood was diluted with 10% DMSO in phosphate buffer pH 6.8 (1:1 ratio) and was smeared, stained on a clean glass slide and subjected to differential count.
3. Fresh human whole blood from multiple donors (five) was pooled, smeared and stained before and after diluting with 10% DMSO (1:1 ratio). The stained slides were subjected to differential count.
4. Fresh human whole blood collected for cryopreservation (Single donor and multiple donors) was stained before dilution and subjected to WBCs count.
5. Diluted cryopreserved blood from single donor was thawed at 1, 10 and 25 days after storage, smeared, stained and was subjected to differential count.
6. Diluted cryopreserved blood from multiple donors was thawed at 1, 10, 25, 45 and 75 days after storage, smeared, stained and was subjected to differential count.

2.3.4. Cryopreservation of human whole blood (single donor)

Fresh human whole blood was collected with a sterile pyrogen free syringe and was transferred to a depyrogenised, sterile, Erlenmeyer flask containing EDTA (2mg/ml of blood). This blood was diluted by adding equal volume (1:1 ratio) of 10% DMSO in phosphate buffer (pH 6.8) with gentle shaking to a total volume of 60ml. After dilution of the blood, 10ml each was transferred in to a 15ml sterile, pyrogen free cryotubes. The cryotubes were then immediately snapfreezed using liquid nitrogen (by dipping the tubes into the cylinder for 1 minute) and stored at -80°C. The freezed blood was subjected to evaluate the cytokine release at 1, 10 and 25 days.

2.3.5. Cryopreservation of pooled human whole blood (multiple donors)

Blood collected from five healthy donors separately in sterile pyrogen free tubes containing EDTA (2mg/ml of blood). Each donor's blood was pooled slowly to a depyrogenised Erlenmeyer flask. The pooled blood was diluted by adding equal volume (1:1 ratio) of 10% DMSO in phosphate buffer (pH 6.8) with total volume of 100ml. 10 ml of the pooled diluted blood was transferred into a 15ml sterile pyrogen free cryotubes. The cryotubes were then snapfreezed using liquid nitrogen by dipping the tubes for 1 minute and then stored at -80°C. The freezed blood was used for evaluating the cytokine release at 1, 10, 25, 45 and 75 days.

2.3.6. Cytokine (IL -1β) induction by LPS in cryopreserved blood

Single and multiple donors (pooled blood) cryopreserved blood was thawed at 37°C and was used within 15 minutes. The single donor (3 different donors individually) cryopreserved blood was evaluated for cytokine induction at 1, 10 and 25 days using LPS. Similarly pooled cryopreserved blood (multiple donors) was evaluated for cytokine induction at 1, 10, 25, 45 and 75 days using LPS. The experimental design is mentioned in the Table 2.1.

Table 2.1: Cytokine (IL-1β) induction by LPS using cryopreserved blood

Components	Control	LPS	
Blood (µl)	240	240	240
RPMI 1640 (µl)	960	959.5	955
Endotoxin units (µl)	-	0.5	5

The total reaction volume was 1200µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic conditions. There were 24 numbers of tubes each for control, 0.5 EU and 5 EU of LPS. All the reaction tubes (72 tubes) with reaction mixtures were incubated in carbon dioxide incubator set with 5% CO_2 at

37°C and increased humidity. Three reaction tubes each were collected at the end of every one hour upto 24 hours. At the end of each hour all the three reaction tubes were taken out and centrifuged at 500g for 2 minutes at 4°C. The supernatant was collected in a tube and immediately stored at -21°C and was used to detect the IL1β using ELISA.

2.3.7. Cytokine (IL- 1β) induction by LPS in fresh pooled blood

Fresh human whole blood was collected (1ml) from healthy from 5 donors. It was then pooled and used for the detection of cytokine induction using LPS. The experimental design is mentioned in the Table 2.2.

Table 2.2: Cytokine (IL-1β) induction by LPS in fresh pooled blood

Components	Control	LPS	
Blood (µl)	150	150	150
RPMI 1640 (µl)	250	250	250
Endotoxin units (µl)	-	0.5	5
Saline (µl)	850	850	845

The total reaction volume was 1250µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic condition. There were 8 numbers of tubes each for control, 0.5EU and 5EU LPS. All the reaction tubes (24 tubes) with reaction mixtures were incubated in carbon dioxide incubator set with 5% CO_2 at 37°C and increased humidity. Three reaction tubes each were collected at the end of every one hour upto 8 hours. At the end of each hour all the three reaction tubes were taken out and centrifuged at 500g for 2 minutes at 4°C. The supernatant was collected in a tube and immediately stored at -21°C and was used to detect the IL-1β using ELISA.

2.3.8. Coating of antibody on ELISA plate

The antibody coated ELISA plate was prepared by in-house method. Briefly, an appropriate concentration of antibody was coated using 50mM carbonate-bicarbonate buffer of pH 9.6. 50µl of diluted antibody added per well of ELISA plate. It was then tightly sealed and incubated for overnight at 4°C and this plate was used for the detection of IL-1β.

2.3.9. Enzyme Linked Immunosorbant Assay (ELISA) to measure IL-1β release

The supernatant solution collected from the reaction mixture with cryopreserved blood (2.3.6) and fresh pooled blood (2.3.7) were thawed at room temperature and was used as antigen for ELISA. Antibody pre-coated plates (SCTIMST developed) were equilibrated to room temperature, washed with deionized water (two times) before performing ELISA. The washed plates were blocked by adding 1% BSA and incubated for 1 hour. The plates were washed two times using PBS (200µl/well) with gentle shaking for 12s. The above supernatant solution was diluted with 1% BSA (1:5). 50µl of this solution was added per well and incubated for 2 hours at room temperature. After incubation, plates were washed for twice using PBS with a gentle shaking. Diluted (1:200) labeled antibodies prepared by in-house method were added 50µl/ well and incubated again for 2 hours in dark. The plates were washed twice after incubation using PBS with a gentle shaking. TMB substrate 50µl/well was added and incubated for 30 minutes in dark. After incubation the reactions were terminated by adding 50µl/well of 1M H_2SO_4 and incubated for 10 minutes in dark. Plates were read at 450nm with the corrective filter at 620nm using ELISA reader.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Results

3.1.1. Analysis of haematological parameters in blood collected from single donor

The blood collected from a single donor for cryopreservation was subjected to analyze the haematological parameters such as WBC, haemoglobin, RBC and platelet count using an automatic hematology analyzer (SYSMEX K4500).

Table 3.1: Analysis of haematological parameters in blood collected from single donor

Sample	Platelet count ($x10^8$/ml)	WBC count ($x10^6$/ml)	RBC count ($x10^9$/ml)	Hemoglobin (g/dl)
Individual-A	2.32	6.3	5.7	16.5
Individual-B	2.52	5.9	5.0	15.0
Individual-C	2.76	6.2	4.64	14.1

3.1.2. Analysis of haematological parameters in blood collected from multiple donors

The blood collected from multiple donors (individual and pooled blood samples) for cryopreservation was subjected to analyze the haematological parameters such as WBC, haemoglobin, RBC and platelet count using an automatic hematology analyzer (SYSMEX K4500).

Table 3.2: Analysis of haematological parameters in blood collected from multiple donors

Sample	Platelet count ($x10^8$/ml)	WBC count ($x10^6$/ml)	RBC count ($x10^9$/ml)	Hemoglobin (g/dl)
Donor A	1.97	4.2	5.22	15.6
Donor B	1.84	7.3	5.36	16.1
Donor C	2.82	4.7	4.93	14.3
Donor D	2.17	8.0	5.9	16.6
Donor E	2.60	7.0	4.68	13.2
Pooled blood (donors A to E)	1.92	5.9	5.11	15.9

3.1.3. Evaluation of differential count of blood

Differential count was done with Leishman's stain in the following conditions (Figure 3. 16- 3.30).

3.1.3.1. Differential count of fresh blood and blood stored at 4 °C, -21 °C and -80 °C

Table 3.3 shows the differential counts of fresh blood, blood stored at 4°C, -21°C and -80°C. It was observed that the morphology of the WBCs was slightly changed and RBCs was totally lysed at -21°C and -80° C.

Table 3.3: Differential count of the fresh blood, blood stored at 4 ° C, -21 ° C and -80° C

WBCs	Fresh blood	4° C	-21° C	-80° C
Lymphocytes	37	44	40	39
Neutrophils	56	49	55	59
Eosinophils	5	5	3	0
Monocytes	2	2	2	2

3.1.3.2. Differential count of fresh human whole blood diluted with 10% DMSO in phosphate buffer pH 6.8 (1:1 ratio)

Table 3.4 shows the differential count of fresh human whole blood diluted with 10% DMSO in phosphate buffer pH 6.8 (1:1)

Table 3.4: Differential count of the fresh human whole blood diluted with 10% DMSO

WBCs	Fresh human whole blood diluted with 10% DMSO
Lymphocytes	48
Neutrophils	50
Eosinophils	1
Monocytes	1

3.1.3.3. Differential count of fresh and cryopreserved blood

Tables 3.5, 3.6, and 3.7 show the differential count of fresh and cryopreserved blood (1, 10 and 25 days after storage) collected from a single donor. There were three individual donors namely Individual A, Individual B and Individual C. The RBCs were completely lysed after storage at -80° C.

