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The Use of Different Blood Sample Preparations in the Development of Biomarkers for the Environmental Monitoring of Domestic Farm Animals

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

National University of Ireland, Cork

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Declaration

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of PhD are the result of my work. The material has not been submitted for any other degree or qualification at University College Cork or elsewhere.

Signed: _____

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Abstract

The application of biological effect monitoring for the detection of environmental chemical exposure in domestic animals is still in its infancy. This study investigated blood sample preparations *in vitro* for their use in biological effect monitoring.

When peripheral blood mononuclear cells (PBMCs), isolated following the collection of multiple blood samples from sheep in the field, were cryopreserved and subsequently cultured for 24 hours a reduction in cell viability (below 80%) was attributed to delays in the processing following collection. Alternative blood sample preparations using rat and sheep blood demonstrated that 3 to 5 hour incubations can be undertaken without significant alterations in the viability of the lymphocytes; however, a substantial (35%) reduction in viability was observed after 24 hours in frozen blood. Detectable levels of early and late apoptosis as well as increased levels of ROS were detectable in frozen sheep blood samples. The addition of ascorbic acid partly reversed this effect and reduced the loss in cell viability.

The response of the rat and sheep blood sample preparations to genotoxic compounds *ex vivo* showed that EMS caused comparable dose-dependent genotoxic effects in all sample preparations (fresh and frozen) as detected by the Comet assay. In contrast, the effects of $CdCl_2$ were dependent on the duration of exposure as well as the sample preparation.

The analysis of leukocyte subsets in frozen sheep blood showed no alterations in the percentages of T lymphocytes and B lymphocytes but led to a major decrease in the percentage of granulocytes compared to those in the fresh samples. The B lymphocytes were more sensitive to EMS and $CdCl_2$ than other leukocytes. The percentages of IFN-γ and IL-4 but not IL-6 positive cells were comparable between fresh and frozen sheep blood after 4 hour stimulation with phorbol 12-myrisate 13-acetate and ionomycin (PMA+I). These results show that frozen blood gives comparable responses to fresh blood samples in the toxicological and immune assays used.

Chapter 1

Introduction and Literature Review

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Environmental Exposures to Chemicals

During the last two centuries, industrialization has played a key role in the pace of society's progress. Until the mid-20th century, only the positive side of manufacturing industries was seen i.e. the economy of a country grew in parallel to its industrial capacity. However, people began to realize that many industrial facilities released hazardous substances in to the environment (Cohen, 1997).

Our lifestyle benefits from the use of manufactured chemicals found every day throughout the world. These chemicals help protect crops (pesticides), aid husbandry (veterinary pharmaceuticals), and are used to preserve, to colour and to improve the quality of materials, packages, textiles, food and beverages and many other products. However, while chemicals may improve the quality of our lives they may also be harmful to humans, domestic and wild animals and to the environment. The most common sources of environmental pollutants are industrial production facilities, power stations, motor vehicles, agriculture and domestic heating, which release environmental pollutants in gaseous, solid or liquid forms (Bearer, 1995). Chemicals may be emitted into the environment during production, transport or use, as well as after waste disposal. After their intended use, many chemical substances are discharged 'down the drain' into domestic sewage (Woltering, 1987). Depending on their physicochemical properties, they will be distributed throughout the environment. Chemicals can be transported over long distances, even to remote areas, via air, water, soil, sediments and even via living organisms (UNEP, 2006, He et al., 2007, ECHA, 2008).

All substances are toxic over a certain dose, as stated by Paracelsus: "*Dosis sola facit venenum*". Toxicity depends on the dose, duration of exposure and on the target organism. Some chemicals are lethal to living species even at very low concentrations and the effects can range from organ toxicity to cancer. Some chemicals are persistent in the environment as they are non-degradable and tend to accumulate in living organisms and spread into the different ecosystems and enter the food chain (Liu et al., 2008).

The health effects of environmental pollutants on animals can be organ specific or systemic and contaminants, such as heavy metals (mercury, cadmium and lead), industrial chemicals e.g. polychlorinated biphenyl (PCBs), persistent organic pollutants

e.g. polycyclic aromatic hydrocarbons (PAHs) and dioxins can cause immediate (acute) and/or long-term damage (e.g. through carcinogenic, mutagenic or teratogenic mechanisms) (US EPA, 2006).

An acute effect is generally caused by a high dose single chemical exposure (hours) through accidental or gross over-exposure. The adverse condition develops quickly (days) with obvious signs and symptoms., After the environmental contamination by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as a consequence of an industrial accident in a small manufacturing plant in Seveso in northern Italy in July, 1976, thousands of small domestic animals (mainly rabbits and poultry) died within a few weeks. Autopsies on dead animals showed various pathological signs, such as hepatic lesions and hemorrhages (Fanelli et al., 1980). However, cancer, for example, may take many years to become clinically apparent following an initial environmental exposure as part of a multistage process in carcinogenesis (Moolgavkar et al., 1999)

Sub-chronic effects occur from low-level exposure to environmental pollutants for prolonged periods and will often remain undetected until exposure has occurred for some time and unless early biochemical changes in target cells in the body are measured. Heavy metals have very long biological half-lives and are toxic at very low doses. Examples include kidney dysfunction in cows and horses after cadmium exposure (Canty, 2009) and lead neurotoxicity in pet animals (Neathery and Miller, 1975). Small rodents, such as the bank vole from an industrialized area, had both hepatic and renal injuries when the levels of tissue cadmium were <10 μg/g (Damek-Poprawa and Sawicka-Kapusta, 2004, Wlostowski et al., 2004).

Chronic effects, on the other hand, usually occur after lower exposures to chemicals repeated over months or years. The adverse condition may develop slowly over years with incremental damage and is often irreversible. Sheep from farms in the provinces of Naples and Caserta (southern Italy) had very high levels of TCDD present in their milk and these correlated with an increase in mortality, abortion and abnormal foetuses when monitored for over four years (Perucatti et al., 2006). Both cadmium and dioxins have been associated with cancer following chronic exposure (IARC, 1993, Bertazzi et al., 1998). Chronic lead exposure suppresses the nervous and reproductive systems and this has been well documented in humans, laboratory animals and wildlife (Wu et al., 2008).

Most chemical emissions may occur as mixtures of different substances and will not be well characterized, and health effects may be due to singular effects as well as interactions between the various components (Sexton and Hattis, 2007). Interaction effects occur when individual chemicals affect the toxicity of one another, either by synergism or antagonism (more or less than an additive effect) (Cassee et al., 1998, Spurgeon et al., 2010). For instance, the combined exposure to metals such as lead, cadmium and arsenic can lead to both additive and synergistic effects but antagonistic effects have also been described (Wang et al., 2008).

Cases of Environmental Contamination Implicated in Animal Health Problems in Ireland

When investigated thoroughly, some animal health problems are caused by poor farm management, biological causes (infections/disease), poor nutritional status of the soil on the farm and, in some cases, exposure to toxic chemicals whose existence can be traced to industrial emissions. There have been cases in Ireland including those in Askeaton Co. Limerick, Castlecomer Co. Kilkenny and the Silvermines area in Co. Tipperary, whereby the presence of an abandoned mine or factory and its emissions in the vicinity of domestic animals has been linked with a decrease in the health status of sheep and cows and resulted in costly investigations (EPA, 2001, Jorgensen et al., 2009, Li et al., 2011, Qin et al., 2011). It has proven difficult to identify whether emissions were contributing to the animals' health problems and which chemicals might have contributed since emissions generally contain a mixture of chemicals of different classes with varying concentrations and with different toxicological properties. The effects caused by environmental exposure to animals can be the result of either acute, sub-chronic or chronic exposure to low levels of compounds and may arise from additive or synergistic interactions. Environmental exposure is usually for a prolonged period at relatively low concentrations and generally won't cause acute clinical symptoms unless there is an accidental uncontrolled release. Agencies including the Environmental Protection Agency (EPA), the Department of Agriculture, Food and Rural Affairs and Teagasc have assessed the health of domestic animals in selected 'contaminated' farms using environmental and biological monitoring. The results obtained from these investigations did not establish a link between chemical exposures and animal health. While it is not known if the sensitivity of the methods used contributed to this outcome,

the establishment of new biomarkers for biological effect monitoring of domestic farm animals in the Irish countryside may be useful in the case of future incidents.

Askeaton

In the early to mid-1990s, animal health problems on two farms near Askeaton, Co. Limerick came to public attention. Both cases were characterised by ill-thrift, reduced milk production and above normal mortality of cows and loss of calves at birth. Initial investigations by Limerick County Council did not indicate the involvement of a common factor and a range of ailments were described. There were suggestions that nutritional factors leading to loss of immune function could be at the base of the problems. The EPA were then invited by the Department of Agriculture, Food and Rural Affairs to investigate whether the unusually high level of animal disease in the Askeaton area was linked to the possibility of the direct or indirect involvement of an environmental pollutant or pollutants from a nearby Aughinish Alumina plant which extracts alumina from bauxite.

In 2001, the EPA and associated agencies compiled a comprehensive ecotoxicological report (EPA, 2001) into human and animal health in the Askeaton area, entitled "Investigation of Animal Health Problems at Askeaton, County Limerick" published by the EPA, Mid-Western Health Board, Teagasc and the Department of Agriculture, Food and Rural Affairs. This report was based on inputs as follows:

- Veterinary Research Laboratory of the Department of Agriculture, Food and Rural Development (animal health)
- Teagasc (soils, herbage and related aspects)
- Mid-Western Health Board (human health)
- Environmental Protection Agency (environmental quality aspects)
- Subsidiary studies by Coillte on tree health and the levels of metals and other substances in vegetable produce.

The ecotoxicological study conducted by the EPA at Askeaton included:

- A retrospective epidemiological survey of the two most severely affected farms and of 25 other farms identified by their owners as affected by a high level of animal ill health
- A longitudinal study of animal health and production on four of the 25 self-identified problem farms (Toner, 2001)
- A contemporary investigation of animal health on the remaining 21 self-identified problem farms
- Studies of the immune function of animals from the two affected farms in Askeaton and the control farm at Abbotstown
- A survey of animal health in the Askeaton area and in other areas in Co. Limerick and adjacent counties
- A study of the effect of feeding soil from one of the most affected farms to laboratory rats
- An investigation of a postulated association between enzymatic anomalies in voles and environmental pollution in the Askeaton area
- Assessments of level of some potential contaminants blood and tissue of animals from Askeaton farms.

The ecotoxicological report (EPA, 2001) concluded that environmental pollution such as fluorine, aluminium, volatile organic compounds (VOCS), toxic substances in the diet, soil composition anomalies and herbage composition anomalies were not the cause of the health problems experienced in cattle on the two farms investigated in Askeaton. Based on historical information and past observations, it was proposed that on-farm infections, nutritional and management factors were involved. Although it was recognised that there was an unusually high incidence of animal disease on a small number of farms in the area, there was no evidence to suggest a trend affecting the wider Askeaton area as a whole (EPA, 2001).

Following the publication of the ecotoxicological report, a review was conducted by Professor Brian Alloway of Imperial College London on behalf of the Irish Farmers

Association (IFA). This review further clarified that there were aspects of pollution which remained unclear or needed further investigation (White, 2004).

After the publication of the ecotoxicological report, additional revelations came to light pertaining to animal health on a stud farm immediately adjacent to one of the study farms in the Askeaton area (Fogarty et al., 1998). A total of 11 horses had died and tissue from 8 of those that died in the period August 1995 to August 1996 were submitted to the Irish Equine Centre for detailed post mortem examination. Elevated levels of aluminium were detected in the tissue of all eight animals and aluminium was associated with the granulomatous lesions and enteritis which developed in the animals. No probable explanation of these lesions was detected. This was the first recorded case where a cluster of cases of equine granulomatous enteritis had been recorded anywhere in the world. This investigation highlights the need for a greater array of toxicological tests, including biological effect monitoring with highly sensitive and relevant biomarkers, at sites of environmental exposure.

Silvermines

Between 1999 and mid 2002 there were at least five recorded incidents of lead poisoning, each of which has resulted in the death of one or two bovines on farms in the Silvermines area, Co. Tipperary. Silvermines is a large historic abandoned/closed base-metal mine site where lead-zinc and barite ore were mined. Following the first incident, an inter-agency group, comprising several organizations, was established and given the task of overseeing a major investigation into the presence and influence of lead in the area (Department of Agriculture Food and Rural Development, 2000). As part of this investigation, Teagasc had responsibility for the investigation into soils, herbages, fodder and water. A comprehensive investigation was co-ordinated by the EPA and a report published in 2004 (EPA, 2004).

This investigation included monitoring of the health of humans and animals and the environmental and the toxicological data summarised below is from the EPA report which gives guideline values for the acceptable amount of lead in the environment (below which there are no hazardous effects) and provides information on the effects of metal contaminations on human health, animal health and the environment (EPA, 2004).

Environmental monitoring, which was undertaken in Silvermines, included:

■ Analysis of soils and herbage samples taken by Teagasc on farms where animal health problems had been identified

■ Sampling and analysis of surface water and stream sediments from farms and other areas of concern by the EPA and North Tipperary County Council (NTCC)

■ On-going physico-chemical and biological monitoring of the Kilmastulla and Yellow rivers

The metals detected in the soil of the farms in the vicinity included lead, cadmium, copper and arsenic. Based on the levels of lead detected in the blood samples of cattle, it was concluded that lead was the most likely hazard in this area. It was also concluded that cattle could consume a lethal dose of lead during a single day's grazing and this is consistent with the results from a floodplain study (Aslibekian and Moles, 1999). The expert group concluded that it was unlikely that arsenic, cadmium or copper contributed to the adverse health effects in the cattle.

Animal health monitoring in Silvermines was sub-divided into animal health and food safety. In relation to animal health, the EPA report concluded that the investigation of animal health incidents as they arise would the best way forward in monitoring of this area. However, the maximum levels for certain contaminants should be more stringently controlled in the future and the levels of lead, especially, should be monitored in meat, offal and milk (EPA, 2004).

Due to the lack of evidence linking the environmental lead exposure and the ill health of the animals, a possible alternative approach would be to measure a lead specific biomarker, aminolevulinic acid dehydratase (ALAD) in the blood (Martinez et al., 2013). This investigation suggests that the use of constant biological effect monitoring of the animals at sites of environmental exposure may be useful in establishing the detection the effects on health that problematic exposure to lead and other heavy metals could have on animal populations.

Castlecomer

Throughout the late 1990s and 2000s calf deaths on a farm near Castlecomer, Co. Kilkenny, came to the attention of the public and mortality was particularly high in the years 1998 to 2001 (approximately 70 calves were reported to have died in that period). As well as the high calf mortality rates, stunting in calves and growing animals and poor milk production in cows were also identified.

A farm was located near an industrial source of atmospheric emissions – a brick factory therefore a protocol (Protocol for the Investigative Approach to Serious Animal and Human Health Problems; EPA, 1997) was activated in early 2004 to allow for a coordinated investigation involving Department of Agriculture and Food and Rural Affairs, the EPA, Teagasc and the South Eastern Health Board (Health Service Executive from January 2005). The culmination of this investigation was a report, which was presented in two parts and comprises the results of the Department of Agriculture and Food Veterinary Laboratory Service investigations to date. Part 1 of the report covers the possible involvement of fluoride toxicity and Part 2 covers the general animal health investigations (Department of Agriculture and Food, 2006, EPA, 2006, Centre for Veterinary Epidemiology and Risk Analysis, 2009).

The focus of the environmental component of the investigations was looking at the possible presence of fluorides in brick-firing emissions, which are known to have caused environmental problems in other countries; cadmium toxicity was looked at to a lesser extent about the possibility of these contaminants, contributing to animal health problems on this farm. Characterisations of the emissions from the factory conducted by the EPA, did not indicate any other pollutants of significance which might have contributed to animal health problems (Department of Agriculture and Food, 2006, EPA, 2006, Centre for Veterinary Epidemiology and Risk Analysis, 2009).

The results of extensive environmental and animal investigations found no evidence to indicate that environmental pollution caused ill thrift and poor growth rates in animals on this farm. There was no concrete evidence that cadmium or fluoride were solely involved with ill health of the animals and the health problems were likely to have been

multifactorial in origin, even though these chemicals were used in abundance at the nearby brick factory. The Department of Agriculture and Fisheries and Food Veterinary Laboratory Service investigations, up to mid-2006, identified a number of common disease conditions which could account for much of the occurrence and severity of poor animal performance. The epidemiological studies carried out by the Centre for Veterinary Epidemiology and Risk Analysis (based in University College Dublin) also concluded that the problems were likely to be multi-factorial in nature – while at the same time finding no evidence to suggest involvement of fluoride or cadmium toxicity (Department of Agriculture and Food , 2006, EPA, 2006, Centre for Veterinary Epidemiology and Risk Analysis, 2009). Teagasc investigations concluded that while the soils in question were difficult to farm, the nutrient status of soil and herbage and the general farm practices were not radically different from that found on many farms in Ireland and could not explain the poor thrift that was experienced by the animals. The Health Service Executive investigations did not identify any increase in unusual patterns of human illness in the area. The unsatisfactory nature of environmental and biological monitoring based on certain compounds in biological samples or the surrounding flora is highlighted in studies like that in Castlecomer (Department of Agriculture and Food, 2006, EPA, 2006, Centre for Veterinary Epidemiology and Risk Analysis, 2009). Maybe the addition of biological effect monitoring, which could provide a deeper analysis of the biochemical and physiological effects on the animals, could have revealed if any or all of the chemicals involved caused the poor animal health on this farm (More, 2005).

Human versus Animal Biological Monitoring

Realistic assessment of health risks associated with exposures to chemicals in the diet and the environment depends on adequate knowledge and understanding of both the identity of exposures and their associated effects. Currently there is much more research performed on humans compared to domestic animals in the area of diet and its connection to the environment so the level of exposure is much easier to interpret in humans than animals. However, the concept that animals may serve as sentinels of environmental hazards that have implications for public health is not new. As far back as 1962, cases of lead poisoning in cattle and horses living in the vicinity of a smelter alerted the Minnesota State Health Department to conduct surveillance for lead

exposure in the local human populations (Hammond and Aronson, 1964). The primary goal of an animal sentinel system is to act as early warning for new hazards, as an indicator of potential human exposure to complex mixtures or in complex environments, before they might otherwise be detected through human epidemiology studies or toxicology studies in laboratory animals (Hornshaw et al., 1983). Biological markers measured in wild and domestic animals can directly contribute to the detection, quantification, and understanding of the significance of the exposure to chemicals in the environment and help to assess the potential for human exposure to environmental pollutants and to predict human health risks (Shugart, 2005). The advantages of using animals as sentinels is in part due to their relative freedom from concurrent exposures and the influences of cigarette smoking, alcohol, or occupational exposures that may obscure an effect of exposure to environmental hazards. Human exposure monitoring is well established in an occupational capacity but biological effect monitoring for domestic animals like sheep, cows and pigs is still in its infancy. Many authors agree that, among domestic farm animals, sheep and cows are the most suitable environmental bioindicators (Parada and Jaszczak, 1993, Rubes et al., 1997) In fact, they are very sensitive to many environmental pollutants and accumulate xenobiotics in their bodies (Garcia-Repetto et al., 1997). Mammals, including mink and river otters have revealed numerous human health hazards, such as the contamination of the Great Lakes–St. Lawrence basin of the United States and Canada with dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and dioxin (Fox, 2001). Unfortunately, a number of scientific issues need to be resolved in the application of animal data in risk assessment for it to be used with the same confidence as human occupational data. This is because there is a lack of evaluated biochemical tools and relevant toxicology information and pharmacokinetic data available for animals compared to humans.

General Approaches in the Investigation of Environmental Exposure to Chemicals

Large ruminants are generally regarded as the most important domestic livestock species in the world. In developed countries, their contributions are mainly commercial products such as meat and milk. In developing countries, they are a source of food, particularly protein for human diets and they provide income, employment, transport, draft power and organic fertilizer for crop production (Perry, 2005, Rushton, 2009).

Morbidity and mortality in domestic animals may be associated with chemical exposure. The first indications of environmental exposure are generally based on observations and normally followed up by the clinical investigation of the animals to give an initial indication of health status of each animal. Venous blood samples are collected for assessment of biological effects. As well as the standard haematology and clinical biochemistry analysis, the blood can also be used to test for the presence of any infection or disease in the animals. The presence of infectious agents should to be ruled out before the possibility of poisoning with a chemical substance is considered. Post-mortem gross- and histopathological investigations of deceased animals are routinely performed and these investigations can be expanded by sacrificing further animals to support them but this is a last step as it would, in most cases, lead to the destruction of these very valuable domestic animals.