Individual-A

Table 3.5: Differential count of Individual- A

WBC	Fresh sample	1day stored		10 days stored		25 days stored	
		1 set	2 set	1 set	2 set	1 set	2 set
Lymphocytes	35	43	38	38	36	42	56
Neutrophils	59	53	61	58	60	56	42
Eosinophils	3	0	0	1	1	0	0
Monocytes	2	4	1	3	2	2	3

Individual-B

Table 3.6: Differential count of Individual- B

WBC	Fresh sample	1 day stored		10 days stored		25 days stored	
		1 set	2 set	1 set	2 set	1 set	2 set
Lymphocytes	36	47	38	34	35	39	38
Neutrophils	61	53	62	66	64	60	60
Eosinophils	2	0	0	0	0	0	0
Monocytes	1	0	0	0	1	1	2

Individual-C

Table 3.7: Differential count of Individual- C

WBC	Fresh sample	1 day old		10 days old		25 days old	
		1 set	2 set	1 set	2 set	1 set	2 set
Lymphocytes	45	57	51	40	42	53	50
Neutrophils	52	41	48	56	53	42	49
Eosinophils	1	0	0	1	0	0	0
Monocytes	1	2	1	3	5	5	1

3.1.3.4. Differential count of fresh human whole blood used for cryopreservation (pooled)

Table 3.8 shows the differential count of fresh human whole blood (multiple donors) used for pooled cryopreserved blood.

Table 3.8: Differential count of fresh human whole blood used for cryopreservation (Pooled)

WBC	Donor A	Donor B	Donor C	Donor D	Donor E
Lymphocytes	55	40	51	33	47
Neutrophils	40	51	45	58	49
Eosinophils	3	7	2	8	4
Monocytes	2	2	2	1	0

3.1.3.5. Differential count of pooled blood (five donors) stained before and after diluting with 10% DMSO (1:1 ratio)

Table 3.9 shows the differential count of fresh human whole blood collected and pooled from multiple donors (five), stained before and after diluting with 10% DMSO (1:1 ratio)

Table 3.9: Differential count of pooled blood (Multiple donors) before cryopreservation

WBC	Pooled fresh human whole blood without dilution	Pooled fresh human whole blood with dilution
Lymphocytes	40	47
Neutrophils	56	48
Eosinophils	3	1
Monocytes	1	4

3.1.3.6. Differential count of pooled cryopreserved blood

Table 3.10 shows the differential count of pooled cryopreserved blood at different time intervals i.e., 1st, 10th, 25th, 45th and 75th days after cryopreservation. The RBCs was completely lysed after storage at -80° C.

Table 3.10: Differential count of pooled cryopreserved blood

WBC	1 day old		10 days old		25 days old		45 days old		75 days old	
	1 set	2 set	1 set	2 set	1 set	2 set	1 set	2 set	1 set	2 set
Lymphocytes	39	38	51	39	52	40	48	44	47	51
Neutrophils	58	59	45	58	48	59	52	56	50	45
Eosinophils	0	0	0	0	0	0	0	0	0	0
Monocytes	3	3	4	3	0	1	0	0	3	4

3.1.4. Detection of IL-1β by ELISA

Lipopolysaccharide (LPS) induced pro-inflammatory cytokine IL-1β was measured from the cryopreserved blood of Individuals A, B, and C. The study was conducted upto 24 hours; to check at what time period the cells are getting activated and stimulated to release IL-1β. The IL-1β released after treatment with 0.5EU and 5EU of LPS was detected by sandwich ELISA using cryopreserved blood stored at 1, 10 and 25days.

3.1.4.1. Detection of IL-1β from cryopreserved blood (single donors)

3.1.4.1.1. *Individual A*

The results of the study are mentioned in the Figures 3.1, 3.2, 3.3 and found that the maximum release of IL-1β (5EU of LPS) was at 10h, 6h, between 9h and 10h, when used with cryopreserved blood after 1, 10 and 25 days respectively. When treated with 0.5 EU of LPS, the maximum release was found at 12h (I day), 10h (10 days) and 11h (25 days) after storage.

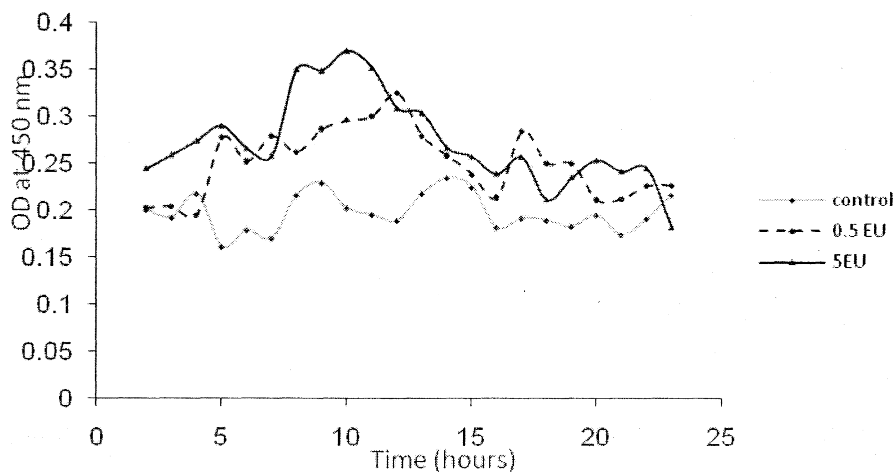


Figure 3.1: IL-1β detection from 1 day stored cryopreserved blood of Individual A.

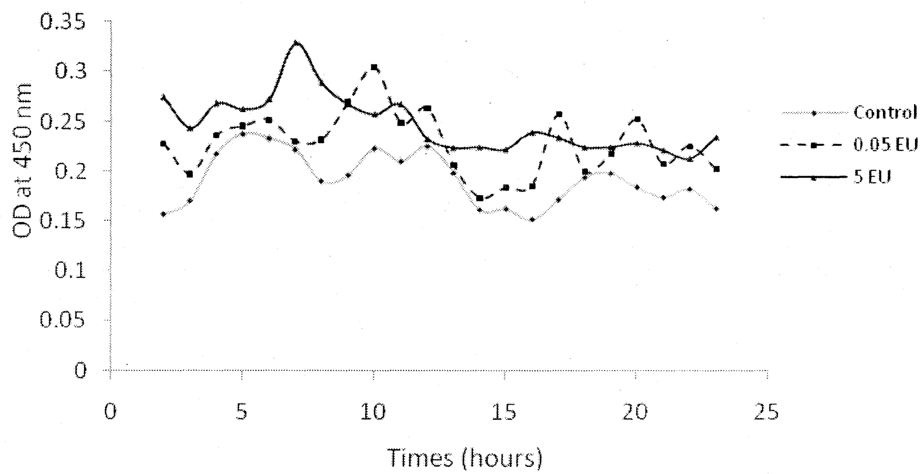


Figure 3.2: IL-1β detection from 10 days stored cryopreserved blood of Individual A.

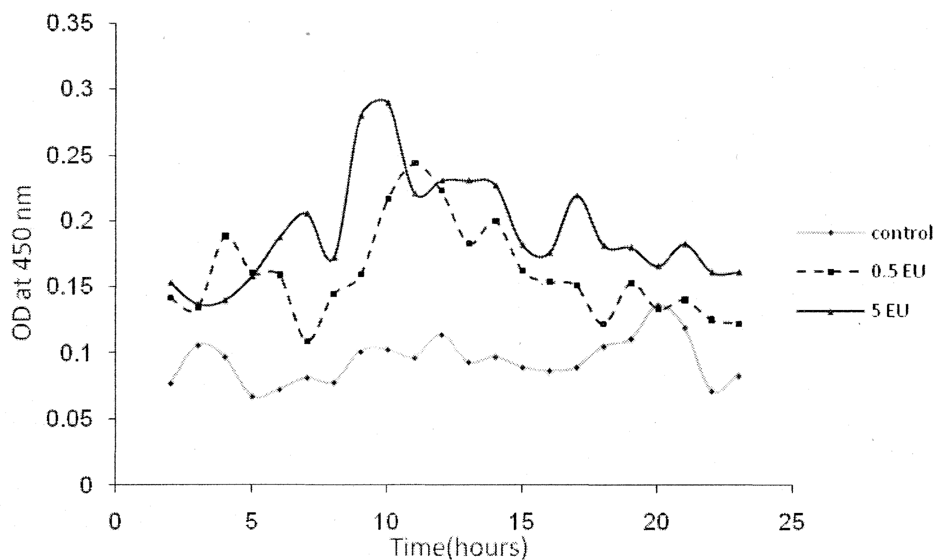


Figure 3.3: IL-1β detection from 25 days stored cryopreserved blood of Individual A

3.1.4.1.2. *Individual B*

Figures 3.4, 3.5 and 3.6 indicated that the maximum release of IL-1β (0.5EU) was found at 9h, 9h and 10h when used with cryopreserved blood stored at 1, 10 and 25 days. Similarly the maximum IL-1β release was observed at 5h (I day), 5h (10 days) and 6h (25 days after storage) when treated with 5EU of LPS.

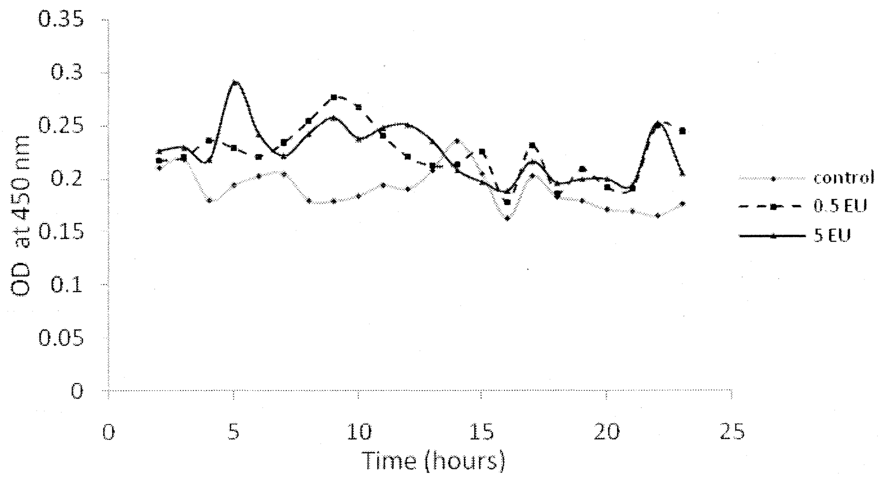


Figure 3.4: IL-1β detection from 1 day stored cryopreserved blood of Individual B

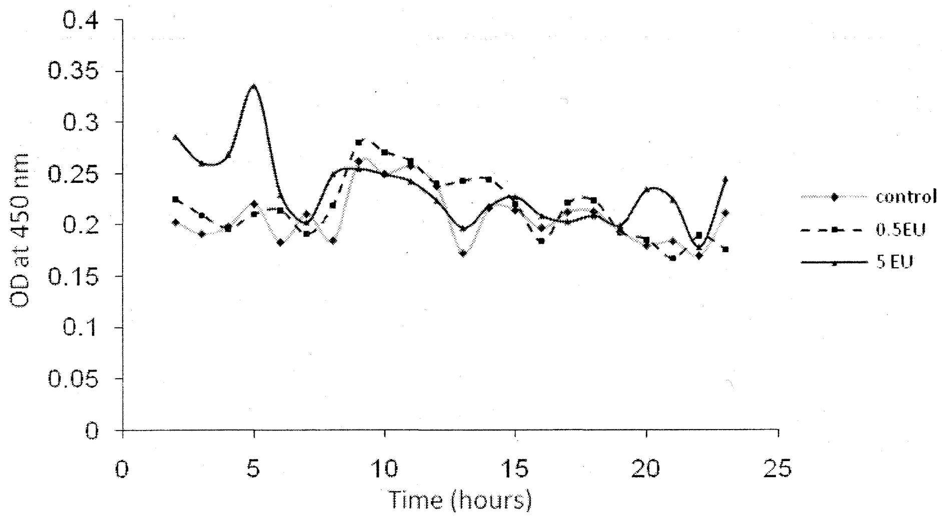


Figure 3.5: IL-1β detection from 10 days stored cryopreserved blood of Individual B

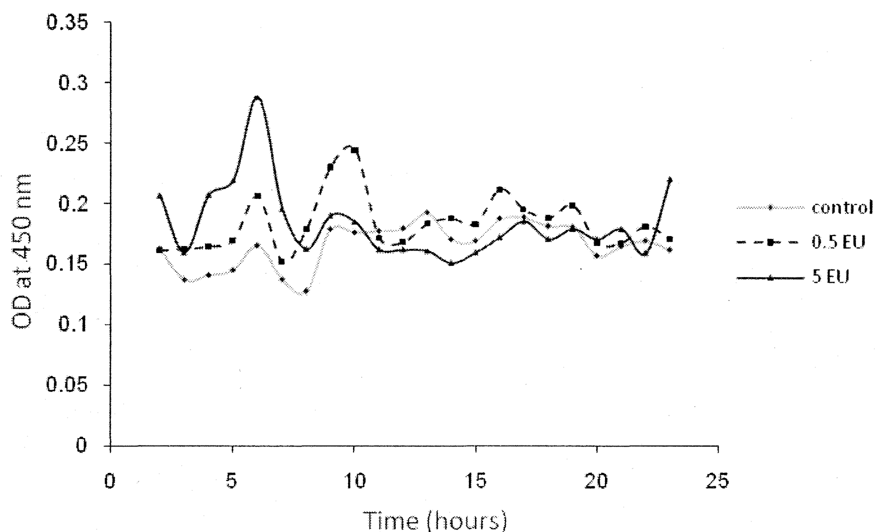


Figure 3.6: IL-1β detection from 25 days stored cryopreserved blood of Individual B

3.1.4.1.3. *Individual C*

Results of the Individual C shown that the maximum release of IL-1β (5EU LPS) was at 10h, 6h and 5h when used with cryopreserved blood stored at 1, 10 and 25 days, where as the maximum IL-1β release was observed at 17h (1 and 10 days stored) and 9h (25 days after storage) when treated with 0.5EU of LPS (Figures 3.7, 3.8 and 3.8).

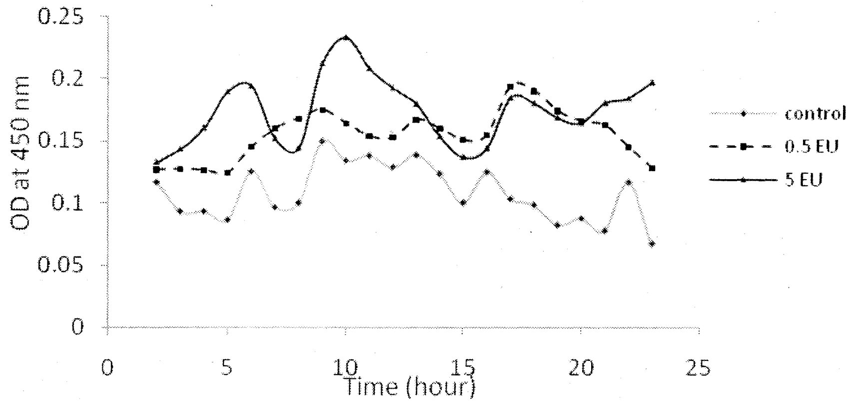


Figure 3.7: IL-1β detection from 1 day stored cryopreserved blood of Individual C

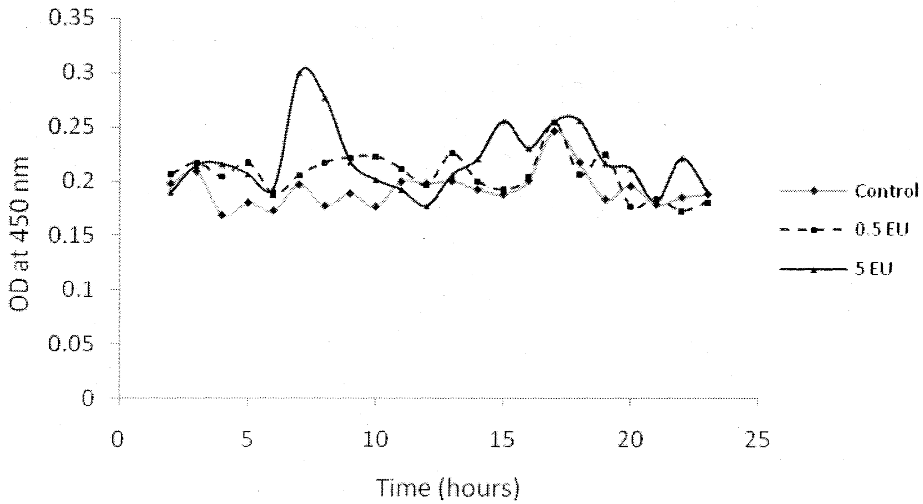


Figure 3.8: IL-1β detection from 10 days stored cryopreserved blood of Individual C