Clinical Investigation

Clinical examination of animals always involves measurement for a subnormal temperature, a weak pulse, tachycardia, depressed respirator rate, lethargy and abdominal discomfort with reduced ruminal sounds and cold extremities. Further checking is normally undertaken to see whether the animals are grazing or ruminating normally and whether if there is frequent extrusion and retraction of the tongue, frothing at the mouth, curling of lips, grinding of teeth and facial twitching (Jackson and Cockcroft, 2002).

Infectious Agents

Infectious diseases and parasites are common causes for poor animal health and reduced productivity of livestock. Animal diseases have great impact on food supplies, trade and commerce, and human health globally. Two of the main infections looked for in large ruminants are foot-and-mouth disease (FMD) and Brucellosis. FMD is a highly contagious, clinically acute, viral disease of cloven-hoofed animals. Brucellosis is one of the most prevalent bacterial zoonoses worldwide and, in particular, in developing countries where this disease may have important economic, veterinary and public health consequences. It is caused by the bacterium, *Brucella* and affects humans, large and small ruminants, and pigs. Respiratory diseases in ruminants are often due to a combination of different causes such as viral and bacterial infections, physiological

stress, poor management and environmental factors as well as adverse weather conditions (Bell, 2008, Scott, 2011). Diagnosis is often delayed and based on post-mortem findings that need to be confirmed by serological markers. In addition, infections can make the animals susceptible to exposure to environmental contaminants.

Clinical Biochemistry and Haematological Profiles

Clinical biochemical analysis and haematological profiles are routine investigations which are used for the clinical diagnosis and provide an indication of the health status of animals (Payne et al., 1970, Unshelm, 1983, Martin and Lumsden, 1987).

Blood collected in serum tubes is used to establish clinical biochemistry profiles. These include the levels of selected biochemical analytes, namely calcium, inorganic phosphate, glucose, blood urea nitrogen, creatinine, uric acid, total protein, albumin, globulin, c reactive protein, direct and indirect bilirubin, as well as the serum concentration of enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate amino transferase (AST), creatinine kinase (CK), gamma glutamyl transpeptidase (GGT), amylase transaminases, sorbitol dehydrogenase, glutamate dehydrogenase and lactate dehydrogenase (LDH). Electrolytes such as sodium, potassium and chloride also are routinely measured (Jackson, 2002). If an organ is damaged, the tissue enzymes will be released into the blood, causing elevated enzyme levels; the pattern of enzymes levels provide an indication of affected organs. For instance, liver injury can be diagnosed by certain biochemical markers like ALT, AST, ALP, GGT and bilirubin. Elevations in serum enzyme levels are indicators of liver toxicity, whereas increases in both total and conjugated bilirubin levels are measures of overall liver function.

Blood samples are normally collected in potassium EDTA from domestic animals to determine the haematological profiles. Total white blood and red blood cell counts, neutrophils (mature), neutrophils (band cells), lymphocytes, monocytes, eosinophils, haemoglobin, haematocrit (packed cell volume) and fibrinogen can be measured.

Blood variables such as erythrocyte sedimentation rate, haemoglobin, haematocrit, ALP, fibrinogen and albumin are parameters for discriminating between healthy pigs and pigs with inflammatory conditions (Odink et al., 1990).

Approaches Used for Monitoring of Environmental Chemical Contamination

Both environmental monitoring and biomonitoring methods are used to measure the levels of harmful chemicals present in the environment. Historically, the focus was on the quantity of physical and chemical substances in the environment and only occasionally was the biological measurements of the chemicals incorporated (EPA, 2002). Biomonitoring can involve the measurement of chemical residues in the tissues of living organisms (biological monitoring) or the evaluation of specific biological endpoints (biological effect monitoring) (Seifert et al., 2003). For all biomonitoring studies it is very important to use the correct biological samples, i.e. samples which are easy to collect, enable fast processing, are preferably non-invasive for repeated sampling to avoid stress to the subjects and can be stored in conditions which will not impact on the assays being performed on these samples (Okazaki et al., 2008).

The design of any monitoring programme depends largely on the goals and objectives of the project. In selecting control sites as references for exposed regions, any historical data must be careful considered before commencing the study. Ideally, multiple test and reference sites must be selected since this will give the greatest information and allow proper comparison of results and elimination of confounding factors. It is necessary to establish baseline or background conditions which could affect the interpretation of the data such as differences in soil composition between the control and the test sites (Belfroid et al., 1995).

Environmental Monitoring

Environmental monitoring refers to the assessment of environmental quality or condition by the measurement of a set of selected parameters on a regular basis. In the past decades, measuring the chemical residues in water, soil, air and flora of the countryside has been used to assess the levels of contamination. However, these traditional approaches start to become less useful when mixtures or low levels of contaminants are in the environment (D'Surney et al., 2001).

An environmental monitoring programme gives useful information on the levels of contamination but, generally, does not give information on the effects of the contaminants on biological systems defined at the outset. The renowned environmental toxicologist Stegeman (Stegeman et al., 1993), outlined the following general questions that should be answered by environmental assessment:

What are the levels of contamination?

Are the levels of contamination biologically significant?

Which species, populations or communities are affected?

How severe and widespread are the effects of the contamination?

Environmental monitoring has a number of advantages and as well as limitations. Historically, analytical chemistry procedures for monitoring soil, air and water were developed much faster than biological assessment techniques. These chemical methods tended to be more reliable, repeatable and less expensive than biochemical methods. Quality assurance and quality control procedures were more developed for chemical analytical methods as compared to their biological counterparts. The lack of validation of standard operating procedures in biologically-based assessment methods, led to chemical analyses taking the centre stage in the environmental monitoring arena. With the realization that many contaminants could cause severe effects to the health of animals at low environmental concentrations, research attention turned to biological-based monitoring, rather than chemical contaminant-based monitoring (EPA, 2002).

Biological Monitoring: Measuring Chemicals in Biological Samples

Biological monitoring involves the measurement of chemicals and/or their metabolites in the tissue or fluids. Improvements in our knowledge of pharmacokinetics and the pharmacodynamics of environmental chemicals and advancements in analytical biochemistry have made this technique more widespread. Thus it is becoming possible to measure minute quantities of chemicals in biological materials and to interpret the results with a level of confidence which was previously reserved for measuring chemicals in the soil and water, especially when the contaminant and its metabolites are known (Jakubowski and Trzcinka-Ochocka, 2005).

Biological techniques can also account for absorption by routes other than inhalation, such as skin contact or ingestion (Jakubowski, 2012). There are also some important disadvantages and limitations in the use of biological monitoring which must be considered before this approach can be applied in biological samples other than blood.

Most sampling procedures are invasive i.e. the body boundary must be penetrated to obtain the sample and can cause distress to the animal. Urine sampling is inconvenient and very impractical for domestic animals as they excrete indiscriminately. Non-invasive sampling techniques such as breath, milk or hair sampling are limited by the fact that interpretation of the results is still a problem for most chemicals (Jakubowski, 2012).

There are not yet adequate animal pharmacokinetic data for many different compounds in the environment. Therefore, the interpretation of a biological level of certain compounds is still quite uncertain so other types of biological effect monitoring are required. Also it is extremely difficult to look for the identity of unknown contaminants in the biological samples of exposed animals even with the use of the most advanced analytical methods. This can be further confused by the possibility that a mixture of different contaminants is present in the samples.

Biological Effect Monitoring Using Biomarkers

Biological effect monitoring is a newer advancement in the field of ecotoxicology than environmental and biological monitoring and has drawn significant attention in both academic and industrial circles. Environmental exposure to chemicals may cause changes on a biochemical or genetic level as well as effects on the immune system which can lead to a number of detrimental conditions to the health of the affected individual or organism. Biological effect monitoring is unique in its approach since it measures the effect of chemicals on biomarkers. A biomarker is a xenobiotically-induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample (NRC (National Research Council), 1987).

In addition to providing a true reflection of the effects of contaminants on organisms, biological effect monitoring may act as an early warning signal before irreversible damage at population or ecosystem levels can occur (Hong et al., 2000, Rahman et al., 2000). The lack of funds, research resources and skilled scientists were considered to be the main factors which hamper the implementation of programmes of biological effect monitoring of pollution (Fann et al., 1999). In order to develop biological effect monitoring, new biomarkers need to be established and validated. Rapid, reliable, cost-effective and easy to use biomarker techniques are, therefore, in demand, particularly in third world countries where pollution is becoming a growing concern (Paustenbach and Galbraith, 2006) and also in developed countries where air pollution is increasing and chemical monitoring techniques are not effective (Rylance et al., 2013). Active biological effect monitoring allows the exposure period of the animals to be controlled over space and time, and provides an accurate comparison between sites, particularly if some sites have a limited population of species that can be studied (Shugart and Theodorakis, 1998, Shugart and Goldgar, 1999). A very effective approach in biological effect monitoring is the use of biomarkers that measure physiological, cytological and biochemical responses to indicate environmental stress due to anthropogenic contamination. However, in order to objectively evaluate biomarkers for their suitability for inclusion into a biological effect monitoring programme, a set of criteria should be developed to evaluate their strengths (benefits) and weaknesses (drawbacks) (Kniazeva et al., 2000, Xu et al., 2000).

Biological effect monitoring provides early warning signals of adverse biological effects while chemistry-based surveillance system cannot do this. Biological effect monitoring is more effective and more economical in revealing overall toxicities of complex mixtures. The use of a suitable biomarker with different degrees of specificity is an important aspect in biological effect monitoring (Sarkar et al., 2006). Biomarker studies conducted at molecular levels tend to be more biologically relevant than the measurement of chemicals in the tissues or fluids of animals. The biological responses of organisms to contaminants are long-lasting and biomarkers can reveal occurrences of contamination that intermittent chemical monitoring would miss (Handy et al., 2003).

The fact that only a few biomarkers are specific enough to allow the precise identification of exposure to the specific environmental chemicals is a limitation in biological effect monitoring. Finally, at the cellular level, some animals have the ability to repair damage and so even though the biomarker levels have increased due to exposure, this might not have any permanent physiological damage at the whole animal level, thus increasing the chance of false negatives. Furthermore, it is presently very difficult to find animals which have been sufficiently exposed to environmental toxins to study dose-effect and dose-response between the levels of chemicals in the body, the levels of biomarkers and the early health effects (Lam, 2009).

Therefore, the selection of appropriate biomarkers for use under specific ecological circumstances will have to be determined by their relevance and cost-benefits (Lam, 2009). A suite of biomarkers, in combination with chemical analysis of both the soil and plants and biological tissues, would provide the perfect combination for successful monitoring of the environment.

Biomarkers

Biomarkers are measures reflecting an interaction between a biological system and a chemical, biological, or physical environmental agent. The literature usually considers three classes of biomarkers: *biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility* (NRC (National Research Council), 1987). It may be possible to measure biomarker responses by several different approaches. For example, a particular biomarker can be measured by mRNA and protein levels or by its catalytic activity. Interpretations by each approach may be different but preference for one type of measurement over another should be considered in light of the specificity, sensitivity and technical challenge of measurement (Amara et al., 2010).

The measurement of biomarkers can often be more valuable in the environmental assessment of contaminated sites than chemical analysis. Biomarkers reflect the concentration of contaminant that is biologically available and active, giving a true sense of the contaminant is affecting the biochemistry and physiology of the organism.

In the case of environmental contaminants, such as dioxins and PCBs, which persist in biological organisms, the use of biomarkers has enabled scientists to evaluate progress in reducing exposures, to show the sources of contamination and to clarify the route of exposure through food consumption or through inhalation or skin exposure. In contrast, biomarkers of a compound that is metabolized relatively quickly provide only limited opportunity for inferring sources or exposure pathways (Handy et al., 2003).

Common Biomarker Categories

Biomarkers can be divided into different categories such as biomarkers of genotoxicity, chemical specific enzyme/protein biomarkers and also markers of immunotoxicity.

Genotoxicity

Genotoxicity is described as a deleterious action on a cell's genetic material that affects its integrity and that may have long-lasting and profound consequences which can initiate many pathological conditions. This damage may be manifested in the form of DNA base alterations, DNA adduct formation, DNA strand breaks and DNA cross linkages. General biomarkers of genotoxicity such as DNA adducts or damage to DNA integrity in surrogate tissues or cells, have been used for measuring effects of the combined exposure to air pollutants as well as known genotoxicants (McCarthy et al., 1991, Sheen et al., 2005, Vineis and Husgafvel-Pursiainen, 2005, Rippel et al., 2006, Avila Junior et al., 2009). Formation of adducts between reactive chemical species and cellular DNA is the primary event of mutagenesis and carcinogenesis. The metabolism of genotoxicants by the cytochrome P450 enzymes often results in the production of chemically reactive intermediates that are highly electrophilic and can covalently bind to the bases of DNA forming adducts (Costa et al., 2002, Vyshkina et al., 2005).

DNA single strand breaks are the most prevalent type of genetic damage following exposure to chemical contaminants. These breaks may be introduced directly by chemical contaminants in the environment through the induction of cell death by either apoptosis or necrosis, or through secondary interactions with oxygen radicals or other reactive intermediates (Maynard et al., 2009). In addition to a linkage with cancer, studies have demonstrated that increases in cellular DNA damage precedes or corresponds with higher order cellular effects such as cell cycle arrest, or the induction of apoptosis and in whole test organisms, reduced growth, abnormal development and reduced survival (Su, 2006). Single stranded DNA breaks can be measured by the alkaline Comet assay which is a microelectrophoretic technique for direct visualization of DNA damage in cells (Collins, 2003). The alkaline treatment facilitates the unwinding and denaturation of DNA molecules and is, therefore, used to detect single

strand damage (Nandhakumar et al., 2011). The advantages of this technique are its ability to evaluate DNA damage and repair in proliferating and non-proliferating cells, its ability to detect intercellular differences in DNA damage and the fact that it requires very small numbers of cells (McKelvey-Martin et al., 1993). Comet assay analysis is performed on a cell by cell basis, making it possible to measure the heterogeneity of response within a cell population (Fairbairn et al., 1995, Tice et al., 2000). The percentage of tail DNA seems to be the most useful marker for DNA damage. It measures the percentage of DNA that has migrated from the head of the comet and is directly related to the frequency of breaks over a wide range of damage (Collins et al., 1996). The percentage of tail DNA appears to be a more reliable measure of DNA damage than using the tail moment parameter as described previously (Fairbairn et al., 1995) and which tends to be prone to variability in DNA migration and does not have a linear response to the amount of actual DNA damage (Rojas et al., 1999). Single stranded DNA damage is caused by environmental pollutants such as heavy metals, polycyclic aromatic compounds, poly-halogenated hydrocarbons and pesticides (De Coster S et al., 2008).

An example of an environmental chemical that causes changes in DNA is the heavy metal, cadmium. Cadmium has been shown to intercalate between DNA strands and disrupt transcription by destabilizing the helical structure of DNA. These interactions are probably responsible for cadmium-induced single strand breaks in mammalian cells (Satarug et al., 2004). Therefore, the mechanism of cadmium genotoxicity may involve cadmium directly binding to DNA bases, interfering with the repair of DNA lesions causing DNA strand break induction which in turn enhances reactive oxygen species production (Waalkes and Poirier, 1984, Yamada et al., 1993, Stohs and Bagchi, 1995, Stohs et al., 2001).

Enzyme/Protein Biomarkers

The direct action of a pollutant on target tissues can cause biochemical changes at the enzyme and protein level which in turn can cause genetic changes leading to DNA damage and, finally, cell death (Gil and Pla, 2001). Protein and enzyme biomarkers have been measured in organisms exposed to toxic contaminants in order to monitor the environment. Cells adopt biochemical defence strategies to counteract the damaging effects of oxygen or nitrogen-free radicals or other reactive species induced by toxic chemicals. Protein structure or function may be affected and there are several ways to measure this stressful response. Protein/enzyme markers include (1) variations of specific enzyme activities, such as, cytochrome P450 activity or glutathione S-transferases with phase II biotransformation capabilities (Bonacci et al., 2007, Liu et al., 2007, Ma et al., 2007), (2) toxin binding protein levels, e.g. metallothioneins (Yoon et al., 2007) or (3) heat shock proteins (Brant et al., 2007).

Cytochrome P450 (CYP) is the name given to a family of enzymes which catalyse the metabolism of a wide range of endogenous lipophilic compounds (steroids, fatty acids and cholesterol derivatives) as well as a number of xenobiotics. The CYP1 family consists of 3 subfamilies and 3 genes. These enzymes are inducible and activated by certain polycyclic hydrocarbons and dioxins (Camus-Randon et al., 1996, Schrenk, 1998, Inoue et al., 2000, Nelson et al., 2004). Increased expression of CYP1A1 can facilitate the transformation of non-polar hydrocarbons into epoxide or hydroxyl metabolites through the addition of molecular oxygen. The resulting metabolites can react with intracellular proteins and DNA, resulting in deleterious DNA lesions, aberrations and dysfunction (e.g. the formation of benzo(a)pyrene diol epoxide from benzo(a) pyrene) (Kurachi et al., 2002). The complex halogenated mixtures produced by pulp mills and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) induce the mixed function oxidases (MFO) enzyme system via the induction of mRNA and protein levels of CYP1A1 which is initiated through the binding of these xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR). Ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) activities can be used to measure enzyme activity of

the CYP1A (Larsen et al., 1998). Therefore, CYP1A1 is best viewed as a potential early warning indication of exposure, a predictive tool for contaminant risk assessment.

Extensive evidence indicates that the toxicity and genotoxicity of cadmium is ameliorated by binding to cysteine clusters in metallothionein, a sulfhydryl-rich, low molecular weight metal-binding protein that is involved in the high affinity binding, transport and detoxification of excessive cadmium (Klaassen et al., 1999, Ohrvik et al., 2007). It is proposed that intestinal cadmium absorption may be limited by the metallothioneins, which are synthesised in the intestinal epithelium following oral cadmium exposure and are important in cadmium retention by tissues, primarily in liver and kidney (Min et al., 1992, Klaassen et al., 2009).

Glutathione S-transferases are a multigene family of enzymes of the phase II biotransformation system which inactivate toxins by chemically bonding them to the tripeptide, glutathione, making them soluble for easy excretion. Glutathione levels are responsive to metals, a number of organic hydrophilic contaminants, including polycyclic aromatic hydrocarbons (PAH) and some types of pesticides (organophosphate, thiocarbamates and triazines). Glutathione S-transferases have been measured in cattle, deer and horses after exposure to B[a]P and all three animals had high levels of activity with horses having the highest activity (Darwish et al., 2010).

However, the levels of CYP1A and GST activity and the expression of MT in peripheral blood lymphocytes are low and extremely difficult to measure compared to measurement in microsomes of the liver and in the kidneys and also there is a lot of interindividual variation in expression and inducibility (van Duursen et al., 2005, Chang et al., 2009, Sharma et al., 2013).

Immunotoxicity

The immune system is highly organised and involves cooperation and regulation of various cells by soluble mediators (immunoglobulins, immunohormones, and cytokines) and by intercellular interactions at the level of the membrane receptors such as cluster designation (CD) antigens. Harmful effects of environmental chemicals can arise from (1) direct or indirect action of chemicals on the immune system or of products obtained by their biotransformation, (2) induction of the immune response to chemicals or their

metabolites and (3) modification of host immune cell antigens by chemicals or their metabolites (Berlin, 1987). Immunotoxicants are factors of the external environment which cause significant changes (modulation) in the immune mechanisms in humans and animals i.e. pesticides, industrial emissions and heavy metals (Zbinden, 1987, Dietert, 1996).

The methods commonly used to evaluate immunotoxic effects of harmful environmental chemicals are functional and non-functional tests (Vandebriel, 1995). Non-functional tests provide information mainly about the changes in the lymphoid tissue, number of peripheral lymphocytes and monocytes, level of total immunoglobulins, cytokines, etc. (Vos, 1977, Vandebriel, 1995, Luster and Kimber, 1996). Functional tests reflect in greater detail the situation *in vivo* because they focus on the direct assessment of phagocytic and antigen-specific components of immunity. The evaluation of the immunotoxicological action of xenobiotics requires a spectrum of methods that can provide detailed information about the immune system status. This is suggested by a range of results obtained in the course of sub-chronic intoxication of sheep with some pesticides and during acute intoxication of sheep with heavy metals (Mikula et al., 1992, Mikula, 1992b, Pistl et al., 1995).

Chemical agents that affect the immune system can cause agent specific or species specific immunity damage which, in the majority of cases, results in immune suppression (e.g. decreased resistance to infectious agents and development of tumours) caused by PCB, lead and cadmium (Hadden, 1986, Safe, 1994) or hypersensitivity (autoimmune or allergic diseases) caused by nickel, mercury, polycyclic aromatic compounds and organophosphate insecticides (Bigazzi, 1988, Henry et al., 1988, Luster and Rosenthal, 1993, Luster and Kimber, 1996). Heavy metals such as mercury and lead have been reported to be cytotoxic to numerous immune cell types and through occupational and environmental exposure cause sub clinical effects (cellular and humoral immunologic variable modifications). One risk posed by excessive mercury consumption is that of damage to the central nervous system through its interaction with the immune system (National Research Council, 2000).