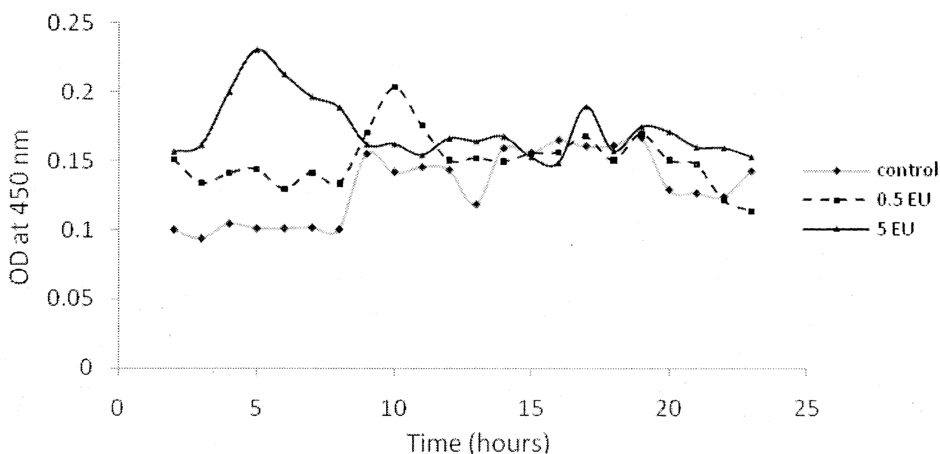


Figure 3.9: IL-1β detection from 25 days stored cryopreserved blood of Individual C

3.1.4.2. Detection of IL-1β from fresh pooled blood

Fresh pooled blood from 5 donors was induced with LPS for the detection of IL-1β. A maximum release of IL-1β was observed at 2h when treated with 5EU (LPS), where as the maximum release of IL-1β was observed at 5h when the concentration of LPS was 0.5EU (Figure 3.10).

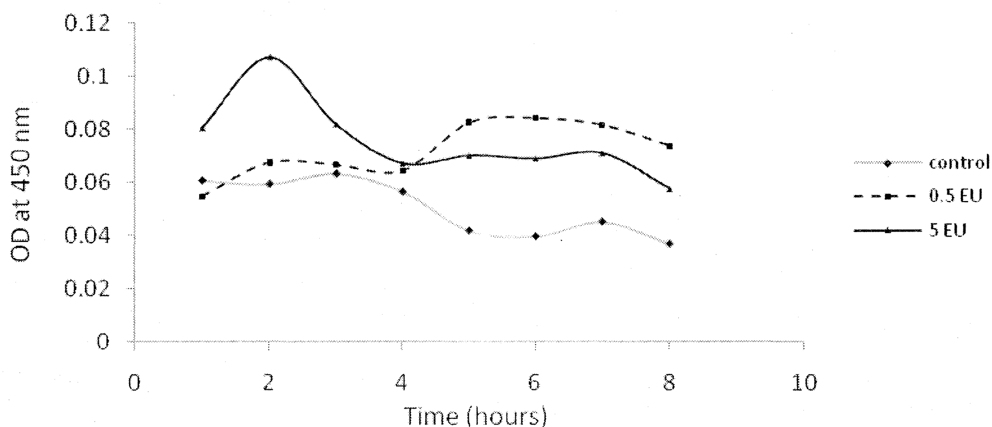


Figure 3.10: Detection of IL-1β from fresh pooled blood

3.1.4.3. Detection of IL-1β from pooled cryopreserved blood

3.1.4.3.1. *1 day stored pooled cryopreserved blood*

Figure 3.11 suggest that the maximum release of IL-1β was at 6h and 12h when treated with 5EU and 0.5EU of LPS respectively using pooled cryopreserved blood at 1st day.

3.1.4.3.2. *10 days stored pooled cryopreserved blood*

When cryopreserved pooled blood at 10th day was used to detect the IL-1β release, the maximum peak was observed at 6h (5EU LPS) and 9h (0.5EU) (Figure 3.12).

3.1.4.3.3. *25 days stored pooled cryopreserved blood*

The maximum IL-1β level was obtained at 5h and 10h when treated with 5 and 0.5 EU LPS using cryopreserved pooled blood at 25th day (Figure 3.13).

3.1.4.3.4. *45 days stored pooled cryopreserved blood*

The 45th day pooled cryopreserved blood when treated with 0.5 and 5EU of LPS induced a maximum level of IL-1β at 7h and 12h respectively as shown in the Figure 3.14.

3.1.4.3.5. *75 days stored pooled cryopreserved blood*

Figure 3.15 showed that, when treated with 5 and 0.5EU of LPS with 75th day pooled cryopreserved blood has a maximum release of IL-1β at 9h and 15h of reaction.

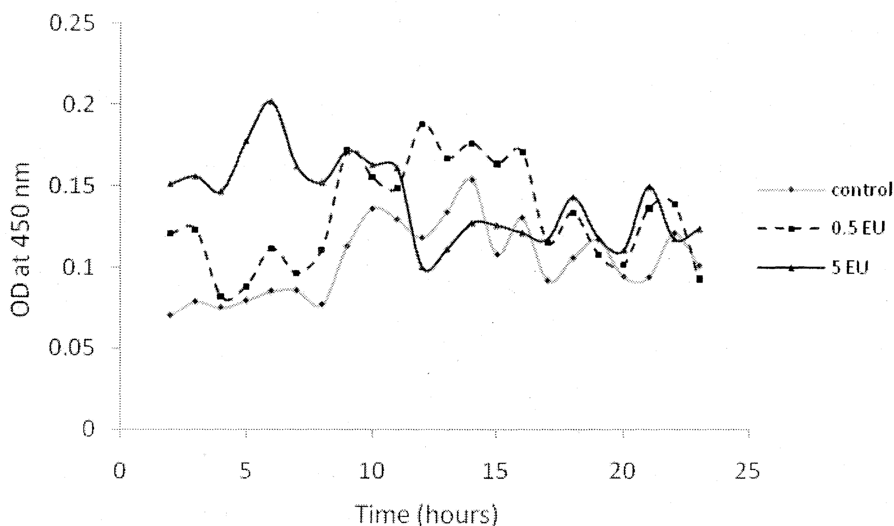


Figure 3.11: IL-1β detection from 1 day stored pooled cryopreserved blood

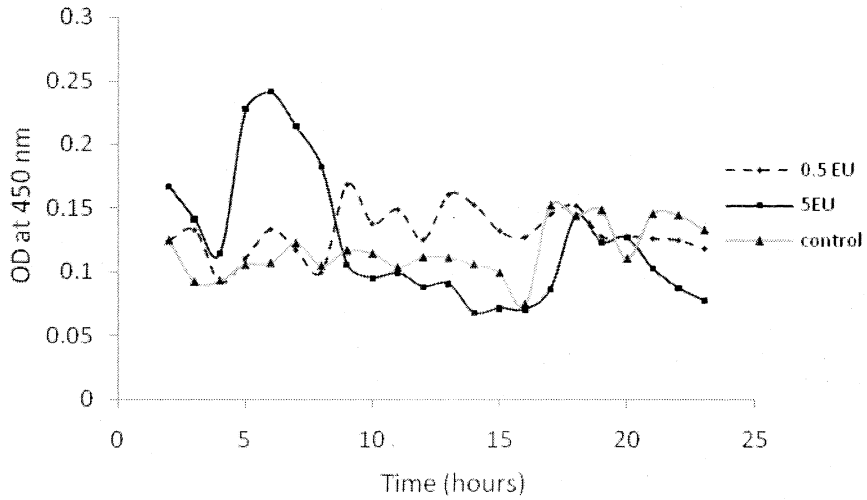


Figure 3.12: IL-1β detection from 10 days stored pooled cryopreserved blood

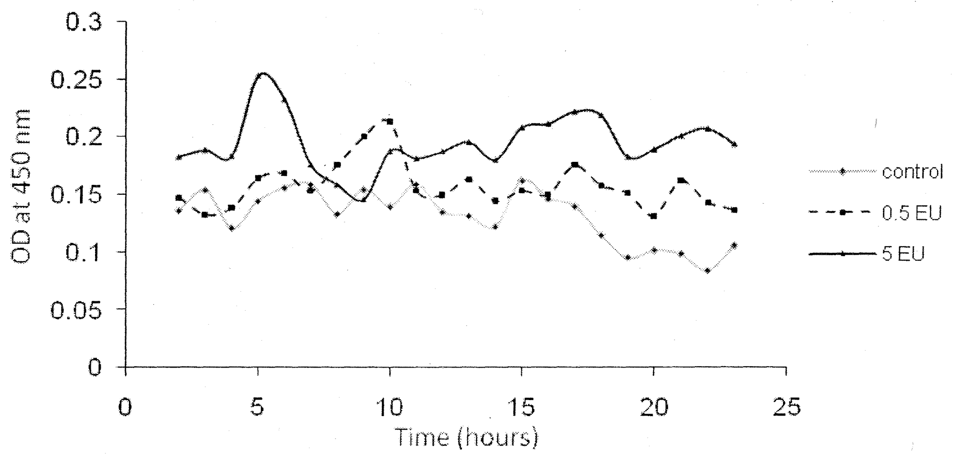


Figure 3.13: IL-1β detection from 25 days stored pooled cryopreserved blood.