The Biological Samples used for Biological and Biological Effect Monitoring

Blood is a common biological specimen of choice used to monitor the effect and level of environmental contaminants with its composition, constituents and importance to the immune system but there are other biological specimens available. Both invasive (blood) and non-invasive (urine, exhaled air, hair, skin, saliva and milk) biological sampling are used for measuring biomarkers. Metals, solvents and pesticides have been measured as parent compounds or as metabolites in blood, urine, exhaled air and hair (Jakubowski and Trzcinka-Ochocka, 2005).

Urine has been the historically favoured specimen of choice for biological monitoring, since it is relatively easy to collect. A major problem in interpreting urinary concentrations is the fact that urine is available at intervals, even where exposure is continuous (Barr et al., 2005). Thus the timing of the sample is important but it cannot be controlled easily in animals that do not excrete in a regular fashion.

For compounds which are volatile, exhaled air is a useful specimen for analysis. The portion of each breath coming from the alveolar region of the lungs is nearly in equilibrium with the blood leaving the lungs. Thus the alveolar gas concentration of a compound is related to that in arterial blood by the solubility of the compound in blood. Breath sampling is much less invasive than blood or even urine sampling and it can be done rapidly and at predetermined times, so this form of biological monitoring is receiving increased attention (Corradi and Mutti, 2005). However, for animals that are normally housed in the open air this will render breath sampling obsolete as the detection of the relatively low concentrations of environmental contaminants in the lung would require analytical sensitivity which is not common place at the present.

Hair has also been used for monitoring biological levels of selective compounds, based on the fact that the hair follicle is perfused, so that chemicals in blood may deposit in part of the hair shaft as it grows from the follicle. A few studies have demonstrated that since the hair grows continuously, the chemical concentration profile along with the length of the hair provides a historical record of blood concentration (Harkins and Susten, 2003). Hair analysis has also been applied to the study of certain environmental

chemicals such as mercury, arsenic, and manganese (Paustenbach and Galbraith, 2006). Hair has also been used for biological effect monitoring, but the biomarkers available are limited (Esteban and Castano, 2009).

Milk has been used to monitor the presence and concentrations of selective compounds in domestic farm animals. For instance 12,000 sheep, cattle and river buffalo from farms in the provinces of Naples and Caserta (southern Italy) were culled based on the fact that the level of TCDD present in the milk of these animals monitored over four years was higher than the permitted level (Perucatti et al., 2006). Also high concentrations of beta-hexachlorocyclohexane (Beta-HCH), a POP (persistent organic pollutant), were measured in the milk of cows located along the Sacco river (Sacco Valley, Central Italy) (Porta et al., 2013) and this has been linked to the nearby location of a chemical plant that has been manufacturing pesticides since the 1950's.

Saliva represents a simple and readily obtainable fluid which is not extensively used as a biomonitoring matrix, but has been used to evaluate a number of environmental contaminants including heavy metals and pesticides (Joselow et al., 1968, Nigg and Wade, 1992, Lu et al., 1997, 1998). The ease of collection makes saliva ideally suited as a non-invasive approach. However, compliance might be difficult with some animals. There are a number of limitations that would have to be noted before it can be used quantitatively to assess chemical exposure (Nigg and Wade, 1992). The use of saliva in biological effect monitoring has been shown previously where a dose-dependent inhibition of saliva choline esterase (ChE) was detected in rats following chlorpyrifos exposure (Timchalk et al., 2004). Salivary cortisol measurements in sheep and cows are effective at quantifying stress activation of the hypothalamic–pituitary–adrenal (HPA) axis (Gibbs, 2011) and cortisol can be increased by heavy metals and pesticides in different species.

Blood

Blood consists of 55% plasma and 45% cellular elements. The cellular elements include red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (Fox, 1999). Blood is used for biological and biological effect monitoring because it contains cells such as erythrocytes which are involved in oxygen transport and leukocytes which reflect changes in the immune system and which contain proteins, enzymes and nucleated cells which can be used to measure genotoxicity (Liew et al., 2006, Angerer et al., 2007).Venous blood is commonly used as a surrogate for direct action on organs as the levels of the chemicals and/or their metabolites can be measured in blood (Liew et al., 2006, Angerer et al., 2007). Blood concentrations of agents or metabolites or biomarkers are often relatively high, making analysis easier, but blood is also a very complex fluid containing numerous suspended and dissolved materials in addition to the analyte of interest. Thus, the analysis often involves extraction and other processing steps that add to cost and effect the interpretation of the results. Also, blood collection is inconvenient and can be controversial because of concern over possible transmission of blood-borne diseases such as hepatitis C and HIV (Psychogios et al., 2011).

Blood Plasma

Blood plasma is the matrix in which blood cells and platelets are suspended. All the metabolites used by blood cells, including glucose, amino acids, and vitamins are dissolved in the plasma, as are hormones that regulate cellular activities, wastes such as nitrogen compounds and CO_2 produced by metabolizing cells. Blood plasma is a dilute salt solution. The predominant plasma ions are sodium, chloride and bicarbonate. In addition, there are trace amounts of other ions such as calcium, magnesium, copper, potassium and zinc. The liver produces albumin, which comprises most of the plasma protein, the alpha (α) and beta (β) globulins, which serve as carriers of lipids and steroid hormones and fibrinogen, which is required for blood clotting. Blood plasma which has had fibrinogen removed is called serum (Frandson, 2013).

Erythrocytes

Each cubic millimeter of blood in domestic farm animals contains about 7 million erythrocytes. The fraction of the total blood volume that is occupied by erythrocytes is called the blood's haematocrit and is normally 35-45% in mammals. The erythrocytes of domestic farm animals contain haemoglobin, a pigment which binds and transports oxygen and carbon dioxide and is found in erythrocytes at a level 11 to 13g /100ml for domestic animals (Frandson, 2013).

Erythrocytes develop from stem cells and circulate for 3 to 4 months after being released from the bone marrow. When plasma oxygen levels decrease, the kidney converts plasma protein into the hormone, erythropoietin, which then stimulates the production of erythrocytes in bone marrow. In mammals, maturing erythrocytes lose their nuclei through a process called erythropoiesis. As mammalian erythrocytes age, they are removed from the blood by phagocytic cells of the spleen, bone marrow and liver (Frandson, 2013).

Leukocytes

Leukocytes consist of lymphocytes, monocytes, eosinophils, dendritic cells, basophils and neutrophils. Analysis of blood provides information on the different cell types, activity and number of leukocytes in the periphery and thus reflects cell trafficking in the immune system at the time of sampling. However, leukocytes in the periphery are unlikely to reflect their levels in the tissues from where they originated or resided. There is some variation in the number of the different types of leukocytes in the blood of different animals. Sprague-Dawley male rats have lymphocytes (75%-85%) and neutrophils (25%-15%), respectively, while for sheep there are four major groups of leukocytes: lymphocytes, granulocytes, monocytes, and auxiliary cells with 40-75 % being lymphocytes and neutrophils comprising the majority of the rest (Petterino and Argentino-Storino, 2006). These cells have membrane receptors which help them to recognize both foreign and self-antigens called cluster designation (CD) markers. The most widely used sample preparation for biological effect monitoring is peripheral blood mononuclear cells (PBMCs) which are isolated from blood by density centrifugation through Ficoll-gradients (Boyum, 1968) and these cells are mainly

lymphocytes (90%) and monocytes (6%) in the case of domestic animals (Gurtoo et al., 1975).

Lymphocytes

Lymphocytes are generated in the bone marrow during haematopoiesis; they leave the bone marrow, circulate in the blood and lymph and reside in the various organs of the lymphatic system and have longer lifetimes compared with granulocytes. Lymphocytes show attributes of specificity, diversity, memory and self/non self-cell recognition. The major peripheral blood lymphocyte populations in sheep are the T cells (56-64%) and the B cells (30-50%) (Thorp et al., 1991, Smith et al., 1994, Tizard, 2000). B and T lymphocytes differ in functions and the secretion of molecules such as cytokines and immunoglobulins (Owen, 2012). B lymphocytes are durable and have an average half-life of 2 years with some surviving as long as 20 years (Macallan et al., 2005) whereas the T lymphocytes have a half-life of less than 2 weeks since they are continuously produced (Freitas and Rocha, 2000). However, subsets of around 10% of all circulating T lymphocytes may live for almost 9 months or more (Freitas et al., 1986). Due to their biological half-life survival in the blood, B and T lymphocytes are useful sentinels for studies on sub-chronic environmental exposures.

B Lymphocytes

Naïve B lymphocytes mature within the bone marrow and migrate to the periphery with an antigen-binding B cell receptor which is either a membrane-bound glycoprotein or immunoglobulin. Immune response occurs when there is a first encounter between antigen presenting cell (APC) and either a foreign or self-antigen. This APC comes in contact with naïve T cells and this either initiates the humoral (B cells) or cellular (T cells) immunity. The humoral response happens when the membrane-bound antibody is specific, the naïve B lymphocytes begin to rapidly divide and differentiate into memory B lymphocytes and effector cells called plasma cells. Memory B lymphocytes have a longer life span than plasma cells and continue to express membrane-bound antibody with the same specificity as their parent cells. Plasma cells, in contrast, produce large amounts of the receptor molecules, immunoglobulins (Ig), in a form that can be secreted and these are the major effector molecules of humoral immunity that bind to the specific antigen and induce further immune cell response (Owen, 2012). Different types of Ig,

i.e. IgM, IgG1, IgG2, IgA and IgE, are secreted by bovine and ovine plasma cells. IgG1 and IgG2 are by far the most abundant immunoglobulins in blood and play a major role in antibody dependent cell mediated cytotoxicity (Tizard, 2000, Janeway, 2001). IgA operates mainly on epithelial surfaces as a neutralizing antibody preventing bacterial colonization, whereas IgM is the largest molecule and the major immunoglobulin produced during the primary immune response. Its main function is to activate the complement system. B lymphocytes can also act as antigen presenting cells (APC) and as such they can phagocytose, process and present antigens in combination through the (major histocompatibility) MHC-II molecules to $CD4^+$ lymphocytes, which in turn secrete IL-2 and induce B lymphocytes proliferation and differentiation into either plasma or memory cells (Janeway, 2001).

T Lymphocytes

T lymphocytes play an important role in the initiation and regulation of the immune response and control in the bone marrow. Unlike B lymphocytes, they originate from bone marrow but mature in the thymus and recognize foreign antigens through membrane receptors (Tizard, 2000, Janeway, 2001). During maturation, the T lymphocytes express a unique membrane bound antigen-binding receptor, the T-cell receptor (TCR). TCRs recognize antigens in association with cell-membrane proteins known as the MHC complex and this helps them to differentiate between normal and altered cells.

The T-lymphocytes can be subdivided into two main classes, αβ and γδ T- lymphocytes, depending on the expression of antigenic markers on the cell surface and cytokine production. αβ T lymphocytes recognize processed antigens presented on MHC molecules on the surface of cells. When naïve T lymphocytes first encounter an antigen associated with an MHC molecule on a cell, they proliferate and differentiate into memory T lymphocytes and effector T lymphocytes. T lymphocytes can be further subdivided into functionally distinct populations by cell surface marker expression of membrane glycoproteins, also known as CD markers, which are either T helper (CD4) or cytotoxic /suppressor (CD8) lymphocytes. Resting T lymphocytes require at least two signals for cytokine gene expression and cell proliferation. The first signal is provided by the ligation of the TCR/CD3 complexed with antigenic peptides bound to the MHC molecules on the surface of APCs including dendritic cells (DCs), activated

B-lymphocytes, monocytes and macrophages. The second signal, often referred to as the co-stimulatory signal, is provided by the surface molecules expressed by APCs. These include the co-stimulatory pathway mediated by the interaction of CD28 on T lymphocytes. Activation of specific T lymphocytes by APCs is an early and crucial step in the development of antigen-specific immune responses, resulting in a wide variety of biological events such as production and secretion of cytokines (Arai et al., 1990, Scott et al., 1990, Finkelman, 1995), cell-surface expression or up-regulation of cell adhesion molecules, ligands for molecules expressed on other cells, cytokine receptors and also the proliferation of antigen-specific clones (Kaye and Janeway, 1984, Jeannin et al., 1999). It is now assumed that the cytokine profile during early infection, as well as the strength and the duration of antigen exposure and the type of APCs inducing the response during priming determines the differentiation of naïve T helper (CD4) lymphocytes into Th1, Th2, Th17 or regulatory T lymphocytes (Tregs) effector cells (O'Garra and Arai, 2000, Hori et al., 2003, Ouyang et al., 2008). These 4 subsets of T helper lymphocytes differ in their cytokine, transcription factor expression and biological functions (Bradley et al., 2000, Janeway, 2001, Sordillo and Streicher, 2002, Broere, 2011). The promotion of the Th1 immune response is characterized by increased secretion of interleukin (IL)-2, tumour necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), which enhances cellular responses against intracellular pathogens and viruses, whereas the Th2 immune response is characterized by a higher production of IL-4, IL-5 and IL-10 that supports humoral immunity (Kehrli et al., 1999). Th2 cells activate B lymphocytes by secreting IL-4 and IL-5, which are then induced to aid in B lymphocytes cell division, as well as differentiation and synthesis of neutralizing opsonizing antibodies (Mumberg et al., 1999, Qin et al., 2003). The Th17 subset, characterized by the expression of IL-17A, IL-17F, IL-22 and IL-26, provides host defence against extracellular bacteria, particularly at mucosal sites and is also involved in the pathogenesis of inflammation (Ouyang et al., 2008). Finally, Tregs, characterized by a continuous expression of the transcription factor, FOXP3, have a crucial role in maintaining homeostasis of the immune system and in preventing the autoimmune reactivity of self-reactive T lymphocytes (Hori et al., 2003).

Naïve $CD8^+$ T lymphocytes proliferate and differentiate into cytotoxic T lymphocytes (CTLs) when they recognize an antigen-MHC complex on APCs with the help of cytokines secreted from other Th cells. CTLs can rapidly induce apoptosis in their target

cells (Shresta et al., 1998). They interact with the mononuclear phagocytes and have the capacity to destroy antigens such as intracellular bacteria or specific target cells such as tumour cells, virus-infected cells in combination with the MHC-I associated molecules and tissue grafts (Sad et al., 1995).

T lymphocytes expressing the γδ TCR form a major subpopulation of circulating lymphocytes in young ruminants where they constitute 5-10% of the total peripheral blood lymphocytes and are the cells most susceptible to paratuberculosis infection (Larsen et al., 1975, Hein and Mackay, 1991). γδ T-lymphocytes migrate preferentially to epithelial surfaces especially the skin and have a wide range of functions including secretion of cytokines such as IFN-γ and cytotoxic activity in response to intracellular infections. They may act in the early response to infections, before antigen-specific responses (Bluestone et al., 1995, Baldwin et al., 2002, Ismaili et al., 2002, Pollock and Welsh, 2002).

Granulocytes

Granulocytes consist of eosinophils, basophils and polymorphonuclear neutrophils (PMNs) (Owen, 2012). PMNs are the majority of the granulocytes in the peripheral blood and are phagocytic cells with multi-lobed nuclei and their recruitment to an area of tissue injury to participate in the primary inflammatory response is the first line of defence against bacteria, fungi, parasites and viruses (Grewal et al., 1980, Rouse et al., 1980, Faurschou and Borregaard, 2003). The principle role of the PMNs is phagocytosis with an impressive assortment of preformed antibacterial systems and soluble mediators, especially the initiation of the oxidative burst i.e. superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production (Janeway et al., 2001, Paape et al., 2002).

PMNs are formed in the bone marrow at a rate of about 8 million per minute, although some reports suggest a turnover rate 10 fold higher (Baggiolini and Dewald, 1985, Maianski et al., 2004). Following formation in the bone marrow, PMNs migrate to the bloodstream and about 12 hours later move into the tissues. PMNs have the shortest half-life among leukocytes, with a half-life of around 35 hours before they undergo apoptosis, thus limiting their potential to participate in long lasting immunological interactions. PMNs constitute approximately 60-75% of the blood leukocytes in most

carnivores, 50% in horses and 20-30% in ruminants and laboratory rodents (Bertram, 1985).

The Immune system

The immune system in ruminants can be divided into the innate and the adaptive immune systems. Innate immunity is the predominant defence during early stages of infections. It is activated by antigens, but the response is not amplified by repeated exposure to the same antigen (Tizard, 2000). In contrast, the adaptive immune system recognizes specific antigens and is mediated by APCs. If the host encounters the same antigen more than once, an enhanced immune reactivity occurs due to immunological memory (Janeway et al., 2001, Sordillo and Streicher, 2002). Both parts of the immune system have the capacity to recognize conserved components of pathogens called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, flagella and bacterial and virus DNA (Hornef et al., 2002). The host cell recognition of these molecules relies on a number of membrane receptors, i.e. the Toll-like receptors (TLRs) or C- lectin receptors, which provide cellular signalling during the initiation of the immune response (Medzhitov et al., 1997, Janeway et al., 2001).

Biomarkers used in Animals Biological Effect Monitoring using Blood

As part of routine clinical diagnosis in animals, biochemical and haematological parameters are measured in the serum and blood, respectively. These can include biochemical measures of liver function (alkaline phosphatase (ALP), total bilirubin (TB), gamma-glutamyl transpeptidase (GGT), β-globublin, lactate dehydrogenase (LDH), albumin, and aspartate aminotransferase (AST)) (Pagana, 2010, Martinez et al., 2013) as well as the acute phase proteins (APPs) (Alsemgeest et al., 1994). However, AST is not liver specific in any domestic animal species and the reference range in horses is rather broad. The levels of different biochemical parameters of liver function changed in sheep when exposed to different chemicals. LDH-1 and LDH-2 isoenzyme activities increased in the blood after a 90-day exposure to tolylfluanid (Sutiakova et al., 2009) and there was an increase in the serum enzymes GGT and AST following sulphur toxicity (Binta et al., 2012). This was in agreement with an observation in cows where serum albumin and β-globublin were increased in animals exposed to vanadium and

monitored over a 5 year period (Gummow et al., 2006). Acute phase proteins (APPs) such as c-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin (Hp), have become the biomarkers of inflammation and infection for diagnostic prognostic purposes in both farm and companion animals and have been adopted routinely in veterinary clinical chemistry (Alsemgeest et al., 1994, Murata et al., 2004, Petersen et al., 2004). There have not, however, been any reported studies on APP changes in animals following environmental contamination exposure. However, the alterations of these parameters are not specific to animals which have been exposed to chemical contaminants in the environment as there are known to be changed with infection , disease and a number other factors. Therefore, other biomarkers can be measured in the blood including genotoxicity markers, immune markers and enzyme markers related to specific biochemical responses to chemical exposure.

Different genotoxic biomarkers have been measured in lymphocytes from peripheral blood, including chromosome aberrations, sister chromatid exchanges, micronuclei, single and double stranded DNA breaks and DNA adducts to monitor chemical exposure. Among the biomarkers, cytogenetic assays are especially useful, as they permit the detection of damage due to several substances with probable carcinogenic properties (Tucker and Preston, 1996). Thus, cytogenetic assays may be used as helpful and easy instruments to monitor animal exposure and this can give a measure of the biological effects of pollutants before overt disease develops (Staessen et al., 2001). Among the many farm animal species that are of interest to scientists, the ones most frequently used in cytogenetic investigations include cattle, sheep, goats and pigs (Rubes et al., 1992).

The monitoring of chromosome aberrations, which are measured in peripheral lymphocytes by microscopic visualisation of metaphase chromosomes, can be performed in relatively basic equipped laboratory conditions although trained personnel are required for scoring. Chromosome aberrations normally develop following exposure to occupational chemicals, ionising radiation, and metals (Vainio and Sorsa, 1981). Low level exposure to environmental toxicants can induce an increased frequency of aberrations but this is difficult to detect statistically, probably because the noise in the system is larger than the number of compound-induced aberrations. Furthermore, monitoring chromosomal aberrations in circulating lymphocytes is not an indication of

target organ dose, or of toxic effects, since the damaged cells will die. This method has been used in biological effect monitoring of farm animals where cattle located near a large chemical plant revealed significantly higher frequencies of chromosome aberrations at different times of the year (Rubes et al., 1992). As well as this, sheep with high levels of dioxins found in their milk had higher chromosome aberrations than sheep that were 80 km away from the exposed area (Perucatti et al., 2006).

Sister chromatid exchanges, which are measured in peripheral lymphocytes, arise through recombinant events between chromatids and are scored by a variety of techniques. The biological significance of the process is unclear. Agents that cause DNA double strand breaks, such as X-rays and bleomycin, induce chromosomal aberrations but not sister chromatid exchanges (Wojcik, 2007). Sister chromosomal exchanges can be detected at lower concentrations than can chromosomal aberrations. Also, increases in the frequency of sister chromosomal exchanges tend to be small, so that large numbers of chromosomes must be scored to achieve adequate statistical power. Even where there is exposure to potent alkylating agents, only a small elevation in sister chromosomal exchange frequency can be observed. High levels of sister chromatid exchanges were found in sheep herds exposed to high dioxin levels during grazing (Perucatti et al., 2006).

Micronuclei detected in lymphocytes arise from failure to segregate chromosomes normally during mitosis. A small piece of a chromosome might break off as a result of damage and become encapsulated in its own nuclear membrane. A strong correlation has been reported between micronuclei induction and chromosome aberrations (Stich and Rosin, 1984, van Sittert and de Jong, 1985). Rubes et al., (1992) compared genotoxic effects induced in some domestic mammals, such as cows, horses, pigs and deer, by the exposure to different levels of industrial pollution and demonstrated a biological impact on animals bred in the most industrialised areas. In particular, lymphocytes from cows, horses and deer had micronuclei frequencies higher than pigs. Micronuclei frequencies can also be detected in blood erythrocytes but among the domestic species, cows, sheep, rabbits and dogs have a spleen that selectively removes micronuclei from circulation (Udroiu, 2006). On the other hand, the micronucleus test works well on equine blood samples. An increase in micronuclei has been found in horses fed with lead-contaminated hay (Burrows and Borchard, 1982)

Single and double stranded DNA breaks measured in peripheral lymphocytes can be caused by exposure to intracellular reactive oxygen species generated by redox sensitive metals such as copper, iron and cadmium or to UV and ionising radiations which act directly on the DNA (Polle, 1993, Schutzendubel and Polle, 2002). The single cell gel-electrophoresis (SCGE) assay, more commonly called the Comet assay, is one of several techniques that can detect double and single-strand breaks in the DNA (Singh et al., 1988). This technique represents a relatively simple, if indirect, *in vitro* approach to screen for genotoxicity. This technique has not been used to-date in the biomonitoring of environmental chemicals in domestic animals.

DNA adducts arise from the reactions of the oxidation products of poly-aromatic hydrocarbons compounds binding with DNA,which can affect various target organs, such as skin, lungs and liver, can also be detected in the blood. DNA adducts can be determined by various methods, amongst which the ^{32}P-post-labelling assay is the most sensitive and measures a wide range of adducts referred to as "aromatic DNA adducts". Phillips et al., (2000) reviewed the strengths, limitations and potential for inter-laboratory variation of the different assays for measuring DNA adducts and concluded that most variation occurs due to differences in reagents, enzymes and the particular protocol used. Meat-producing animals like cattle, deer and horses might be especially at risk by exposure to promutagenic and procarcinogenic chemicals such as benzo[a]pyrene (B[a]P) due to the high activity of phase I and phase II enzymes which can eventually lead to the formation of DNA adducts (Phillips et al., 2000, Darwish et al., 2010).

Immune responses are mediated by a variety of immune cells and the main focus is on leukocytes and their subsets which have CD markers on their cell surface and can be identified using specific monoclonal antibodies. Some of these CD markers identify the cellular phenotype of CD3 (T lymphocytes), CD4 (T helper lymphocytes), CD8 (cytotoxic T lymphocytes), CD11c (dendritic cells), CD19 (B lymphocytes), $CD4^+$ CD25 (activated T lymphocytes), $CD4^+CD25^+$FOXP3 (T regulatory lymphocytes), CD56 (NK cells) and CD14 (monocytes). When lymphocytes are in the activated state there is an increase in the expression of activation markers such as CD69, CD45RO and CD45RA on the cell surface (Sordillo et al., 1997, Tizard, 2000).

Leukocytes are central to all immune responses, but other cells within tissues also participate by signalling to lymphocytes and responding to cytokines such as IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, TNF-α and GM-CSF released by T lymphocytes and macrophages. Chemokines are a family of small cytokines secreted by a number of cells, especially epithelial cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells and include RANTES, IP-10, MIP-1α, MIP-1β, MDC and TARC. Neutrophils, macrophages, natural killer (NK) cells and soluble factors such as complement and lysozyme mediate the innate immune response, whereas lymphocytes, macrophages and soluble components such as immunoglobulins (IgM, IgD, IgG, IgA, IgE) form the specific adaptive immune response (Sordillo et al., 1997, Tizard, 2000).

Among the most widely studied tests of immune function are the proliferation responses of T and B lymphocytes which are a reflection of the cell-mediated immunity hypersensitivity (Burns and Goodwin, 1997). Mitogens such as phytohaemagglutinin (PHA) (Smith and Allen, 1978), concanavalin A (Con A) (Stavy et al., 1971) and pokeweed mitogen are lectins from plant sources (Barker and Farnes, 1967). Mitogens activate leukocytes by cross-linking glycoproteins on the cellular surface and these mimic the bacterial super-antigen response. Mitogens can induce vigorous secretion of most cytokines in PBMCs and whole blood lymphocytes and also stimulate lymphocyte proliferation and cell division *in vitro* in a large number of T and B lymphocytes independent of T and B cell receptor stimulation. The main immune biomarkers and the constituents of blood in which they are measured are shown in Table 1. However, the analytical method involved processing blood samples within hours of the blood being taken to measure these immune biomarkers and this can prove impractical to deliver the samples to the test laboratory within the required time following exposure.

Table 1. Biomarkers Measured in Blood Used to Investigate Immunotoxicity

Immune Markers	Examples of Endpoints	Biological Samples
CD markers	CD3, CD4, CD8, CD11c, CD19, CD25,CD56, CD14, CD203c (basophils), CD18 (neutrophils), FOXP3	Whole blood/PBMCs
Activation CD markers	CD69, CD45RO, CD45RA	Whole blood
Immunoglobulins	IgM, IgD, IgG, IgA, IgE,	Plasma
Cytokines	IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, TNF-α, GM-CSF	Serum/plasma, peripheral blood,
Chemokines	RANTES, IP-10, MIP-1α, MIP-1β, MDC, TARC	Serum/plasma
Lymphocytes Proliferation markers	Mitogenic stimulation (PHA, Concavalin A, specific antigen)	Peripheral blood

Besides the immune and genotoxicity biomarkers described above, there are other enzyme biomarkers measured in the blood including the enzymes delta-aminolevulinic acid dehydratase (ALAD) and acetylcholinesterase.

ALAD plays a key role in haemoglobin formation and its activity is an indicator of the rate of haemoglobin synthesis. Heavy metals such as lead, mercury and inorganic arsenic can cause the inhibition of ALAD. It is mainly used in biomonitoring studies of occupational exposure to metals in species containing haemoglobin, especially humans, domestic pigs and wild species including mice and voles (Ahamed et al., 2006). It has also served as an important biological marker for lead exposure and injury (McFarland, 2005). The effects of cadmium on ALAD are less clear since it has been demonstrated to increase (Wilson and Bhattacharyya, 1997) or decrease (Abdulla and Haeger-Aronsen, 1971, Lynch et al., 1976) ALAD in cow erythrocytes.

The complexity of the nervous system has hindered the development of biomonitoring strategies for chemicals affecting this organ. The organophosphorous pesticides exert acute toxicity by inhibiting acetylcholinesterase in the neuromuscular junction and in cases of severe intoxication, seizures and respiratory failure are the main causes of death

(Marrs, 1996.). Measurement of peripheral acetylcholinesterase activity is a useful, dose dependent means of monitoring pesticide exposure in domestic food animals such as cattle, sheep and pigs (Carlock et al., 1999, Askar, 2011). Acetylcholinesterase activity in erythrocyte, plasma and serum of sheep and cattle may be a suitable biomarker for anti-cholinesterase compounds (Askar, 2011). However, one of the major limitations of using acetylcholinesterase measurement is that the blood must be collected in heparin tubes and this reduces the number of other biomarkers which can be measured. Another limitation is that there are differences detected among different species of animals and there is also high variability in the activity levels in individual animals.

Main Considerations When Biological Effect Monitoring with Blood

In large biological effect monitoring studies, samples may often require storage for later analysis due to sampling logistics as the collection, processing and assaying of samples are time-consuming. Fresh blood must be processed (i.e. isolation of PBMCs) within hours of collection and this is not feasible for field-collected and/or large sample analysis. This creates limitations for the study as the assaying of samples cannot be done in one big batch and the samples must be assayed on the same day as collection.

Effective sample processing is very important for maintaining the high viability of leukocytes in blood especially when they have been collected in the field. While there is always a time delay between collection and processing of the blood, there is a significant and substantial drop in cell viability when the collection and processing time take over 4 hours (Groeger et al., unpublished data)

There is a need for alternative sample processing methods as some approaches are not practical for the time needed for collecting and processing multiple samples in the field. A number of issues arise when trying to process the samples in a certain way in a limited time frame. Time restrictions have a major effect on sample processing and on which biomarkers can be measured in the blood and whether these biomarkers can be analysed in fresh or frozen blood which had been collected previously. One promising alternative to the time consuming process of isolating cells on the day of collections is to cryopreserve the blood samples. The amount of blood sampling which is possible will depend on the programme objectives, the stability of the contaminants or the half-life of the biomarkers, the biology of the test species (behaviour, physiology, etc.) and

the sensitivity of the biomarker response over time. Blood sampling time points could be either within the range of months or years for long term programmes or daily sampling for monitoring the acute effects of an accidental pollution event.

Many factors may influence analysis and interpretation. Pilot studies should be conducted before the main programme is initiated. Variability can be estimated and the programme designed to be robust and have sufficient accuracy, precision and statistical power to fulfil the objectives of the planned study. For example, if insufficient sampling is performed to detect slight changes in biomarkers the programme will be inconclusive and not protect or effectively monitor environmental health (Lam, 2003).

After blood samples have been taken from the animals exposed to chemicals, the viability of the blood lymphocytes should be assessed by measuring cell death including necrosis and apoptosis. Another important factor leading to cell death is the influence of oxidative stress which should also be measured in the lymphocytes.

General Cytotoxicity

Cytotoxicity refers to the potential of a chemical compound to kill mammalian cells via two specific mechanisms or modes of cell death, either apoptosis or necrosis (Wyllie, 1980, Darzynkiewicz et al., 1997)

Apoptosis is a morphologically and biochemically distinct form of cell death and is an energy-requiring process, characterised by DNA fragmentation, nuclear segmentation, cytoplasmic shrinkage and membrane blebbing (Kerr et al., 1972, Thompson, 1995). This normal physiological process plays a fundamental role in allowing multi-cellular organisms to control unwanted cell numbers during development and other normal biological processes and can protect cells against malignant transformation and subsequent cancer development (Kerr et al., 1972, Wyllie, 1987). Apoptosis is caused by various physiological signals such as CD95 (Fas), tumour necrosis factor alpha and growth factor withdrawal (IL-2), oxygen free radical formation, or by a number of non-physiological stimuli such as heat shock (Elmore, 2007), UV lights and gamma radiation (Reed, 2000) and anti-neoplastic drugs (Hickman et al., 1992, Thompson, 1995).

Cells undergoing the process of apoptosis have a characteristically shrunken look due to a decrease in cellular volume which results in blebbing of the plasma membrane into membrane-bound condensed bodies without a loss of membrane integrity. Internally, these cells maintain organelle integrity but DNA becomes condensed and fragmented resulting in DNA laddering that can be detected by TUNEL staining in nuclei (Gavrieli et al., 1992). Other internal characteristics include aggregation/condensation of nuclear chromatin with nuclear fragmentation, shrinkage of cytoplasm via cell dehydration, changes in cell morphology, loss of microtubules and mitochondrial leakage due to pore formation. There is mobilization of intracellular ionized calcium, non-random mono and oligonucleosomal fragmentation of DNA (ladder effect), release of factors (such as cytochrome-c) into the cytoplasm from the mitochondria and an activation of caspase cascades (Elmore, 2007).

An early event in apoptosis is the alteration of membrane symmetry manifested in the translocation of phosphatidylserine from cytoplasm to the extracellular side of the membrane and occurs prior to loss of plasma membrane integrity and DNA fragmentation (Martin et al., 1995, Vermes et al., 1995). In functional terms, phosphatidylserine at the cell surface serves as a signal to macrophages that they are to phagocytose the cells, enabling them to be lysed in a non-inflammatory manner (Wyllie, 1980, Cohen et al., 1992, Fadok et al., 1992, Darzynkiewicz et al., 1997, Leist and Nicotera, 1998, Fadok et al., 2000). The translocation of phosphatidylserine from the cytoplasm to the extracellular side of plasma membrane can be detected by using annexin V, a phospholipid-binding protein with a high affinity for phosphatidylserine in the presence of calcium ions, Ca^{2+}. Therefore, to detect early apoptotic cells, a combination of annexin V and propidium iodide (PI) staining is used. Propidium iodide is a fluorescent dye which stains DNA but it cannot enter through the intact membrane of viable or early apoptotic cells. Staining cells simultaneously with annexin-FITC V (green fluorescence) and the non-vital dye PI (red fluorescence, bivariate analysis) allows the discrimination of intact cells ($FITC^{-}PI^{-}$), early apoptotic ($FITC^{+}PI^{-}$) and late apoptotic or necrotic cells ($FITC^{+}PI^{+}$)(Vermes et al., 1995).

Necrosis is an “accidental” cell death and is a pathological process which occurs when cells are exposed to a serious physical or chemical insult, such as in hypoxia, ischemia, temperature fluctuations, disruption of membrane structure and exposure to toxins (Fink

and Cookson, 2005). The digestion of DNA and post-lytic random DNA fragmentation (late event) are also part of the process of necrosis. Necrosis is a violent and non-discriminating process characterised by rapid cytoplasmic swelling, destruction of cell organelles (mitochondria and endoplasmic reticulum) and plasma membrane rupture which leads to expulsion of intracellular contents, including lysosomal enzymes, and their release into the extracellular fluid or the surrounding medium (Wyllie, 1980, Darzynkiewicz et al., 1997, Halestrap et al., 2000). The major biochemical process of necrosis begins with the loss of ion homeostasis leading to an influx of water and extracellular ions (Darzynkiewicz et al., 1997). There are different biomarkers that can be used for detecting a loss of membrane integrity in necrosis including PI, lactate dehydrogenase (LDH) which is normally sequestered inside cells and the live-cell protease, which is only active in cells that have an intact cell membrane. The live-cell protease loses activity once the cell membrane integrity is compromised and a dead-cell protease can then be measured in culture media (Decker and Lohmann-Matthes, 1988, Riss and Moravec, 2004, Niles et al., 2007). Researchers have developed assays that use ATP and NADH contents as markers of viability based on mitochondrial health (Riss and Moravec, 2004).

Oxidative Stress

Polycyclic aromatic hydrocarbons (PAHs) (Penning et al., 1999) and transition heavy metals like cadmium, copper, lead and mercury (Ercal et al., 2001, Gaetke and Chow, 2003) have been found to cause oxidative stress. A three-tier hierarchical cellular response model has been proposed to explain the role of oxidative stress in mediating its biological effects (Li et al., 2003). This model suggests that low levels of oxidative stress induce protective effects (tier-1) by the activation of antioxidant enzymes. If these responses fail to provide adequate protection, then a further increase in reactive oxygen species (ROS) production will result in pro-inflammatory (tier-2) and cytotoxic (tier-3) effects. Free radicals are highly reactive molecules containing one or more unpaired electrons that are capable of an independent existence, occupying an atomic or molecular orbital (Kehrer, 1993, Halliwell, 1996). There are many forms of free radicals, such as oxygen- and nitrogen-centred species (Kehrer, 1993, Halliwell, 1996). Therefore, a free radical species may be highly reactive with other compounds and can initiate chain reactions by either donating the unpaired electron (i.e. reduction) or

extraction of an electron (i.e. oxidation) to complete its own orbital. Because most molecules present in living organisms are non-radicals, a reaction with a free radical will most likely create a new radical (Kehrer, 1993, Halliwell, 1996).

ROS arise from cellular respiration which involves the reduction of molecular oxygen (O_2) to water in the mitochondrial electron transport chain (Yu, 1994). Upon subsequent addition of electrons to oxygen, the superoxide anion radicals ($^{\bullet}O_2^-$), hydroperoxyl radical (HO_2), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) are all formed (Kelly et al., 1998).

The $^{\bullet}OH$ species produced are highly toxic and are claimed to cause most of the pathology induced by ROS (Lieu et al., 2001). The $^{\bullet}OH$ radical is the potentially most potent oxidant encountered in biological systems and can react readily with a variety of molecules, such as lipids, DNA and proteins at close to diffusion limited rates (Yu, 1994). Normal metabolic products such as H_2O_2, heme and free iron can act as strong pro-oxidants, because of their ability to generate extremely reactive $^{\bullet}OH$ species through non-enzymatic reactions. However, due to its high reactivity, the $^{\bullet}OH$ radical is a non-selective oxidant and the specificity of its reactions is dictated by the site of radical generation and it has a short half-life in biological tissue (Siesjo et al., 1989). $^{\bullet}OH$ species are formed via the Fenton reaction: which is the iron-salt-dependent decomposition of H_2O_2, which is extremely slow unless catalyzed by a transitional metal such as Fe^{2+}.

When red blood cells are lysed after cryopreservation there is a release of iron into the culture medium. Iron has the capacity to accept and donate electrons easily, changing between ferric (Fe^{2+}) and ferrous (Fe^{3+}) oxidation states and participating in the redox reactions such as Fenton reaction leading to an increase in $^{\bullet}OH$ molecules (Kasprzak, 2002). Other biomarkers such as oxidative stress, altered heme biosynthesis and increases in different stress proteins could be more suitable for evaluating toxicity of metals at lower doses (Wang and Fowler, 2008).

Biological structures or biomolecules, such as polyunsaturated membrane lipids, DNA and amino acids, are the target molecules reacting with ROS. The damage that occurs includes mitochondrial swelling and lysis, calcium influx, protein damage and lipid peroxidation and consumption of reducing molecules e.g. nicotinamide adenine

dinucleotide phosphate-oxidase (NADPH). These may lead to cellular apoptosis or necrosis (Bus et al., 1974, Cagen and Gibson, 1977, Burk, 1991, Berlett and Stadtman, 1997, Cheng et al., 1998, Cheng et al., 1999)

Antioxidants including ascorbic acid and ascorbate are used to scavenge ROS. The L-enantiomer of ascorbic acid is vitamin C and it has a hydrophilic nature and wide distribution in the body. Vitamin C is the main water-soluble antioxidant in the aqueous phase and also acts as a reducing agent in biological fluid by scavenging pathologically relevant ROS ($^{\bullet}O_2^-$ and $^{\bullet}OH$) and RNS and protecting DNA against ROS damage (Frei et al., 1989, Duthie et al., 1996, Halliwell, 1997, Carr and Frei, 1999).

Aims of the Thesis

Biological effect monitoring in domestic animals is in its infancy where the biomarkers are not well developed and validated and blood sample processing may hinder investigations. Therefore the overall aim of this study was to develop *ex vivo* biomarker assays that would be suitable for biological effect monitoring of the exposure of sheep to chemicals in the environment.

The specific aims were:

1. To establish suitable blood sampling procedures for *ex vivo* assays using a combination of field-collected samples from sheep and laboratory-collected samples from rats and their characterisation by measuring markers for viability, early apoptotic or late apoptotic/necrotic events and reactive oxygen species in both short and longer term cultures.

2. To determine the responses of lymphocytes from different blood sample preparations to common positive controls for genotoxic assays i.e. ethylmethane sulphonate (EMS) and cadmium chloride ($CdCl_2$) using markers for viability, early apoptotic or late apoptotic/necrotic events and DNA damage.

3. To immunologically characterize sheep blood preparations using leukocyte subpopulation analysis and immune function as measured by cytokine production.

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Chapter 2

Development of Basic Protocols and Methodology: Sample Collection and Processing of Blood from Sheep and Rats.

Table of Contents

Introduction

Due to the increased industrialisation in the world, there is a growing amount of harmful chemical emissions into the environment (Cohen, 1997). Therefore, it has become extremely important to investigate the levels of chemicals in our surroundings and their potential adverse health effects on human and non-humans. This is normally done by performing environmental monitoring studies i.e. measurement of pollutants in environmental samples. This approach can be limited as the biological effects of the chemicals are not assessed and the composition of the chemical emissions are not fully characterized which makes targeted analysis difficult because it is not always apparent what to measure. Sometimes environmental monitoring is done in parallel with biological and biological-effect monitoring. This is also called biomonitoring and encompasses the measurement of chemicals and residues of their metabolites in the tissues obtained from living organisms as well as assessing specific biological endpoints in biological samples (Lam and Gray, 2001).