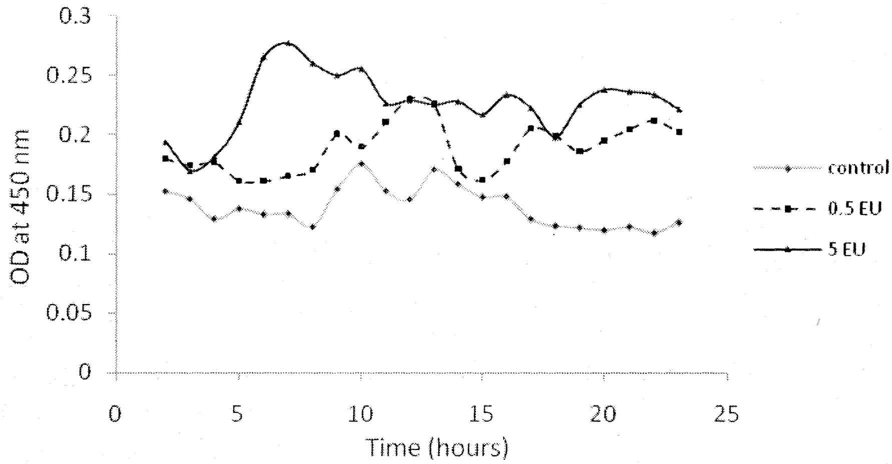


Figure 3.14: IL-1β detection from 45 days stored pooled cryopreserved blood

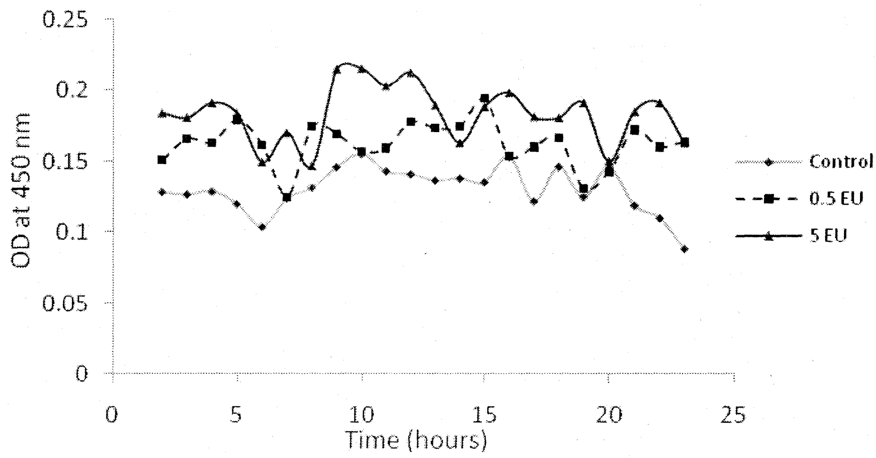
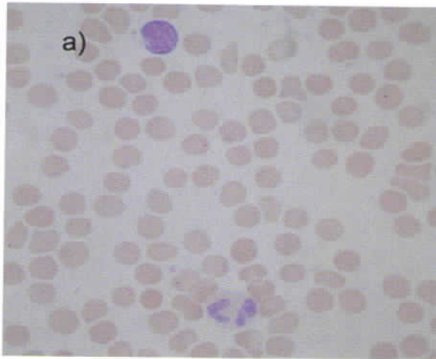


Figure 3.15: IL-1β detection from 75 days stored pooled cryopreserved blood

Leishman's Stained blood smears



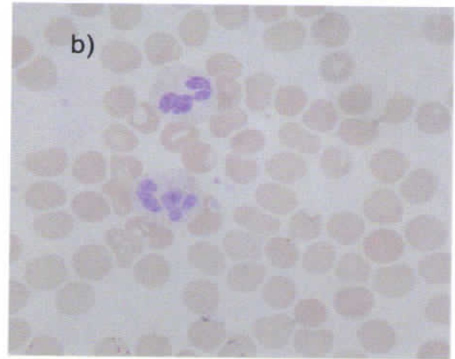
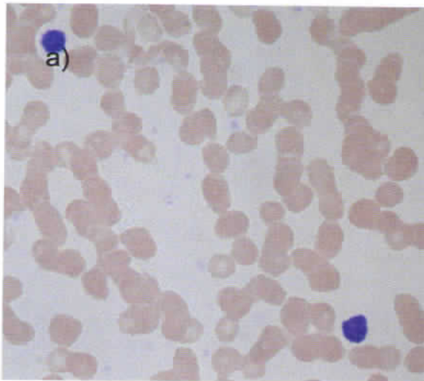


Figure 3.16 Photomicrographs of Leishman's stained fresh human whole blood (a &b)



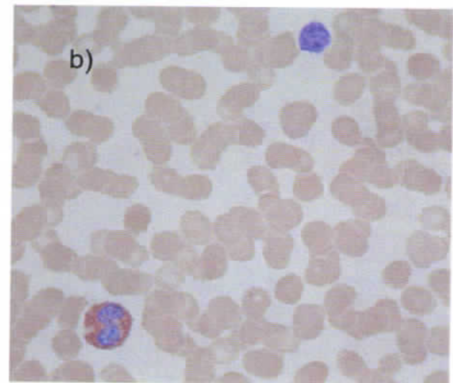


Figure 3.17 Photomicrographs of Leishman's stained blood stored at 4 degree celcius (a & b)

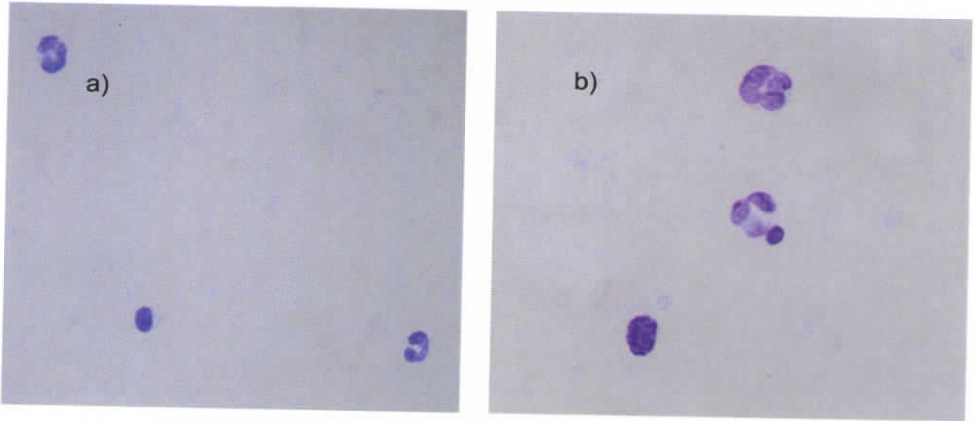


Figure 3.18 Photomicrographs of Leishman's stained blood stored at -21 degree celcius (a & b)

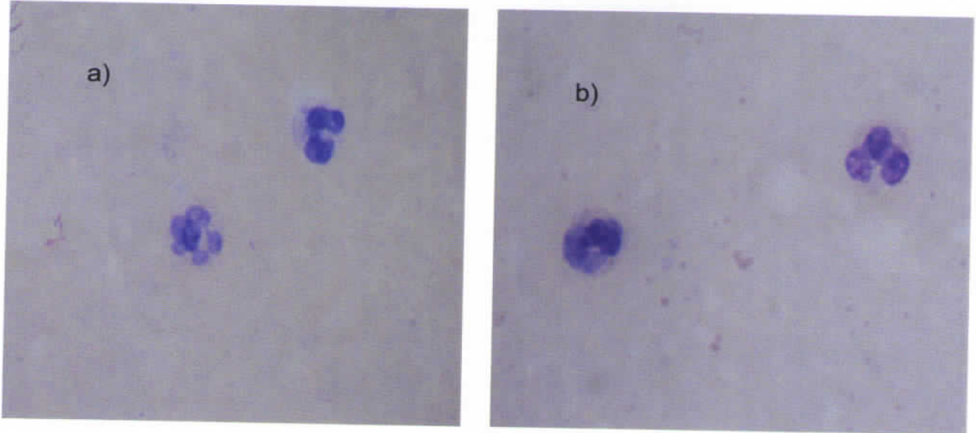
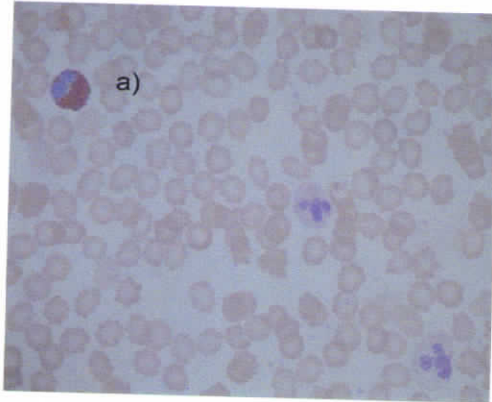