Blood is a common biological sample used for biomonitoring studies due to its ease of collection and availability, but also because of the possibility for repeated sampling (Salama et al., 1999, Faust et al., 2004b). It is generally accepted that blood cells can be used as sentinel cell types to provide early warning signals for adverse health outcomes (Salama et al., 1999, Faust et al., 2004a, Faust et al., 2004b). In biomonitoring studies, the traditional blood cell type used to document biomarkers of effects are the peripheral blood mononuclear cells (PBMCs) (van Leeuwen et al., 2008). PBMCs have advantages compared to other tissue specimens such as their accessibility (due to the less invasive nature of their extraction, compared to internal tissue biopsies) and they contain cells affected by chemicals such as circulating monocytes and T and B lymphocytes (Liew, 2005). PBMCs are the most transcriptionally active cells in the blood and represent a popular model system that serves as a circulatory mirror of the *in vivo* systemic changes in the physiological, immunological and metabolic activities of the body (Fan and Hegde, 2005, Liew, 2005). Among the plethora of available primary cells, PBMCs and their individual subsets (CD4 and CD8 T lymphocytes (up to 70% of all cells), B lymphocytes (15%), natural killer cells (10%), monocytes/ macrophages (5%), dendritic cells (1%) and basophils (1%)) have facilitated a broad spectrum of applications from *in vitro* cell-based assays to the monitoring of *ex vivo* changes before and after drug

treatments or environmental exposures (Mohr and Liew, 2007). As a result, many studies conducted thus far in areas of research such as immunology, infectious and cardiovascular diseases, cancer and in toxicological biomarker research, have featured PBMCs (Whitney et al., 2003, Chon et al., 2004, Lampe et al., 2004). PBMCs are also the common sample used to study DNA damage in lymphocytes in genotoxicity assays (Tuo et al., 1996). For such studies, various endpoints have been measured such as the formation of micronuclei (Ranaldi et al., 1998), sister chromatid exchanges, chromosomal aberrations (Tompa et al., 1994) and, of particular interest to this study, DNA strand breaks as measured by the Comet assay (Tuo et al., 1996, McNamee et al., 2001, Braz and Favero Salvadori, 2007, Sirota and Kuznetsova, 2008). In addition, PBMCs are often used to measure DNA adducts that have been used as surrogates for target tissues such as the lung and as biomarkers of cancer risk (Wiencke, 2002, Lee et al., 2010).

In practice, once the PBMCs are isolated various aspects of acquired and innate immunity can be quantified by *in vitro* tests of cell-mediated immune responses which are useful indicators of immune health. After *in vitro* stimulations of PBMCs, the only blood cell type analysed by flow cytometry or the Comet assay is lymphocytes as they are the only cell type which has not adhered to the plastic in the tissue culture plates. These indicators of immune health can be measured using a variety of methods, including proliferation assays (Firbas et al., 2006), cytotoxic-T lymphocyte assays (Koibuchi et al., 2005, Firbas et al., 2006), intracellular cytokine staining (Asanuma et al., 2000, Maecker et al., 2005), the cytokine enzyme-linked immunospot (ELISPOT) assay (Arlen et al., 2000, Smith et al., 2001, Mwau et al., 2004, Goepfert et al., 2005) and tetramer staining (Maecker et al., 2005, Firbas et al., 2006).

Assays involving PBMCs are labour-intensive by nature and their isolation must to be done within a narrow time-frame in order to avoid sample deterioration. This time-frame for the separation and storage of PBMCs is about 8 hours after the blood is drawn (Bull et al., 2007). However, large sample numbers and low measurement errors in the assays are required for biological testing. When the number of samples and the number of subjects are increased on the day of the experiment, prolonged processing time and possible sample deterioration can result. Therefore, it is necessary to develop reliable, efficient and standardised techniques to increase the number of samples processed

(Leyland-Jones et al., 2008). Traditionally, biomarker investigations in laboratory animals and humans have been well established and there are not too many challenges to face compared to field-based studies.

The study of the effects of environmental contaminants on farm animals and wildlife presents challenges regarding sample collection and storage when studies are in remote locations, especially for *ex vivo* assays that require viable cells. In field-studies, whole blood or isolated blood cells must often be held for long periods of time or shipped overnight before reaching the laboratory and blood samples are known to degrade very quickly (Lahvis et al., 1995). Furthermore, analysis in a laboratory may not be possible for days or weeks due to a backlog of samples or a limit of sufficiently trained staff.

The cryopreservation of cells is a potentially beneficial way to preserve them and to study the *in vitro* responsiveness at a later date (Weinberg et al., 2000, Finkelstein et al., 2003). Notably, cryopreservation is used for long-term storage of immortal cell lines and PBMCs which have been applied in immunotoxicology studies of harbour seals (Ross et al., 1993, Levin et al., 2005), chickens and American coots (Finkelstein et al., 2003) as well as in multicentre Acquired Immunodeficiency Syndrome (AIDS) studies (Weinberg et al., 2000). There is still a limited understanding of factors that affect the viability of cryopreserved lymphocytes and, thus, their use in field-studies. The factors which may affect them include transport temperatures, times between sample collection, processing, freezing and transfer to long-term storage in liquid nitrogen and potential variation in the final concentration of cryopreservative in each sample (Weinberg et al., 2000, Disis et al., 2006, Bull et al., 2007, Kierstead et al., 2007, Smith et al., 2007). In this study, the cryopreservation and thawing protocol was adopted from that of Reimann et al, (2000) which took into account factors such as cryoprotectants, the type of protein additive in the cryopreservation medium, storage tank, cryovials, thawing method and the temperature of the medium at the time of thawing the cells. Other studies reported the effects of altering several key steps in the cryopreservation process such as the volume of washes, number of cells frozen per tube, media components and temperature during the thawing process (Kleeberger et al., 1999, Betensky et al., 2000, Fowke et al., 2000, Maecker et al., 2005, Disis et al., 2006). Other important additions to the cryopreservation procedure include the removal of DMSO by slow dilution and centrifugation and the addition of DNAase to remove the DNA from

dead granulocytes to avoid the clumping of lymphocytes as demonstrated by Reimann et al., (2000) and Steven et al., (2007).

Studies have previously reported the cryopreservation of isolated lymphocytes (Pero et al., 1998, Kleeberger et al., 1999, Beck et al., 2001) in which the samples were handled by a limited number of laboratories, ranging from one (Pero et al., 1998, Beck et al., 2001) to four (Kleeberger et al., 1999) and all reported successful cryopreservation and subsequent use. The major focus of these studies was to establish that the responses of cryopreserved samples were similar to those of fresh samples in several important characteristics including viability and transformability with Epstein Barr Virus (EBV) and the influence of delayed blood processing on cell viability. In contrast to the other characteristics, transformation by EBV was consistently found to be unaffected by the length of time (from 40-60 weeks) samples were stored in liquid nitrogen (Pero et al., 1998, Beck et al., 2001, Hayes et al., 2002).

While cryopreservation has its advantages, there are also some drawbacks to the use of this technique. Studies in bone marrow, hepatocyte and lymphocyte systems have suggested that cryopreserved cells exhibit higher levels of apoptosis compared to fresh cells in culture (Schmidt-Mende et al., 2000, Yagi et al., 2001, Sarkar et al., 2003). Cryopreservation-induced apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages of the apoptotic process (Fadok et al., 1992, Vermes et al., 1995). The use of annexin V and propidium iodide (PI) as cell death markers has become an established method for differentiating between the two major forms of cell death, namely, apoptosis and necrosis and is a reliable method to measure cryopreservation–induced apoptosis (Sarkar et al., 2003, Jeurink et al., 2008). It has been shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membranes and expose phosphatidylserine (PS) which is translocated to the outer layer of the cell membrane. This process only occurs in the early phases of apoptotic cell death during which the cell membrane remains intact (Fadok et al., 2000). Annexin V preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyelin. PI binds to DNA by intercalating between the bases. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold and can be measured by flow cytometry (Moore et al., 1998). The PI exclusion

assay was used as an objective and sensitive approach to assessing the viability of cells in various studies (Weinberg et al., 2000, Bailey et al., 2002, Doherty et al., 2005, Disis et al., 2006). The PI exclusion assay has been shown to be a rapid, highly linear, functionally correlated assay (Mascotti et al., 2000). Thus annexin V can be used as a marker for early apoptosis while double staining with annexin V and PI can differentiate between early and late apoptotic cells since viable lymphocytes with intact cell membranes are impermeable to PI (PI-negative) whereas necrotic and late apoptotic lymphocytes, with deficient cell membranes, are permeable to PI (PI-positive). Given the rising health issues associated with the increasing amount of contaminants in the environment and the number of alarming incidences reported, it would be highly beneficial to develop techniques to properly assess the effects of chemicals in biological tissues or samples and to store them for multiple endpoint analysis at a later date. The overall aim of the present study was to investigate the effects of blood sample preparations and cryopreservation on cell viability and responses in *ex vivo* biomarker assays. While the ultimate goal being to develop biological effect monitoring methodologies for domestic animals. At the onset, the potential to cryopreserve PBMCs collected from multiple sheep under field-conditions, without affecting the number of viable cells needed for the *ex vivo* assays, was assessed. The effect of the time interval between the sampling of blood from individual sheep and the cryopreservation step was investigated because this aspect has not been addressed in the literature. The objective was to use a cryopreservation protocol that would not include additives, such as cytokines, that could have the potential to artificially augment antigen-specific immunity and the response to genotoxic compounds.

Following the initial field-based studies using sheep blood, rat blood was used for a detailed protocol development/evaluation of fresh and frozen PBMCs because it is very easy to collect, relatively cheap and provides sufficient volume for *ex vivo* assays. The impact of the cryopreservation procedure used to store PBMCs in the viability of lymphocytes in culture was examined. The extent to which the loss of viability observed was due to early apoptotic or late apoptotic/necrotic events were examined. In addition, the effects of the mitogens, phytohaemagglutinin (PHA-P) and pokeweed mitogens (PWN), on the survival and the apoptotic induction of freshly isolated and frozen PBMCs were assessed.

Materials & Methods

Chemicals & Reagents

All chemicals used were of the highest grade available. RPMI 1640 medium with 2mM L-glutamine, heat-inactivated foetal bovine serum (FBS), HEPES, phosphate-buffered saline (PBS) , dimethyl sulphoxide (DMSO), propidium iodide (PI), Ficoll-Hypaque density gradient (Histopaque 1083) , DNAse, phytohemagglutinin P (PHA-P) and pokeweed mitogen (PWM) were purchased from Sigma-Aldrich, Ireland. The antibiotic gentamycin was procured from GIBCO, UK. 96-well sterile tissue culture plates were purchased from Sarstedt (Ireland). Ammonium chloride red blood cell lysis solution buffer (Pharmlyse), annexin V-FITC apoptosis detection kits and 9 ml sodium heparin blood collection tubes were procured from BD Biosciences, UK.

Animals

Sheep

Blood samples were taken from four 2-year-old Cheviot-cross ewes (with tag numbers 405, 445, 555 and 645) that were provided by and maintained in the grass paddock of the Central Veterinary Laboratory (CVL), Abbotstown, Co Dublin

Rats

Sprague-Dawley male rats, 1-year-old, weighing 250 to 400 g, were obtained from the Biological Services Unit of University College Cork, Ireland and had been purchased from Harlan, Bicester, U.K. The rats were housed in groups of 6 under standard controlled conditions (21+/-1°C) on a 12 hour light/dark cycle (lights on at 8.00 a.m.) and received standard water and diet, provided *ad libitum*.

Blood Collection

Sheep

Jugular blood samples were extracted from the ewes after they were restrained in small pens so that they could not turn around but were able to move forwards and backwards. 70-80 ml blood was collected from each ewe in 9x9 ml sodium heparin tubes. The blood was pooled together from each individual sheep and then processed for the isolation of PBMCs and cryopreservation as shown in Figure 1.

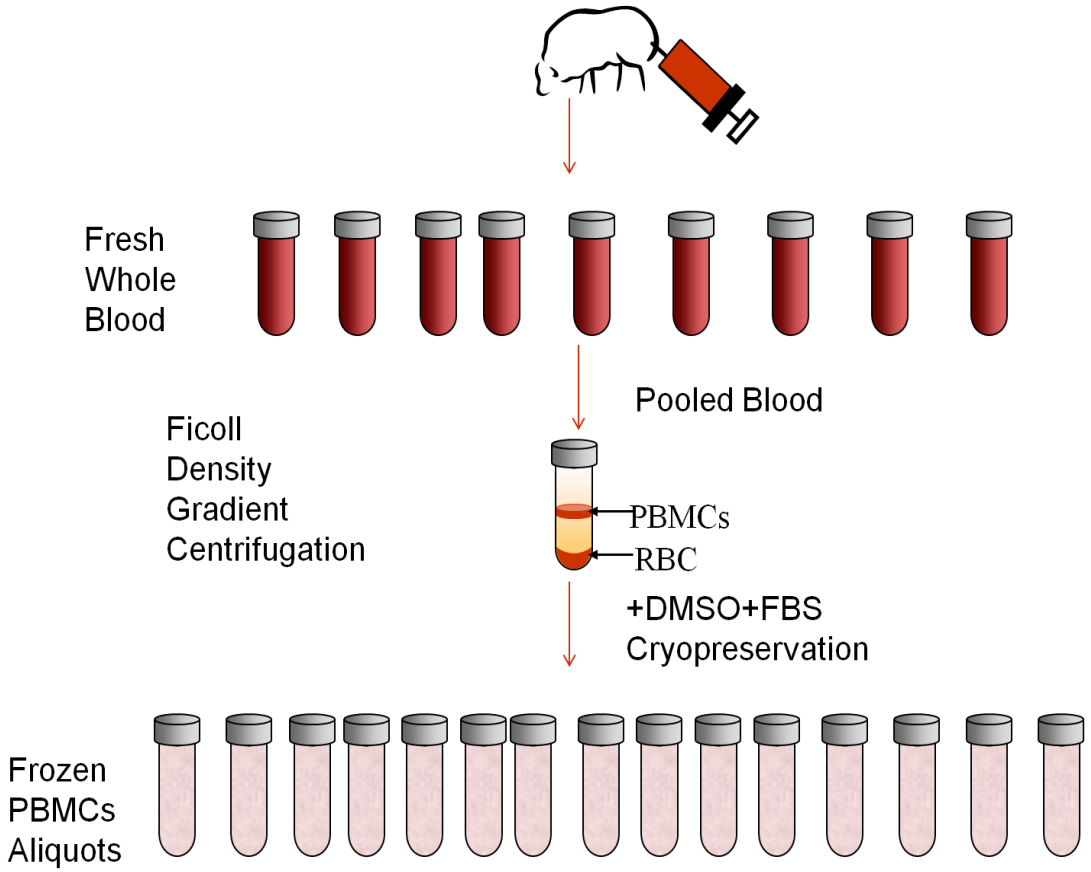


Figure 1. Schematic of the Sampling Procedure Methods used when Blood was Collected from Sheep in the Field.

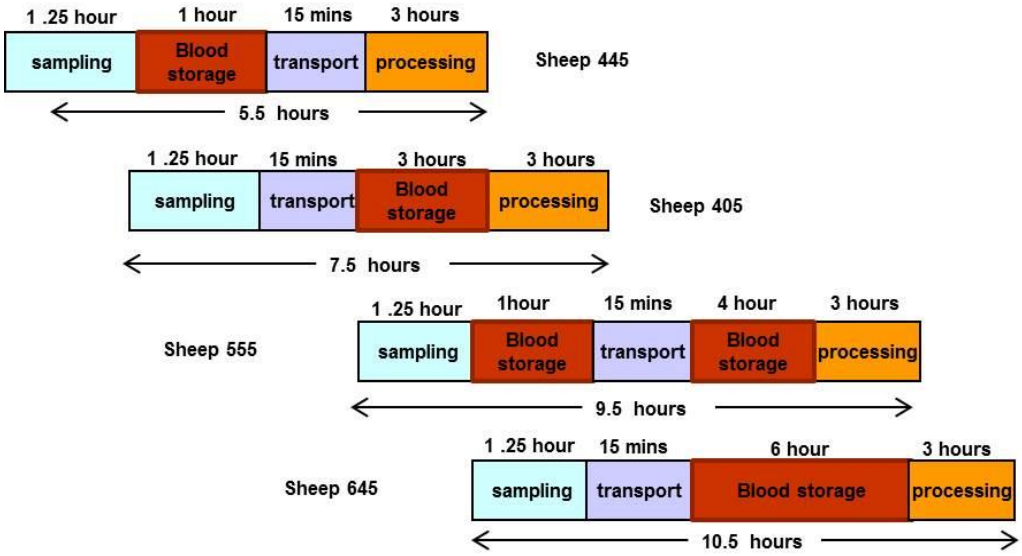


Figure 2. Schematic of the Schedule of Sampling, Transport, Storage and Processing when Blood was Collected from Sheep in the Field.

Because the blood was collected under field-based conditions and multiple samples were taken from each sheep, the blood sampling draws were staggered in the order sheep 445 405, 555 and 645. The veterinarian took samples in the field and following 15 minute transport time the laboratory-based technician processed the blood and performed Ficoll-density gradient centrifugation to isolate PBMCs (as described in the section "Isolation of PBMCs from Blood"). Another confounding factor was that blood was stored at room temperature in between sampling and processing. While biological samples containing proteins should be normally stored on ice to maintain activity for a long time, the method for isolation of PBMCs requires both the blood samples and the reagents to be stored at room temperature for optimal isolation efficiency. The duration for which each sample was kept before processing was recorded and together with the schedule of sampling is shown in Figure 2. The PBMCs in all samples were counted using trypan blue stain and a haemocytometer and cryopreserved by placing them in cryovials in a slow-freezing-rate container (Mr Frosty, Nalgene, UK) and stored in a -80°C freezer (one container was used for each individual sheep samples). The next day the containers were placed on dry ice, transported to the laboratory in Cork by train and placed in the -80°C freezer for 24 hours. Finally, the cryovials containing the frozen PBMCs were transferred to liquid nitrogen for storage.

Rats

Each rat was placed into a Perspex custom-built anaesthetic chamber and anesthesia was induced with 4% halothane vaporized in oxygen (3l per minute). Then 5-7 ml of blood was extracted from each of 4 rats by cardiac puncture and transferred to 9 ml sodium heparin tubes followed by cervical dislocation. The procedure was performed on 3 separate occasions using a total of 12 rats. The reason for this was to limit the amount of time the blood samples were stored at room temperature to a maximum of about 1 hour.

The blood from each individual rat was then processed for the isolation of PBMCs which were either put into culture straight away or cryopreserved as soon as possible. On the day that PBMCs were isolated from fresh blood, frozen PBMCs were thawed and cultured in parallel with the fresh PBMCs. This process was performed on 3 separate occasions for each rat.

Sample Preparations

Isolation of PBMCs from Blood

Fresh sheep and rat PBMCs were isolated from heparinised blood by routine Ficoll-density gradient centrifugation as described by Boyum (1968). Briefly, 2.5 ml of whole blood was carefully layered on the top of Histopaque 1083 Ficoll-density separation medium in a centrifugation tube at a ratio of 1:1. After centrifugation for 30 minutes at 400 x g**,** the white buffy layer of PBMCs was formed at the interface between blood plasma and the Histopaque medium and the PBMC fraction was carefully transferred using a Pasteur pipette into a tube containing 5 ml of RPMI 1640 cell culture medium. The PBMCs were then washed twice with RPMI 1640 and centrifuged at 300 x g for 7 minutes. The cell pellet was either resuspended in 5ml culture medium (RPMI 1640 medium supplemented with 2mM L-glutamine, heat-inactivated 10% foetal bovine serum (FBS), 10 mM HEPES and 20 µg/ml gentamycin) or further processed for cryopreservation as described below. At this stage a viable cell count was performed using trypan blue stain and a haemocytometer. The cell suspension was diluted until a cell concentration of $1x10^6$ cells/ml was achieved and 500 µl of the PBMC cell suspension were added per well in a 24-well plate for further analysis (Boyum, 1968).

Cryopreservation of PBMCs

At the final step in the PBMC isolation procedure, the cell pellet isolated from the 9 ml sodium heparin aliquots of blood was resuspended in freezing medium (80 % FBS 20 % DMSO) at a concentration of 5 x 10^6 viable cells/ml. The samples were mixed gently to avoid cell damage. The cryovial was placed in a slow- freezing-rate container (Mr Frosty, Nalgene, UK) and transferred to a −80 °C freezer for 24 hours. This simplified method of controlled-rate freezing lowered sample temperatures by approximately 1°C per hour. After 24 hours, frozen specimens were transferred to a liquid nitrogen dewar flask at (-196ºC) and maintained for 14 days before being thawed and put into culture as described below.