Figure 3.19 Photomicrographs of Leishman's stained blood stored at -80 degree celcius (a & b)



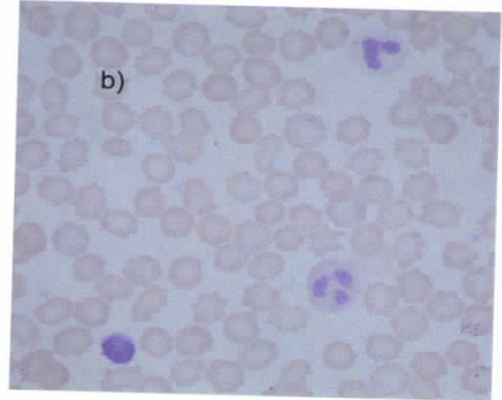
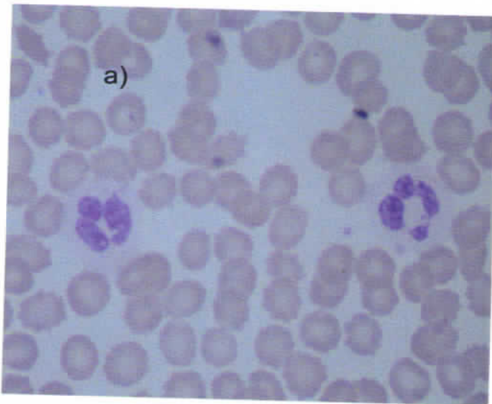


Figure 3.20 Photomicrographs of Leishman's stained fresh human blood diluted with 10% DMSO (a & b)



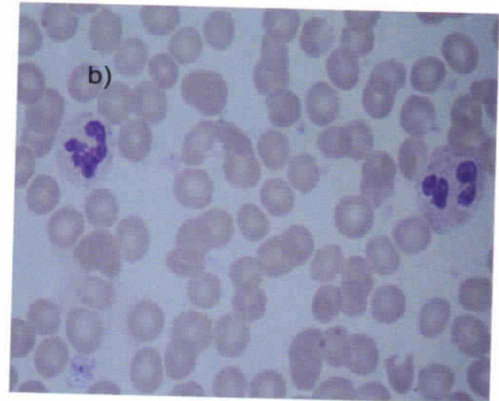
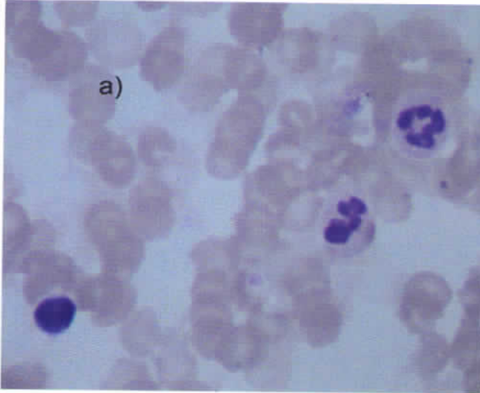


Figure 3.21 Photomicrographs of Leishman's stained fresh pooled blood from five donors before diluting with 10% DMSO (a & b)



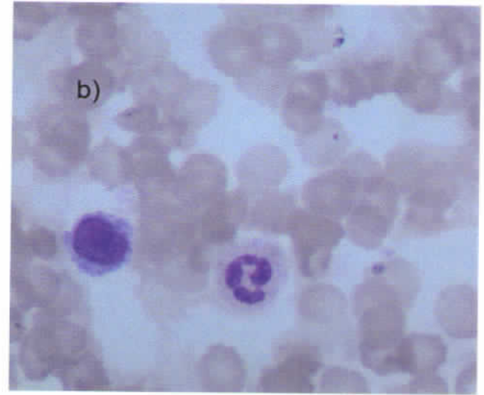
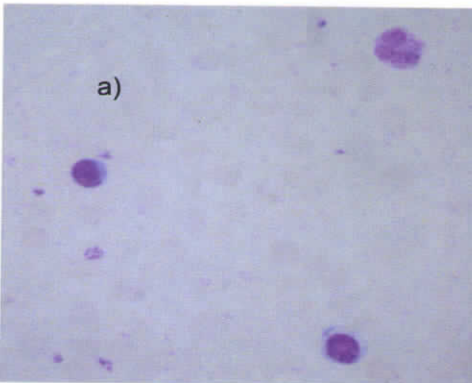


Figure 3.22 Photomicrographs of Leishman's stained fresh pooled blood from five donors after diluting with 10% DMSO (a & b)



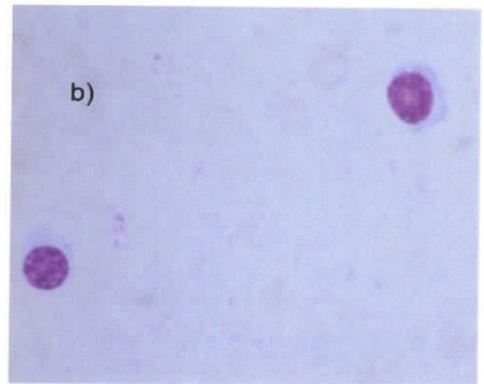
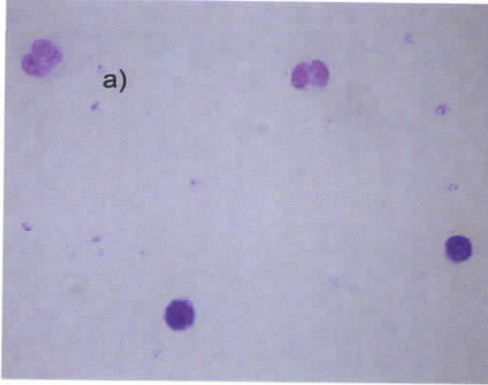


Figure 3.23 Photomicrographs of Leishman's stained 1 day stored single donor cryopreserved blood (a & b)



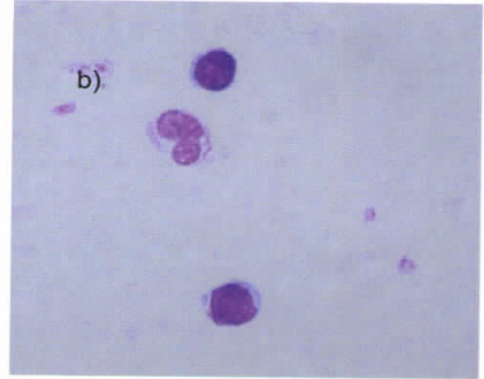
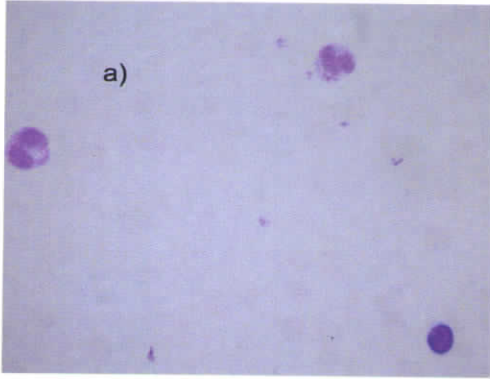


Figure 3.24 Photomicrographs of Leishman's stained 10 days stored single donor cryopreserved blood (a & b)



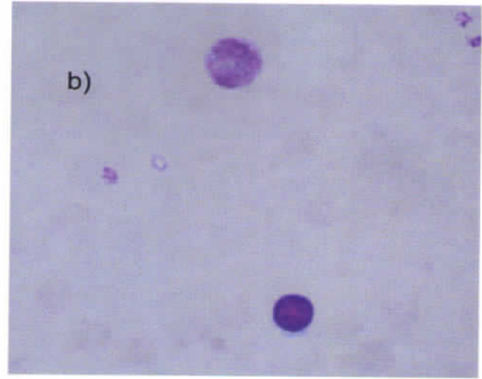


Figure 3. 25 Photomicrographs of Leishman's stained25 days stored single donor cryopreserved blood (a & b)