Thawing of Frozen PBMCs

Frozen PBMCs were thawed according to a procedure adapted from protocols described by Reimann and colleagues (Reimann et al., 2000). A frozen cryovial containing PBMCs was placed in a 37°C water bath with continuous agitation until completely melted and then placed on ice for 2 minutes. All dilution media were heated to 37°C before being added to the thawed cell suspension. One ml of the thawed cell suspension was slowly diluted with RPMI 1640 medium supplemented with 20 % FCS 0.2 µg/ml DNAse 25 mM HEPES buffer (thawing medium) at room temperature. To accomplish this slow dilution, 0.1, 0.2, 0.4, 0.8 and 2 ml of thawing medium were added sequentially at 1-minute intervals with further gentle agitation. Five minutes after the last addition of medium, the total volume was brought to 10 ml with thawing medium and centrifuged at 300 x g for 7 minutes. The supernatant was then removed and this wash step was repeated and the samples resuspended in 10 ml culture medium. At this stage a viable cell count was performed using trypan blue stain and a haemocytometer. The cell suspension was diluted until a cell concentration of 1x10^6 cells/ml was achieved and 500 µl of the PBMC cell suspension was added per well in triplicate in a 24-well plate for further analysis.

Cell Culture

Both fresh and frozen PBMCs were cultured at a concentration of $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, heat-inactivated 10 % foetal bovine serum (FBS), 10 mM HEPES and 20 µg/ml gentamycin. All PBMCs were maintained at 37°C in a 5% CO_2 humidified incubator with a volume of 500 µl/well in a 24-well plate and all treatments were performed in triplicate for the duration of the experiment. The plates were pre-incubated for 1 hour 30 minutes at 37°C prior to exposures as described below

Mitogen Exposures

The mitogens, phytohemagglutinin P (PHA-P) (1 µg/ml) and pokeweed mitogen (PWN) (0.5 µg/ml), were added in together to triplicate wells (20 µl) based on an adapted protocol (Bansal et al., 1993). Control wells contained similar volumes of RPMI added to the PBMCs. The plates were incubated for a further 48 hours at 37°C. This was repeated for each sample preparation from each animal.

Propidium Iodide Exclusion Assay

The viability of lymphocytes in both fresh and frozen PBMCs was determined by propidium iodide (PI) exclusion. 500 µl of the cell suspension ($1x\ 10^6$ ml) from the different samples was taken from the 24-well plate at each specific time point and transferred to flow cytometer tubes. In addition, all biological samples and PBS buffers were kept on ice unless otherwise indicated. The cell suspensions were diluted in 2 ml of PBS and centrifuged for 7 min at 300 x g. The supernatant was then aspirated off and this procedure was repeated twice. The supernatant was discarded and the cells were resuspended in a volume of 100 µl PBS in the tubes to which 5 µl of PI (50 µg/ml in PBS) was added. The cells were incubated for 15 min in the dark at room temperature and the volume was brought to a total of 500 µl with PBS. These samples were subsequently analysed by flow cytometer on a FACS Calibur instrument (Becton

Dickinson, UK). As stated previously, the only cell types that were analysed in the PBMC fraction were lymphocytes as the monocytes, dendritic cells and basophils were still attached to the plastic tissue culture plate. A minimum of 10,000 events (in the lymphocytes gate) were analysed for each sample. Lymphocyte gating was determined by analysing the forward scatter (FSC) and side scatter (SSC) profile of the PBMCs on a dot plot as shown in Figure 3A. Objective gating was used based on those cell populations identified from the Cell Quest Pro manual (Becton Dickinson, UK) and also by the guidelines set out by Calvelli et al., (1993). The criteria for gating included lymphocytes which were both positive and negative for PI staining. The same gate was used for all samples from the same animal species. Different settings were needed for sheep and rats. Data from this lymphocyte gate were displayed as a histogram with PI (red fluorescence, X axis) vs. the number of events (Y-axis) as shown in Figure 3B. The number of positive cells (PI-positive) was measured, reflecting the number of necrotic and late apoptotic cells; the % of viable cells was calculated from this using Cell Quest Pro software (Becton Dickinson, UK).

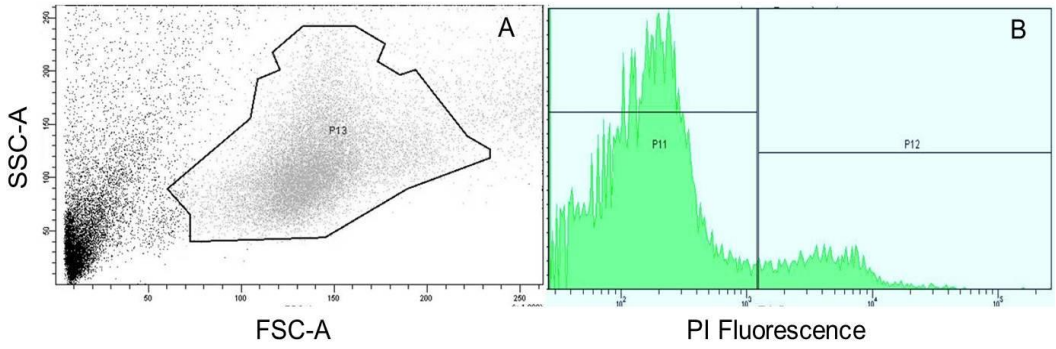


Figure 3A and 3B. Flow Cytometry Profiles of Rat Lymphocyte for Live and Dead Cell Determination using Different Parameters.
A plot of rat PBMCs when gating around lymphocytes using forward scatter (FSC) vs. side scatter (SSC) is shown in Figure 3A.
A histogram of rat PBMCs when gating around lymphocytes with propidium iodide fluorescence (PI) (X-axis) vs. frequency of viable and non-viable cells (Y-axis) is shown in Figure 3B.

Annexin V-FITC Apoptosis Assay

Following culture, PBMCs were prepared and stained with annexin V-FITC (excitation 488nm/emission 530nm) and propidium iodide (PI) (excitation 488nm/emission 585nm) according to the manufacturer's instructions (Becton Dickinson, UK). Staining cells simultaneously with annexin V-FITC (green fluorescence) and the DNA intercalating dye, propidium iodide (red fluorescence), allows (bivariate analysis) the discrimination of viable cells (AnV-FITC$^-$PI$^-$), early apoptotic (AnV-FITC$^+$PI$^-$) and late apoptotic or necrotic cells (AnV-FITC$^+$PI$^+$). Staining of cells for flow cytometric analysis was done in flow tubes (Falcon, BD Biosciences, UK) containing 500 µl of the PBMCs ($1x10^6$cells/ml). Cell pellets were washed with PBS and centrifuged for 7 minutes at 300 x g and the supernatant discarded. The cell pellet was then resuspended in 100 µl of freshly prepared binding buffer (The 10X buffer [0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$] was diluted to a 1X solution) and the cells were stained with 5 µl of PI (50 µg/ml in PBS) and 5 µl of annexinV-FITC (1µg/ml in binding buffer) and incubated for 15 minutes in the dark at room temperature. After the incubation was complete, 400 µl of binding buffer was added and cells were analysed (Moore et al., 1998). Data were displayed as a two-colour dot plot with annexin V-FITC (AnV-FITC) (green fluorescence, X axis) vs. propidium iodide (red fluorescence, Y axis) as shown in Figure 4.

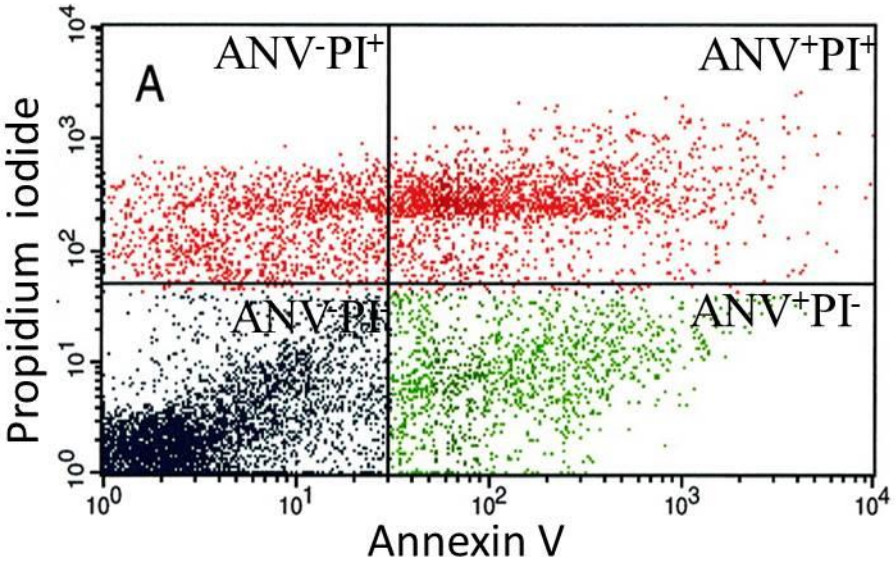


Figure 4. Annexin V (AnV) /Propidium Iodide (PI) Bivariate Analysis of Rat Lymphocytes Used to Identify the Different Cell Sub-Populations. The lower left quadrant contains AnV-FITC$^-$/PI$^-$(black-stained) viable, non-apoptotic lymphocytes. The lower right quadrant contains AnV-FITC $^+$/PI$^-$ (green-stained) early apoptotic lymphocytes. The upper left (AnV-FITC $^-$/PI$^+$) and right (AnV-FITC $^+$/PI$^+$) quadrants (red-stained) contain late apoptotic or necrotic lymphocytes

Statistical Analysis

Results are expressed as the means ± standard deviation (SD) for three independent experiments with the means based on triplicate analysis for each experiment. All statistical analyses used Sigma Stat software (version 2.03). Data were tested for normality using the Kolmogorov-Smirmnov test. Differences between group means were analysed using a one-way ANOVA followed by Tukey post-hoc test for multiple comparisons. Data significance was set at *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

The Effect of Sample Processing on Lymphocyte Viability of Sheep PBMCs over 24 Hours in Culture

The viability of the cells in the various sheep PBMC samples as measured by the trypan blue stain on a haemocytometer was between 91% and 85%, depending on what sheep sample was measured, before the samples were placed in the slow-rate freezing container (data not shown). There was no change in viability as measured by this method when the samples were measured immediately after thawing or in the case of samples after 3 hours in culture that were measured by the propidium iodide (PI) exclusion assay (data not shown).

A comparison of the lymphocyte viability as measured by the PI exclusion assay in the frozen PBMCs from four sheep in culture after 3 hours and 24 hours is shown in Figure 5. The viability of the lymphocytes after 3 hours in culture was 90% for Sheep 445; 82% for Sheep 405; 74% for Sheep 555 and 72% for Sheep 645 and correlated with the duration time between blood sampling and processing for each sheep i.e. 5.5 hours, 7.5 hours, 9.5 hours and 10.5 hours, respectively. The number of viable lymphocytes in frozen PBMCs from all four sheep decreased when they were cultured for 24 hours. The sample viabilities dropped by 13% ($p<0.01$) for Sheep 405 to 69%; by 14% ($p<0.001$) for Sheep 445 to 77%; by 18% ($p<0.001$) for Sheep 645 to 54% and by 22% ($p<0.001$) for Sheep 555 to 52%. Duplicate wells were used for each time point and the experiment was performed in triplicate (3 individual frozen PBMC aliquots were thawed out for each sheep in independent experiments on different days).

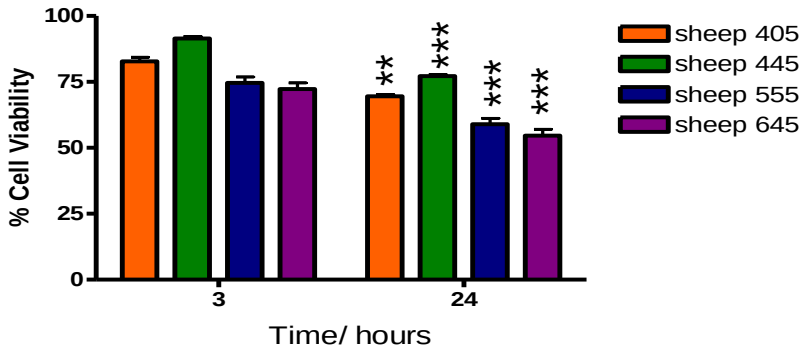


Figure 5. Comparison of the Lymphocyte Viability in Frozen PBMCs from Different Sheep over 24 Hours in Culture. Data are represented as the percentage of viable lymphocytes in various frozen sheep PBMCs over culture periods of 3 and 24 hours assessed by PI staining and measured by flow cytometry. Data are represented as means +/- SD for three independent experiments (frozen aliquots), with 3 replicates per experiment. 3 hours vs 24 hours (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

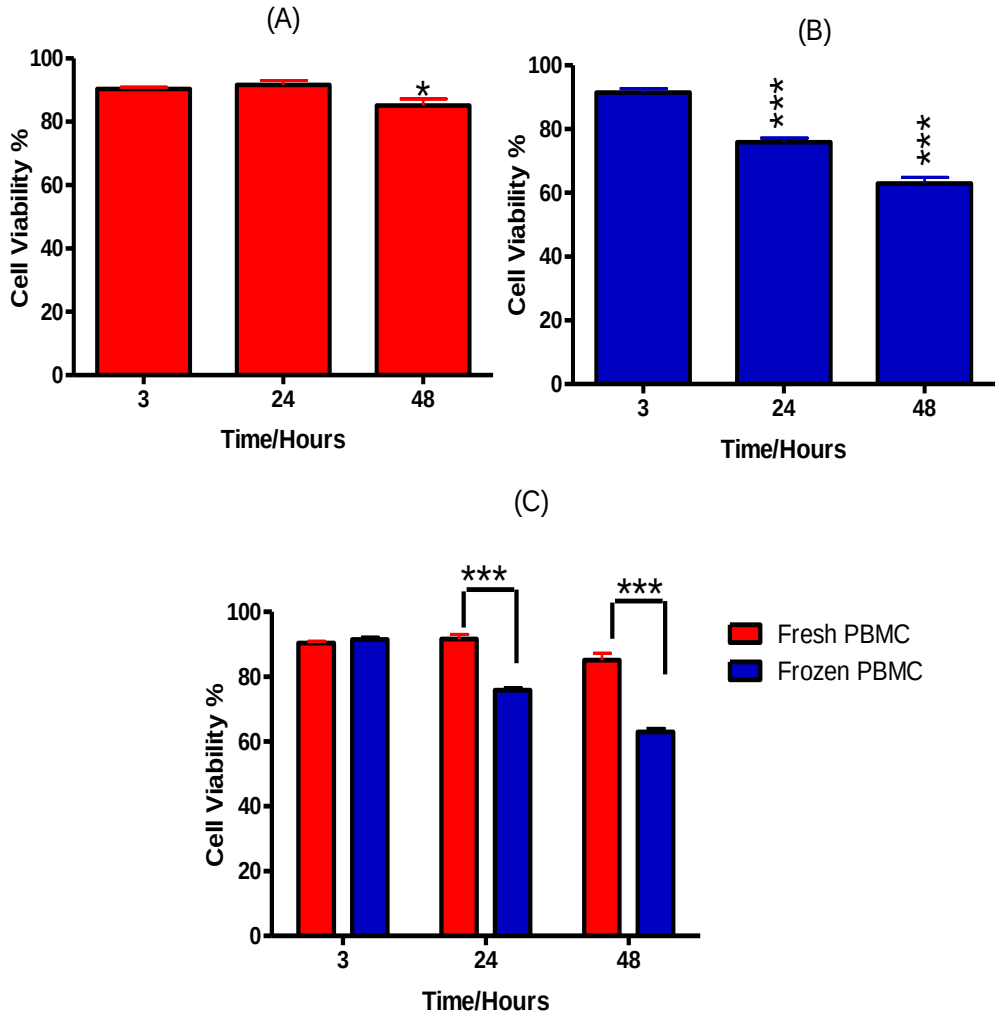
Comparison of the Lymphocyte Viability of Fresh and Frozen Rat PBMCs over 48 Hours in Culture

The levels of viability as measured by propidium iodide (PI) staining in the lymphocyte gated subsets of fresh and frozen PBMCs from rats at 3, 24 and 48 hours are shown in Figures 6A and 6B.

In the case of fresh PBMCs, duplicate wells were analysed for each time point and 3 separate fresh blood draws were taken from three different rats. At 48 hours there was about a 5% loss in viability in fresh PBMCs from the initial 90% measured at 3 hours ($p<0.05$)(Figure 6A).

In the case of frozen PBMCs, duplicate wells were used for each time point and 3 individual frozen PBMC aliquots were thawed out from separate rats. The viability was reduced by 16% at 24 hours and by 29% at 48 hours from the initial 90% measured at 3 hours ($p<0.001$) (Figure 6B).

There was no difference in viability in fresh compared to frozen PBMCs at 3 hours but at 24 hours and 48 hours, the viability in the frozen samples was reduced by 15% ($p<0.001$) and 22% ($p<0.001$), respectively. Therefore, after 48 hours in culture, lymphocyte viability was 85% for fresh PBMCs and after cryopreservation and culture this decreased to 62% (Figure 6C).



Figures 6A and 6B. The Viability of Lymphocytes in Fresh and Frozen Rat PBMCs over 48 Hours in Culture. Data are represented as the percentage of viable lymphocytes in fresh (A) and frozen (B) PBMCs over culture periods of 3, 24 and 48 hours as assessed by PI staining. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment. (frozen aliquots or fresh blood draws). Statistical analysis compares 3 hours vs other time points (* $p < 0:05$, ** $p < 0:05$ and *** $p < 0:001$).

Figure 6C. Comparison of the Viability of Lymphocytes in Fresh and Frozen Rat PBMCs over 48 Hours in Culture. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares fresh vs frozen PBMCs (* $p < 0:05$, ** $p < 0:05$ and *** $p < 0:001$).

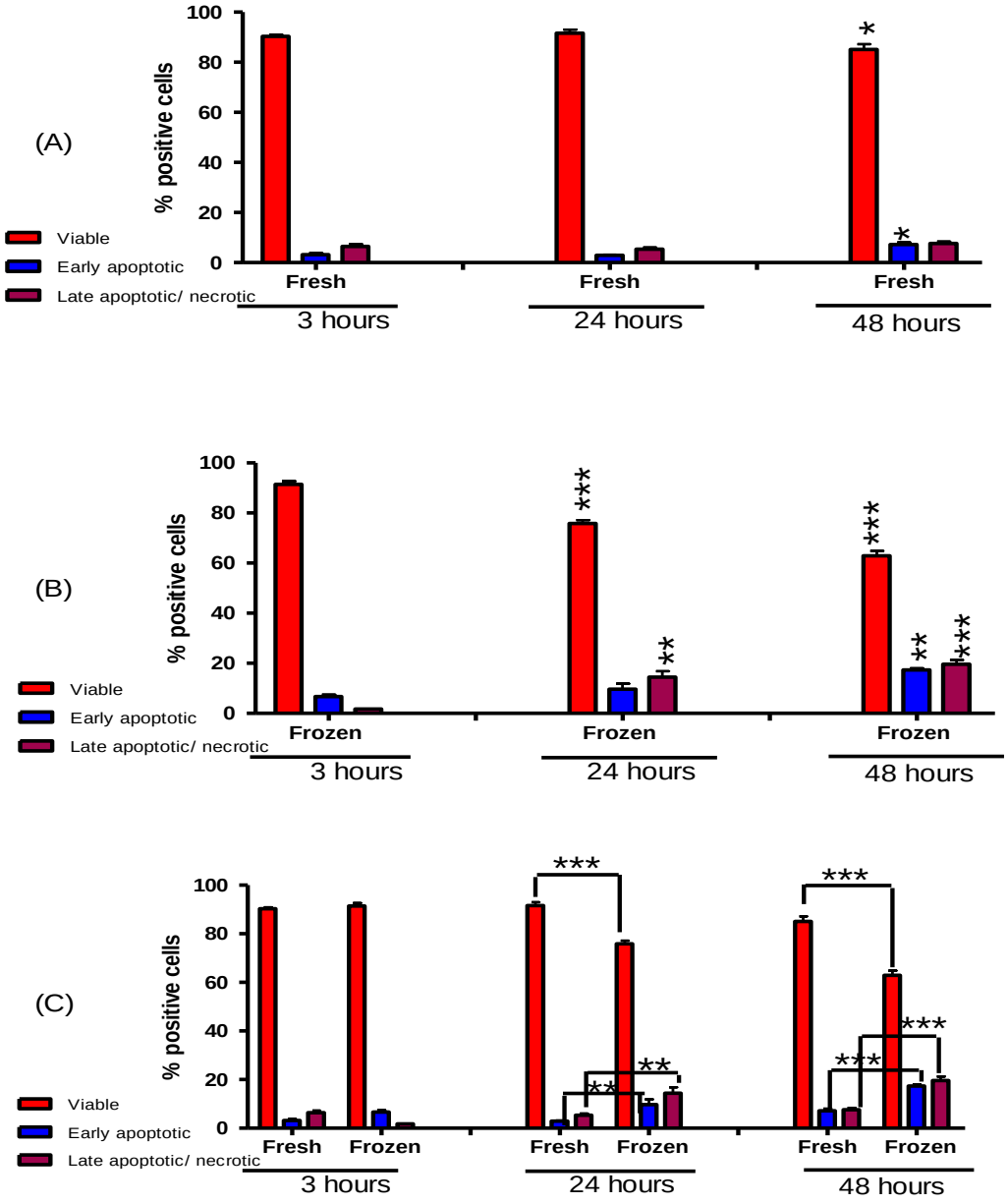
Comparison of the Levels of Apoptosis Induction in Fresh and Frozen Rat PBMCs over 48 Hours in Culture

An investigation into the contribution of apoptosis to the total decrease in viability after 48 hours was undertaken. The levels of viability, early apoptosis and late apoptosis/necrosis in the lymphocyte gated subset of fresh PBMCs from rats at 3, 24 and 48 hours, as assessed by annexin V (AnV-FITC) and PI staining, are shown in Figure 7A. The reduction in viability does not appear to be the result of early apoptosis. The lymphocyte viability at 3 hours for fresh PBMCs was 90% and there was a 5% drop in viable lymphocytes (AnV-FITC$^-$ PI$^-$) from 3 hours to 48 hours in culture ($p<0.05$). The level of early apoptotic lymphocytes (AnV-FITC $^+$PI$^-$) was 3% after 3 hours and after 48 hours it increased by 4% to 7%.($p<0.05$). The levels of late apoptosis/ necrosis (AnV-FITC $^+$PI$^+$)/ (AnV-FITC $^-$PI$^+$) in fresh PBMCs were 6% and 7% at 3 hours and 48 hours, respectively.