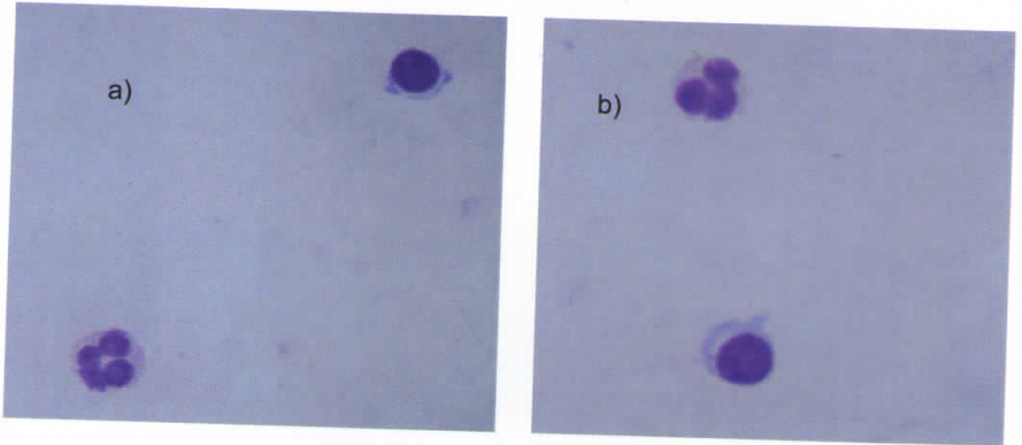


Figure 3.26 Photomicrographs of Leishman's stained 1 day stored pooled cryopreserved blood (a & b)

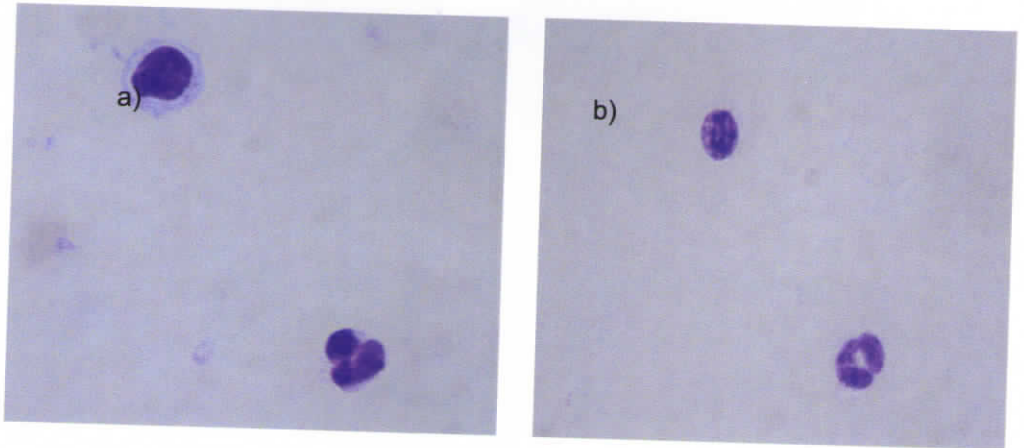


Figure 3.27 Photomicrograph of Leishman's stained 10 days stored pooled cryopreserved blood (a & b)

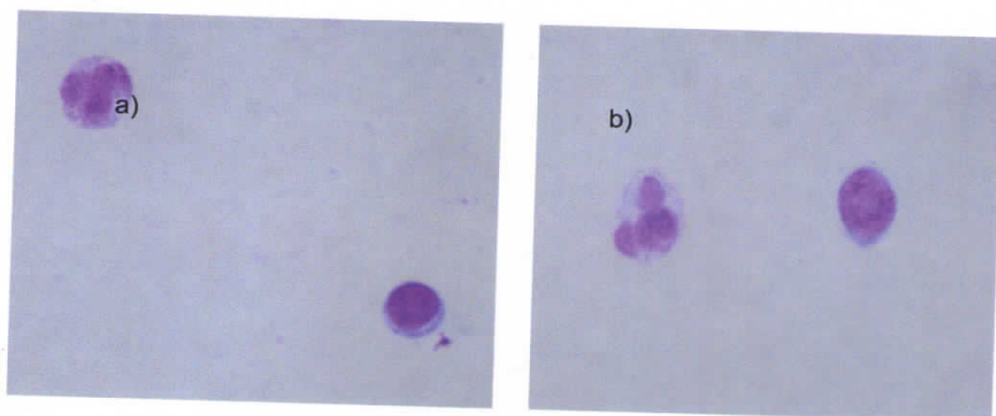


Figure 3.28 Photomicrographs of Leishman's stained 25 days stored pooled cryopreserved blood (a & b)

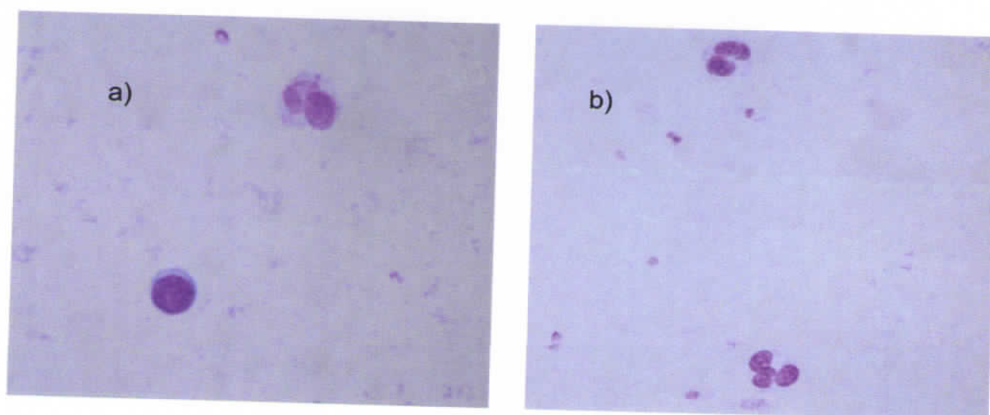
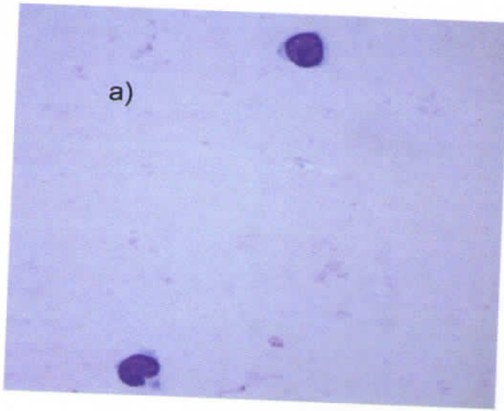


Figure 3.29 Photomicrographs of Leishman's stained 45 days stored pooled cryopreserved blood (a & b)



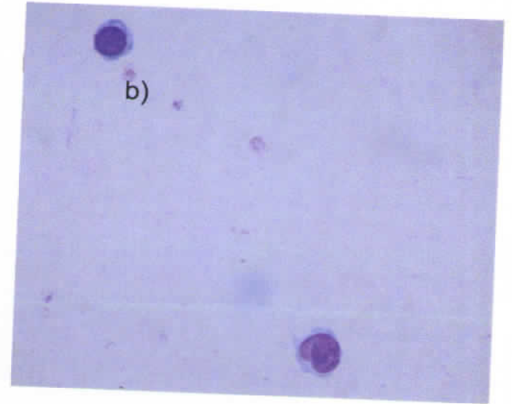


Figure 3.30 Photomicrographs of Leishman's stained 75 days stored pooled cryopreserved blood (a & b)

3.2. Discussion

In the present study an effort was made to evaluate IL-1β as a marker for pyrogenicity induced by lipopolysaccharide (LPS) with cryopreserved blood from single donor or pooled blood from multiple donors. This evaluation was done using the *in vitro* ELISA method developed by SCTIMST, Thiruvananthapuram.

Fresh human whole blood from different donors was initially evaluated for haematological parameters such as WBC, haemoglobin, RBC and platelet count. This was done to check the abnormality or alterations in haematological parameters due to any unexpected pathological conditions before selection for cryopreservation. Human whole blood found suitable was subjected to cryopreservation over varying periods of time as detailed in methodology. Such cryopreserved blood was analysed for differential count using Leishman's staining method. The differential count was targeted because the assay system used here with IL-1β as candidate marker for pyrogenicity, is focused on lymphocytes and monocytes which release IL-1β on stimulation with LPS.