The contribution of apoptosis and necrosis to the cell death of lymphocyte in frozen rat PBMCs at 3, 24 and 48 hours in culture, are shown in Figure 7B. The lymphocyte viability (AnV-FITC $^-$ PI$^-$) at 3 hours was 91% and there was a 15% drop from 3 hours to 24 hours ($p<0.001$) and a 28% drop from 3 hours to 48 hours to 62% ($p<0.001$). The early apoptotic lymphocytes (AnV-FITC $^+$PI$^-$) after 3 hours had a level of 6% and after 24 hours this level increased by 3% to 9% and by 11% to 17%.($p<0.01$) at 48 hours. The level of late apoptosis or necrosis (AnV-FITC $^+$PI$^+$)/ (AnV-FITC$^-$PI$^+$) was 3% after 3 hours and increased by 12% to 15% ($p<0.01$) after 24 hours and by 17% to 20% ($p<0.001$) after 48 hours.

There was no difference between the number of viable lymphocytes (AnV-FITC$^-$ PI$^-$) from fresh and frozen PBMCs after 3 hours. However, there was a reduction of 15% ($p<0.001$) at 24 hours and 22% ($p<0.001$) at 48 hours in the frozen samples. The number of early apoptotic lymphocytes (AnV-FITC$^+$PI$^-$) increased by 3% at 3 hours after cryopreservation and by 6% ($p<0.01$) and 10% ($p<0.001$) at 24 hours and 48 hours, respectively. Likewise cryopreservation caused a 4% decrease in the level of late apoptosis or necrosis (AnV-FITC$^+$PI$^+$)/(AnV-FITC$^-$PI$^+$) in PBMCs at 3 hours. This

decrease changed to an increase of 9% ($p<0.001$) at 24 hours and increased to 12% ($p<0.001$) at 48 hours (Figure 7C).



Figures 7A and 7B. The Level of the Apoptotic Lymphocytes in Fresh and Frozen Rat PBMCs over 48 Hours in Culture.
Data are represented as the percentage of viable lymphocytes, early apoptotic and late apoptotic /necrotic lymphocytes in fresh (A) and frozen (B) PBMCs over culture periods of 3, 24 and 48 hours as assessed by annexin V and PI staining and analysed by flow cytometry. Data are represented as means +/- SD) with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares 3 hours vs other time points (** $p < 0:01$ and *** $p < 0:001$).

Figure 7C. Comparison of the Level of Apoptotic Lymphocytes in Fresh and Frozen Rat PBMCs over 48 Hours in Culture. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares fresh vs frozen (* $p < 0:05$, ** $p < 0:01$ and *** $p < 0:001$).

Comparison of the Lymphocyte Viability of Fresh and Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture

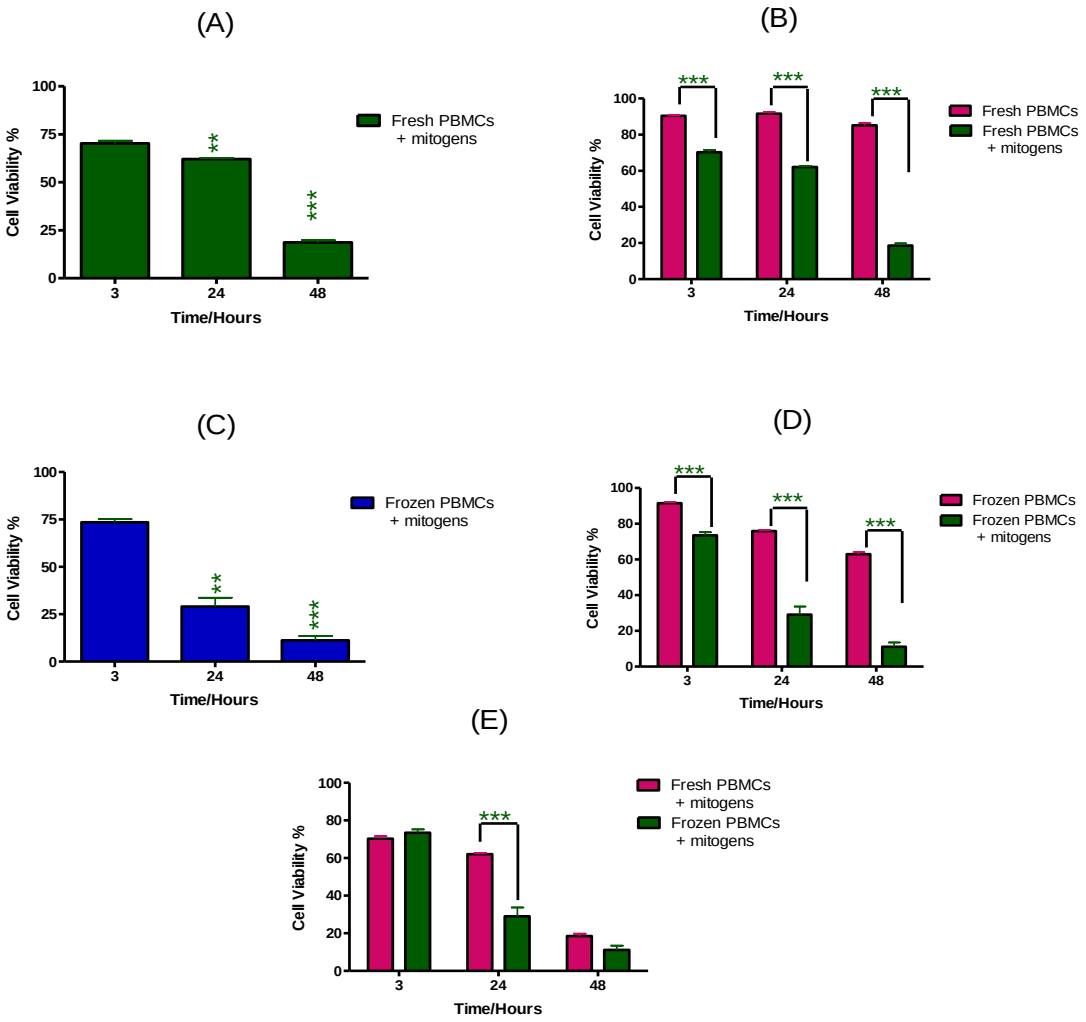
The levels of viability measured by propidium iodide (PI) staining in the lymphocyte gated subsets of fresh and frozen PBMCs from rats in the presence of mitogens, PHA-P and PWN, at 3, 24 and 48 hours are shown in Figure 8A. There was a loss in viability between 3 hours and 24 hours (71 to 63%) ($p<0.01$) and between 3 hours and 48 hours (71 to 19%)($p<0.001$)(Figure 8A).

In the case of fresh rat PBMCs, there was a significant difference in viability in fresh PBMCs compared to fresh samples stimulated by mitogens. At 3, 24 and 48 hours, the viability in the fresh samples in the presence of mitogens was reduced by 20% ($p<0.001$), 15% ($p<0.001$) and 66% ($p<0.001$), respectively. Therefore, after 48 hours in culture, lymphocyte viability was 85% for fresh PBMCs and the addition of mitogens decreased this to 18% (Figure 8B).

In the case of frozen rats PBMCs in the presence of mitogens there was a loss in viability between 3 hours and 24 hours (73 to 27%)($p<0.01$) and between 3 hours and 48 hours (73 to 11%)($p<0.001$) (Figure 8C).

There was a significant difference in viability in frozen PBMCs compared to frozen samples stimulated by mitogens. At 3, 24 and 48 hours, the viability in the frozen samples in the presence of mitogens was reduced by 17% ($p<0.001$), 44% ($p<0.001$) and 52% ($p<0.001$), respectively. After 48 hours in culture, lymphocyte viability was 63% for frozen PBMCs and the addition of mitogens decreased this to 11% (Figure 8D).

When both fresh and frozen PBMCs were stimulated with mitogens, there was no difference in viability at 3 hours but at 24 hours, the viability in the frozen samples was reduced by 32% ($p<0.001$) and by 7% at 48 hours. Therefore, after 48 hours in culture, lymphocyte viability was 18% for fresh PBMCs and this decreased to 11% for frozen PBMCs when both were stimulated with mitogens (Figure 8E).



Figures 8A and 8C. The Viability of Lymphocytes in Fresh and Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture. Data are represented as the percentage of viable lymphocytes in fresh (A) and frozen (C) PBMCs in the presence of mitogens over culture periods of 3, 24 and 48 hours as assessed by PI staining. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares 3 hours vs other time points (* $p < 0:05$, ** $p < 0:05$ and *** $p < 0:001$).

Figures 8B and 8D. Comparison of the Viability of Lymphocytes in Fresh and Frozen Rat PBMCs with or without Stimulation with Mitogens over 48 Hours in Culture. Data are represented as the percentage of viable lymphocytes, early apoptotic and late apoptotic /necrotic lymphocytes in fresh (B) and frozen (D) PBMCs in the presence or absence of mitogens over culture periods of 3, 24 and 48 hours as assessed by annexin V and PI staining and analysed by flow cytometry. Data are represented as means +/- SD. Statistical analysis compares fresh vs fresh + mitogens (** $p < 0:01$ and *** $p < 0:001$).

Figure 8E. Comparison of the Viability of Lymphocytes in Fresh and Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture. Data are represented as means +/- SD Statistical analysis compares fresh PBMCs with mitogens vs frozen PBMCs with mitogens. (* $p < 0:05$, ** $p < 0:05$ and *** $p < 0:001$).

Comparison of the Levels of Apoptosis Induction in Fresh Rat PBMCs Stimulated with or without Mitogens over 48 Hours Culture in Culture

The levels of viability, apoptosis and necrosis of lymphocytes in fresh PBMCs from rats in the presence of mitogens, PHA-P and PWN, at 3, 24 and 48 hours are shown in Figure 9A. The lymphocyte viability (AnV-FITC $^{-}$ PI^{-}) at 3 hours for fresh PBMCs with mitogens was 70% and there was an 8% drop from 3 hours to 24 hours ($p<0.001$) and a 51% reduction to 19% ($p<0.001$) between 3 hours and 48 hours. The level of early apoptotic lymphocytes (AnV-FITC $^{+}$PI^{-}) in fresh PBMCs with mitogens was14% after 3 hours and after 24 hours this level increased by 10% to 24% ($p<0.001$) and by 14% to 29% ($p<0.001$) at 48 hours. The level of late apoptosis/ necrosis (AnV-FITC $^{+}$PI^{+})/ (AnV-FITC $^{-}$PI^{+}) after 3 hours was 16% and stayed at this same level after 24 hours. It increased by 38% to 54% ($p<0.001$) at 48 hours.

The contributions of apoptosis and necrosis to the cell death of lymphocyte in fresh rat PBMCs in the presence or absence of mitogens, PHA-P and PWN, at 3, 24 and 48 hours are shown in Figure 9B. The number of viable lymphocytes (AnV-FITC^{-}PI^{-}) in fresh PBMCs was reduced by 20% ($p<0.001$) after 3 hours in the presence of mitogens. The reduction increased to 29% ($p<0.001$) at 24 hours and to 66% ($p<0.001$) at 48 hours. The level of early apoptotic lymphocytes (AnV-FITC^{+}PI^{-}) increased by 11% ($p<0.001$) at 3 hours in the presence of mitogens and by 21% ($p<0.001$) and 20% ($p<0.001$) at 24 hours and 48 hours, respectively. The addition of mitogens caused a 10 % ($p<0.001$) increase in the level of late apoptosis or necrosis (AnV-FITC^{+}PI^{+})/ (AnV-FITC^{-}PI^{+}) in fresh PBMCs at 3 hours. The increase remained at 10% ($p<0.001$) at 24 hours but increased to 45% ($p<0.001$) at 48 hours.

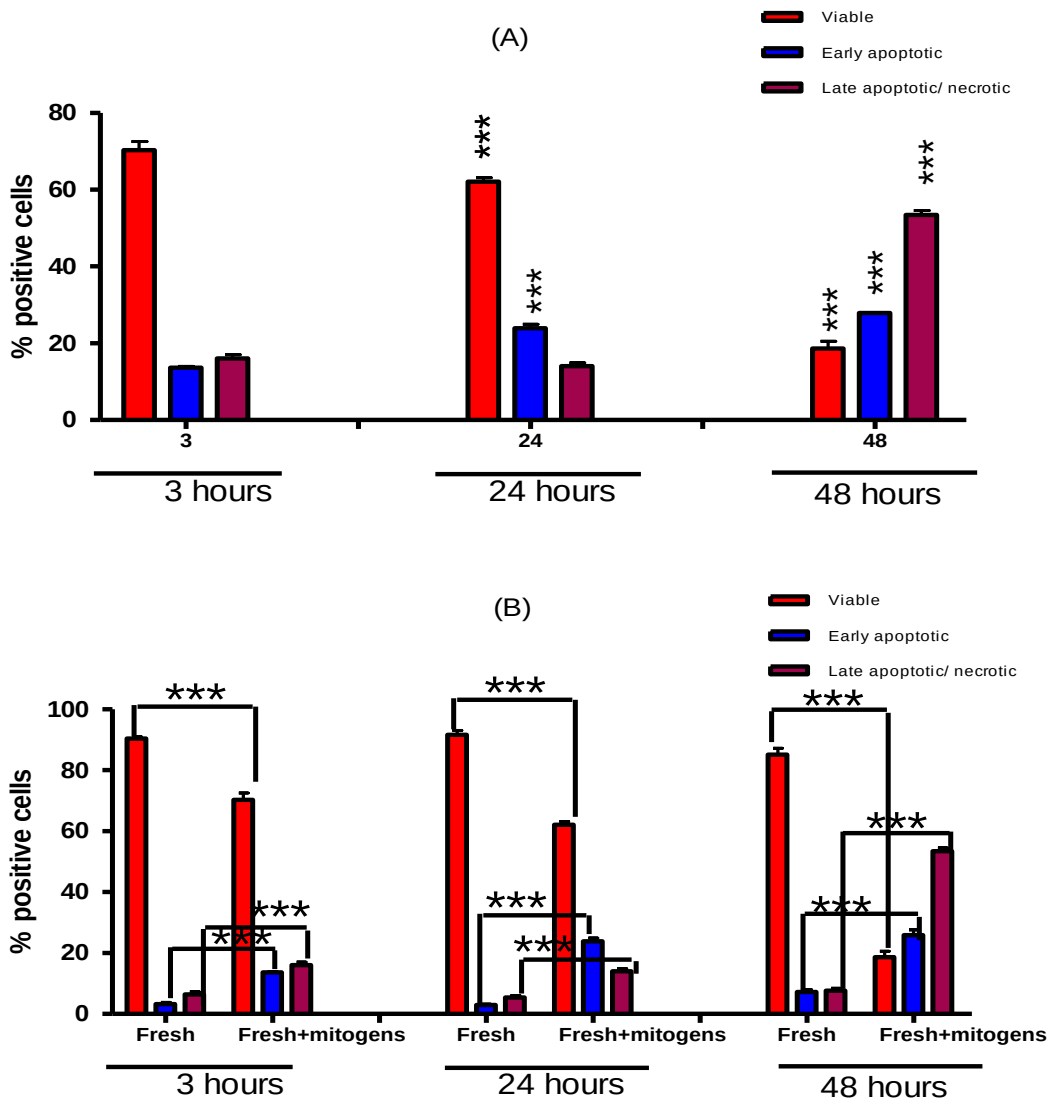


Figure 9A. The Level of the Apoptotic Lymphocytes in Fresh Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares 3 hours vs other time points (** p < 0:01 and *** p < 0:001).

Figure 9B. Comparison of the Level of Apoptotic Lymphocytes in Fresh Rat PBMCs Stimulated with or without Mitogens over 48 Hours in Culture. Data are represented as the percentage of viable lymphocytes, early apoptotic and late apoptotic /necrotic lymphocytes in fresh PBMCs in the presence or absence of mitogens over culture periods of 3, 24 and 48 hours as assessed by annexin V and PI staining and analysed by flow cytometry. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots or fresh blood draws). Statistical analysis compares fresh vs fresh PBMCs + mitogens (** p < 0:01 and *** p < 0:001).

Comparison of the Levels of Apoptosis Induction in Fresh and Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture

The levels of viability, apoptosis and necrosis in the lymphocyte gated subset of frozen PBMCs from rats in the presence of mitogens, PHA-P and PWN, at 3, 24 and 48 hours are shown in Figure 10A. The lymphocyte viability (AnV-FITC$^-$PI$^-$) at 3 hours was 73% and there was a 45% drop from 3 hours to 24 hours ($p<0.001$) and a 62% reduction to 11% ($p<0.001$) between 3 hours and 48 hours. The level of early apoptotic lymphocytes (AnV-FITC$^+$PI$^-$) was 18% after 3 hours and after 24 hours this level decreased by 8% to 10% ($p<0.001$) and by 6% to 11% ($p<0.001$) at 48 hours . The level of late apoptosis/ necrosis (AnV-FITC$^+$PI$^+$)/ (AnV-FITC$^-$PI$^+$) after 3 hours was 9% and increased by 54% after 24 hours and by 71% to 80% ($p<0.001$) at 48 hours.

The contributions of apoptosis and necrosis to the cell death of lymphocyte in frozen rat PBMCs in the presence or absence of mitogens, PHA-P and PWN at 3, 24 and 48 hours in culture are show in Figure 10B. The number of viable lymphocytes (AnV-FITC$^-$PI$^-$) in frozen PBMCs was reduced by 17% ($p<0.001$) after 3 hours in the presence of mitogens. The reduction increased to 46% ($p<0.001$) at 24 hours and to 51% ($p<0.001$) at 48 hours. The level of early apoptotic lymphocytes (AnV-FITC$^+$PI$^-$) increased by 10% ($p<0.001$) at 3 hours in the presence of mitogens but decreased by 2% and 8% ($p<0.001$) at 24 hours and 48 hours, respectively. The addition of mitogens caused a 7 % ($p<0.001$) increase in the level of late apoptosis/ necrosis (AnV-FITC $^+$PI$^+$)/(AnV-FITC $^-$PI$^+$) in frozen PBMCs at 3 hours. The increase remained at 48% ($p<0.001$) at 24 hours but increased to 59% ($p<0.001$) at 48 hours.