The cryopreservation was done by 1:1 dilution of blood with 10% DMSO in 30mM phosphate buffer (pH 6.8). The cryopreserved blood was subjected to differential count using Leishman's stain to observe the morphology and to check the differential count, since the alternations in number of cells and structure may affect the release of pro-inflammatory cytokines. The result of the haematological analysis suggests that the morphology (leucocytes) and differential counts were similar to that of the fresh blood.

LPS was used to induce pro-inflammatory cytokines particularly IL-1β from fresh- pooled, cryopreserved single and multiple donor (pooled) blood. They

were treated with 5EU and 0.5EU of LPS to induce IL-1β release. Control (without LPS) was used in the study to check whether cryopreservation procedure created any alternations in the level of IL-1β. It was documented that the maximum release of IL-1β was observed between 2 to 4h in fresh blood when treated with 5EU of LPS (unpublished data). Similarly the maximum release of IL-1β was observed at 2h and 5h after inducing with 5EU and 0.5EU of LPS in fresh pooled blood (Figure 3.10). The cryopreserved blood after 1st, 10th and 25th day from single donor (3 individuals) was studied for IL-1β release and it was found that (Figure 3.1-3.9) a maximum release of IL-1β was observed between 6h to 10h when treated with 5EU of LPS, whereas the time period was delayed between 9h to 17h when treated with 0.5EU of LPS.

Pooled cryopreserved blood at 1, 10, 25, 45 and 75 days was studied for IL-1β release. In case of pooled cryopreserved blood after 1 and 10 days of storage, the maximum level of IL-1β release was found between 5h to 7h, when treated with 5EU of LPS. IL-1β release was delayed to 9h to 12h when the concentration of LPS was at 0.5EU. In 25th day pooled cryopreserved blood, IL-1β release was at 5h and 10h for 5EU and 0.5EU LPS respectively. The maximum level of IL-1β release was obtained at 7h at 5EU and 12 h at 0.5 EU of LPS in case of 45 days stored cryopreserved blood. After 75 days storage of pooled cryopreserved blood The IL-1β release was maximum at 9h and 15h when treated with 5 EU and 0.5EU of LPS (Figures 3.11-3.15).

In both the studies (cryopreserved blood from single and multiple donors) after 15h of incubation, the IL-1β release was inconsistent in control and treated (5EU and 0.5EU of LPS) groups. The variation seen in the IL-1β detection may be due to the higher sensitivity of sandwich ELISA which may create minor technical difficulties. It was observed that IL-1β level was fluctuating in control sample taken from a particular donor (Individual B). The reason for the inconsistency

may be due to individual variation in cytokine profile which may be caused by the action of the inhibitory factors affecting the IL-1β release. The above findings are supported by the report of Dinarello [Dinarello, 1996] that IL-1β mRNA level increase rapidly on a stimulant and decreases due to the synthesis of transcriptional repressor and/or decrease in mRNA half life. A concentration dependent IL-1β release was observed in both studies with cryopreserved (single and multiple donors) and fresh pooled blood. This finding was similar to the report of Hartung *et al.*, which explained that high concentrations of endotoxin require a shorter incubation period to release IL-1β from diluted blood and a longer incubation period for smaller concentrations of endotoxin (Hartung *et al.*, 1996). In the present study similar observations were noted that the IL-1β level reaches a peak much faster with 5EU, while longer time is needed to release IL-1β when treated with 0.5EU of LPS.

The results of the present study established that the maximum release of IL-1β level was observed at 2h when 5EU of LPS was treated with pooled fresh blood, which is similar to that of fresh blood (un published data). Similarly in cryopreserved blood from single and multiple donors (pooled), the initiation of IL-1β release was delayed to 5h as reported earlier by Daneshian [Daneshian *et al.*, 2009]. It was also found that there was a concentration dependant IL-1β release at 5h when treated with 0.5 and 5EU of LPS. Hasiwa *et al.,* 2007 reported that cryopreserved blood stimulated with various concentrations of LPS in comparison to freshly taken blood showed a much higher cytokine release. Similar finding was observed in the present study that the IL-1β release in cryopreserved blood was better, even on stimulation with low concentration (0.5EU) of LPS. The cryopreserved blood system can also be used to check materials that have been made immunologically inert, depyrogenated or subjected to cleaning for its effectiveness. The large inter-individual variation in case of the cryopreserved blood is reduced by pooling blood from at least five

donors before freezing [Mazzotti *et.al,* 2007]. The cryopreserved blood is more advantageous with respect to WBC isolation and culture because it does not reflect the *in vivo* system. The interactions between different cell types as *in vivo* cannot take place because plasma components often play an important role in immune recognition [Schindler *et al.*, 2004] which will be absent in the *in vitro* WBC culture system.

The adoption of the new pooled cryopreserved blood test system has got more advantageous as it ensures ready availability of standardized blood that is non-allergic and free of pathogenic infections. It was also seen in the cryopreservation procedure, that the cells are highly preserved and are able to release the proinflammatory cytokine IL-1β even with small stimulation. This is dependent on the differential count which is taken care of when using pooled cryopreserved blood.

CHAPTER 4
SUMMARY AND CONCLUSION

Pyrogens are chemically heterogenous group of fever inducing substances derived from Gram negative, Gram positive bacteria, viruses and fungi. Pyrogenic contamination is a major safety risk to various biological and medical products. Proinflammatory cytokines particularly IL-1β plays a major role in the onset of fever and is a marker for pyrogenicity. IL-1β binds to receptors on the blood side of circum ventricular organs of the brain and initiates the expression of enzyme cyclooxygenase-2, which converts arachidonic acid to prostaglandins (PGE2). This process leads to an increase in temperature.

The pharmacopoeia lists two test systems for pyrogenicity: they are *in vivo* rabbit pyrogen test and LAL assay. Rabbit pyrogen test measures the fever reactions following injection of the sample, where as LAL assay measures the coagulation of the lysate prepared from the blood of horse shoe crab specifically initiated by endotoxin, cell wall components from Gram negative bacteria. However, these assays have shortcomings as they are not quantitative, use experimental animals and are species specific.

The first pyrogen assay based on human whole blood was developed by Hartung and Wendel. It involves two parts, incubation of the sample with human whole blood followed by an enzyme immunoassay for the measurement of IL-1β. Absence of continuous supply of fresh whole blood from non-allergic donors was reported to be a drawback of human whole blood assay.

The present study mainly focused on the evaluation of haematological parameters of fresh-pooled, cryopreserved single donor and pooled blood from

multiple donors. The morphology and differential count of cells were checked for normality. After cryopreservation of blood, it was found that the pro-inflammatory cytokine IL-1β was released after induction with LPS (0.5 and 5EU) better than that of fresh blood. Cryopreserved blood was better at release of IL-1β on stimulation with LPS when compared with fresh blood. However the release of IL-1β in cryopreserved blood was delayed in comparison to fresh blood. This may be because of the delay in the activation and stimulation of the concerned cells. Hence in the present study it can be concluded that the pooled cryopreserved blood is a viable alternative to fresh human whole blood. This will definitely help to provide a continuous supply of non-allergic and pathogen free blood which will be readily available to the various segments of health care industries for evaluating the *in vitro* pyrogenicity using ELISA method.

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ANNEXURE

Preparation of Reagents:

Phosphate buffer

Disodium hydrogen phosphate dihydate- 2.853g

Potassium dihydrogen phosphate- 5.083g

The above constituents were ground in pestle and mortar, 0.79g was taken from this mixture and dissolved in deionized water, pH adjusted to 6.8.

30 mM phosphate buffer

a) 0.1M Sodium phosphate monobasic- 13.8g/L

b) 0.1M Sodium phosphate dibasic- 26.8g/L

For pH 6.8 mix, 51 ml of sodium phosphate monobasic and 49 ml of sodium phosphate dibasic. 30 ml of 100mM phosphate buffer was made upto 100ml using deionised water to get 30mM phosphate buffer. The pH was adjusted using a sensitive pH meter, filtered and stored.

50 mM carbonate bicarbonate buffer

Sodium carbonate- 1.59 g

Sodium bicarbonate- 2.93 g

The above constituents were dissolved in 1 litre of deionized water and the pH was adjusted to 9.6. Filtered, sterilized using 45µm filter paper and stored at 4°C.The shelf life of this buffer is one month

10XPBS

Sodium chloride -40g

Potassium chloride -1g

Disodium hydrogen phosphate - 5.75g

Potassium dihydrogen phosphate - 1g

The constituents were dissolved in deionized water and made up to a final volume of 500ml. The pH was adjusted to 7.2. Filter sterilized using 45µm filter paper and stored at room temperature. 10X PBS were then diluted to 1X PBS which was used as working standard.

1%BSA

1g BSA was dissolved in 100 ml of 1XPBS.

1M H_2SO_4

55.6 ml of concentrated sulfuric acid was diluted in 1 litre of deionized water.