When the data for fresh and frozen PBMCs from rats were compared, the number of viable lymphocytes (AnV-FITC$^-$PI$^-$) in frozen PBMCs was reduced by 3% after 3 hours in the presence of mitogens. The reduction increased to 32% ($p<0.001$) at 24 hours and to 7% ($p<0.001$) at 48 hours. The level of early apoptotic lymphocytes (AnV-FITC$^+$PI$^-$) increased by 3% at 3 hours in the presence of mitogens and after cryopreservation but decreased by 16% ($p<0.001$) and 17% ($p<0.001$) at 24 hours and 48 hours, respectively. The addition of mitogens and cryopreservation caused no change in the level of late apoptosis or necrosis (AnV-FITC$^+$PI$^+$)/(AnV-FITC$^-$PI$^+$) in PBMCs at 3 hours but their

level increased by 49% (p<0.001) at 24 hours and by 26% (p<0.001) at 48 hours (Figure 10C).

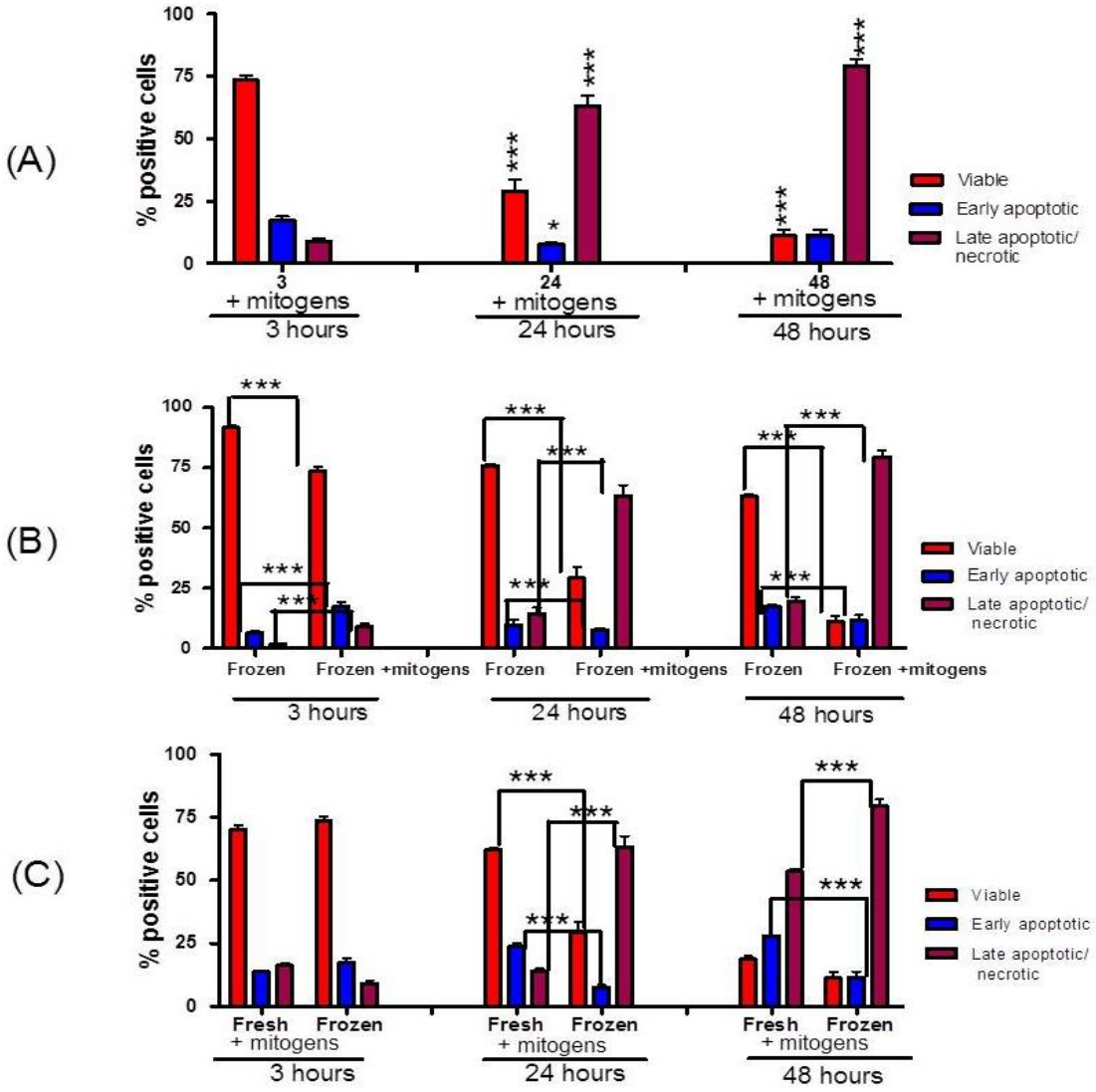


Figure 10A. The Level of the Apoptotic Lymphocytes in Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots). Statistical analysis compares 3 hours vs other time points (** p < 0:01 and *** p < 0:001).

Figure 10B. Comparison of Level of Apoptotic Lymphocytes in Frozen Rat PBMCs Stimulated with or without Mitogens over 48 Hours in Culture. Data are represented as the percentage of viable lymphocytes, early apoptotic and late apoptotic /necrotic lymphocytes in frozen PBMCs in the presence or absence of mitogens over culture periods of 3, 24 and 48 hours as assessed by annexin V and PI. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (frozen aliquots). Statistical analysis compares frozen PBMCs with and without mitogens.

Figure 10C. Comparison of the Level of Apoptotic Lymphocytes in Fresh and Frozen Rat PBMCs Stimulated with Mitogens over 48 Hours in Culture. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment (fresh blood draws or frozen aliquots). Statistical analysis compares fresh PBMCs with mitogens vs frozen PBMCs with mitogens * (* p < 0:05, ** p < 0:01 and *** p < 0:001).

Discussion

The biomonitoring of environmental contaminants involving fresh peripheral blood mononuclear cells (PBMCs) has been successfully conducted in wild animals such as bottlenose dolphins exposed to organochloride contaminants (Lahvis et al., 1995) and tree swallows exposed to pesticides (Bishop et al., 1998) with multiple samples taken from up to 15 animals. In the field, adequate sample collection procedures for analysing immune function, genotoxicity and adduct formation, which are the mainstays of biological effect monitoring, are dependent on a variety of factors affecting the quality of the samples. These include the type of blood containers, speed of processing, stability conditions between transport from sample site and laboratory, minimal time between processing and a manageable quantity of samples. In addition, one has to be aware of the increased length of processing time involved for multiple blood samples. A labour-intensive sample processing method, such as the isolation of PBMCs, takes substantially more time than the much simpler methods normally used for genetic and cytokine analysis for which whole blood is cultured (Connor, 2000).

In the present study, the blood samples were taken from sheep located in the field relatively close (2 kilometres, approx 15 minutes travel time) to the laboratory. In the cases of environment contamination reported in areas such as Silvermines, Co. Tipperary, Castlecomer, Co. Kilkenny, and Askeaton, Co. Limerick, the transport times to a suitable laboratory such as the Regional Veterinary Laboratories in Limerick or the State Veterinary Laboratory in Kilkenny would be greater (approximately 45 minutes).

9x9 ml blood samples were collected from each of four sheep under field-based conditions and the blood was pooled before fresh PBMCs were isolated from each sample and then aliquoted into cryovials and cryopreserved. Therefore, cryopreservation of the fresh PBMCs was used to stabilise the samples and make them available for subsequent assays in the laboratory.

Because of the number of samples collected in the field , there were differences between the samples from each sheep in the time that elapsed between venepuncture, storage , transportation and isolation of PBMCs and their cryopreservation (5.5, 7.5, 9.5, and

10.5 hours for sheep 455, 405, 555 and 645, respectively). Given the number of samples and the resources available in the laboratory adjacent to the field, it was not possible to assess the viability of freshly-isolated PBMCs by the propidium iodide (PI) exclusion assay. Instead, the number of viable cells in the PBMCs before cryopreservation was counted on a haemocytometer using the trypan blue stain. This method gives a measure of the membrane integrity of the cells. There were differences in the levels of membrane integrity in the four sheep samples and, overall, the reduction in the membrane integrity correlated with the increasing time that elapsed between blood collection and cryopreservation of the PBMCs.

The viability of PBMCs, as measured by membrane integrity before cryopreservation, was comparable to those measured by the PI exclusion assay at 3 hours post thawing. While trypan blue exclusion is a routinely used benchmark assay, it is very dependent on operator accuracy as monocytes and other mononuclear white blood cells might be counted as lymphocytes. In contrast, only lymphocytes can be analysed in the PI exclusion assay as one can exclude part of the cell population (monocytes and granulocytes) and debris using objective gating strategies. Despite these considerations there was good correlation between the two methods used.

The viabilities of the frozen sheep PBMCs, ranged between 91 and 72% after 3 hours in culture. When the time in culture was extended to 24 hours, the viabilities of all sheep samples were below 80% and the lowest viability (50%) was that of the samples with the greatest delay before processing.

The observation that viability was greatly reduced by delayed processing is in agreement with the findings of Kristal et al. (2005) who found that when the processing time was 32 hours the viability was around 75% but when blood storage was extended to 144 hours the viability decreased to 52%. Beck et al. (2001) reported that even after 6 days before processing, frozen lymphocytes could be transformed with Epstein Barr virus (EBV) similar to fresh lymphocytes even for multiple sample collections. However, these studies took place in a laboratory environment and did not compare the differences in laboratory versus field-based processes and the impact that extended storage at room temperature would have on the viability of frozen PBMCs. A noticeable difference between these studies was that EDTA was used as the anticoagulant in the former study (Kristal, 2005) while acid-citrate-dextrose (ACD) was used in the latter

(Beck et al., 2001). However, in a further study, no differences were detected in cell recovery, viability and immune function when EDTA, ACD and sodium heparin were used as anticoagulants in the collection of blood samples for the cryopreservation of PBMCs (Bull et al., 2007). The time lag between blood collection, processing and analysis or cryopresvation, which could be a more important factor than the type of anticoagulant used in the blood tubes, was also examined by Bull et al, (2007). They found that when cryopreserved within 8 hours of venepuncture, PBMC recovery, viability and immune function were not significantly affected by the blood anticoagulant used but when the storage was extended to 24 hours these parameters were affected. This effect of extended storage is in agreement with the findings for sheep 405 and 445 compared to sheep 555 and 645 in the present study.

The field-studies with sheep blood showed that cryopreservation was a suitable method for the provision of a supply of viable PBMCs for *ex vivo* assays when processed within 5.5 hours. However, because it is easy to collect, relatively cheap and provides enough volumes, rat blood, rather than sheep blood, was used for more detailed comparative studies on fresh and frozen PBMCs. It is important to have high cell viability in cryopreserved samples so that reproducible, reliable and relevant results can be achieved in assays using immune or toxicological endpoints. The general consensus is that for any cell assay, one should have a starting viability of >80% (Kleeberger et al., 1999). Therefore, as part of control practices, viability tests need to be performed on cryopreserved cells in order to verify that preservation is effective, i.e. that all or nearly all the cells are viable upon thawing. The benefits of this were demonstrated in human clinical studies (Weinberg et al., 2000) and it is known that cryopreservation can have negative impacts on the characteristics of cells (Deneys et al., 1999, Fowke et al., 2000, Reimann et al., 2000, Cavers et al., 2002). In a study by Betensky et al. (2000), consistent proliferative T-cell responses were observed in assays after 72 hours in culture when the viability of thawed PBMCs was greater than or equal to 70%.

In the case of fresh rat PBMCs, there was a small decrease (5%) in the number of viable cells over a 48 hour culture period. This is in agreement with other studies that observed viabilities of 95-90% after isolation and during long term culture (Sarkar et al., 2003, Disis et al., 2006, Garcia-Pineres et al., 2006, Ruitenberg et al., 2006). Interestingly, up to 90% of the lymphocytes in the frozen PBMCs were viable after cryopreservation

when cultured for 3 hours. These results are in agreement with those of Wang et al. (1998) and Fowke et al. (2000) who reported 89% and 86% viability, respectively, for PBMCs after cryopreservation and with those of Venkataraman and colleagues who reported 98% of cells with membrane integrity using the cryobox method (Venkataraman et al., 1992, Wang et al., 1998). However, other studies have reported reduced viabilities of 70 -80% after cryopreservation and these may have been caused by the use of quick freezing rather than the slow-controlled method (Ichino and Ishikawa, 1985, Weinberg et al., 2000, Riccio et al., 2002).

In contrast to the fresh PBMCs, there was a considerable loss of viable lymphocytes (30%) when the frozen samples were cultured for 48 hours. This loss of viability could result from either of two mechanisms of cell death, namely necrosis or apoptosis and the contribution of these was investigated since both processes could affect the interpretation of the data obtained in other assays used in this study.

Annexin V and propidium iodide bivariate analysis detects the breaking up of the phospholipid asymmetry of the plasma membrane (Fadok et al., 2000).There are various markers of apoptosis which can be measured by a number of approaches such as by the nuclease activation of apoptosis or by the formation of a DNA ladder following agarose gel electrophoresis, or by the end-labelling of the newly created DNA ends (the TUNNEL assay) or by the changes in chromatin structure using DNA binding dyes and fluorescent microscopy. Since the events being measured can occur at different stages of apoptosis, inter-study comparisons can be difficult, depending on the technique used (Darzynkiewicz and Traganos, 1998, Mills et al., 1998). Cells undergoing early apoptotic events can still repel trypan blue and propidium iodide and, therefore, may still be considered viable according to the lack of cell permeability but these cells could still be on the way to becoming late apoptotic at a later time point and these cells could be detected with annexin V staining (O'Brien and Bolton, 1995).

When fresh PBMCs were cultured for 48 hours, there was a slight increase (up to 7%) in levels of early apoptotic lymphocytes and the number of late apoptotic / necrosis lymphocytes was less than 10%. These results are in agreement with those of Jeurink et al. (2008) who reported an increase in early and late apoptotics cells of around 20% at 24 hours in fresh PBMCs. However, the results from both studies are in contrast with a

previous report where the number of apoptotic cells was very low (5%) up to and throughout 5 days culture (Sarkar et al., 2003).

Frozen PBMCs had higher levels of early apoptosis (16% at 48 hours) than fresh cells and 21% of the frozen PBMCs were in late apoptosis/necrosis phase at 48 hours. The level of apoptotic lymphocytes was about 40% at 48 hours which is similar to other studies (Sarkar et al., 2003, Jeurink et al., 2008). Cryopreserved cells usually show higher levels of apoptosis compared to fresh cells in bone marrow, hepatocyte and lymphocyte culture systems (Glander and Schaller, 1999, Schmidt-Mende et al., 2000, Yagi et al., 2001, Sarkar et al., 2003). However, Riccio et al., (2002) reported that the frequencies of apoptosis were very low, at about 10 % for both fresh and frozen PBMCs after 24 hours in culture. The discrepancies between the results may be explained by differences between the methods used to measure apoptosis. The breaking up of the phospholipid asymmetry of the plasma membrane, as detected by annexin V binding, happens at an earlier time point than the DNA fragmentation or chromatin condensation described by Riccio et al., (2002).

The main mechanism associated with the reduction in viability when PBMCs were cryopreserved is apoptosis and this was detected by an increase in annexin V positive cells at 24 hours. What is encouraging is that at 3 hours there was no difference in the percentage of viable lymphocytes between fresh and frozen PBMCs. The next step was to examine whether these frozen PBMCs could be used to assess immunotoxicity manifested by either increased immune activity or decreased immune function. Cell mediated immunity can be assessed by the lymphocyte proliferation assay which involves culturing PBMCs with mitogens. The proliferation is necessary to investigate immune functionality of the cells because lymphocytes are quiescent and need to be stimulated (Keller et al., 2005). The normal duration of the lymphocyte proliferation assay is 72 hours but this is dependent on the specific mitogens used. Because of the major loss of viability observed after 48 hours, the 72 hour time point was not used.

The lymphocyte viability of fresh rat PBMCs stimulated with the mitogens, phytohemagglutin (PHA-P) and poke weed mitogen (PWM), decreased substantially by almost 70% compared to fresh PBMCs without stimulation after 48 hours and corresponded to a significant increase in early (23%) and late apoptotic /necrotic (47%) lymphocytes. This is in agreement with the use of the mitogen, concanavalinA (ConA),

which caused a similar decrease in viability and increase in apoptotic lymphocytes (Jeurink et al., 2008). In the case of cryopreserved PBMCs stimulated by mitogens, there was a large reduction (87%) in the level of cell viability at 48 hours in culture but the reduction was not substantial after 3 hours. This high level of cell death is much higher than that previously reported by Jeurink et al, (2008). However, these workers stimulated only T-cells, in contrast to the use of mitogens to stimulate both T-and B-cells and increase the level of cell death in the present study. There was a difference in the level of early apoptotic cells between fresh and frozen PBMCs stimulated by mitogens. For frozen PBMCs, 17% were in the early apoptotic phase at 3 hours but these then became late apoptotic/necrotic lymphocytes after 48 hours. Overall, however, there was very little difference between the inductions of apoptosis in frozen PBMCs (80%) compared to fresh PBMCs (70%) after treatment with mitogens over a 48 hour period.

The phenomenon of mitogen-induced apoptosis has already been described in the case of T-lymphocytes. These cells can receive signals that prime them for apoptosis but they do not actually undergo cell death until the physical or physiological signal is received (Costas et al., 1996). It is possible that, during cryopreservation, cells receive certain physical or physiological signals that prime them for apoptosis but do not initiate this process. By culturing lymphocytes in the presence of mitogens, the primed cells can be driven fully into the death cascade by this activation-induced apoptosis (Yahata et al., 1999, Zhu et al., 1999).

There are reports in the literature that, upon thawing, lymphocyte samples from a number of human subjects did not respond to stimulation with PHA-P and failed to grow in culture, possibly due to low viability (King et al., 1997). After ConA stimulation of frozen PBMCs, proliferation activity was similar to that for fresh PBMCs after 48 hours (Jeurink et al., 2008). Furthermore, frozen PBMCs retained their proliferative ability after over 1 week storage at either -70°C in a freezer or -196°C in liquid nitrogen (Sobota et al., 1997). The cryopreservation protocol for PBMCs used in this study has also been used in an investigation of the responses of fresh and frozen T-cells from humans after stimulation with cytomegalovirus (CMV) in ELISPOT, cytokine flow cytometry and tetramer assays (Maecker et al., 2005) and there were similar responses in all these endpoints before and after cryopreservation.

Cryopreserved PBMCs have also been used routinely in numerous genotoxic assays such as the Comet and micronucleus assays (O'Donovan et al., 1995, Visvardis et al., 1997, Duthie et al., 2002). The option of cryopreservation of biological material is crucial to the success of studies that range from large epidemiological ones mainly interested in genetic analyses (Visvikis et al., 1998) to those focused on Epstein Barr Virus (EBV) transformation because the quantity of samples and the different times at which samples were received make it impossible to have analysed the blood samples in the fresh state (Stevens et al., 2007).

Main Conclusions

The time that sheep blood was kept at ambient temperature without processing had a major impact on the viability of the lymphocytes. The delays in the processing time after blood sample collection led to reduced viability of frozen sheep PBMCs after thawing and culturing for 3 and 24 hours. When the fresh sheep blood samples were left for longer than 5.5 hours at room temperature there was large drop in viability compared to those processed right away.

In the case of rat blood, there was no difference in the viabilities of lymphocytes in fresh and frozen PBMCs after 3 hours in culture. However, there was a significant reduction in the case of frozen PBMCs when the time in culture was extended to 24 and 48 hours. In addition, the levels of apoptosis in frozen PBMCs were much higher than those in fresh rat PBMCs after 24 and 48 hours in culture.

Stimulation of rat PBMCs with mitogens caused a reduced viability due to an increase in early apoptotic cells at 24 hours and late apoptotic cells after 48 hours. Cryopreservation followed by stimulation of PBMCs with mitogens led to a substantial reduction in viability after 24 hours, mainly due to an increase in late apoptotic /necrotic cells. The percentage of apoptotic cells provided the best indication as to whether PBMCs responded to mitogens.

Traditionally, fresh PBMCs isolated from blood have been the bench mark sample supporting most environmental biomonitoring studies. The results from this study specifically demonstrated that the ability to isolate and retain batches of PBMCs from multiple rats, coupled with the use of optimized processing, cryopreservation and

recovery conditions, led to well-preserved characteristics of frozen PBMCs when culturing in the short term up to 3 hours. On the basis of the initial results in rats, the cryopreservation of PBMCs is a promising procedure for long term storage of sample. However, the data in this Chapter indicate that one of the main restrictions of the cryopreservation approach described here is the reduction of viability and induction of apoptosis during prolonged culture time. This will affect the type of *in vitro* and *ex vivo* analysis which can be performed with these samples.

However, further studies are needed to identify sampling procedures, such as the use of frozen blood, which would be quicker than the isolation of PBMCs. These studies should be undertaken initially with rat blood before transferring the methodology to field-based studies.

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Chapter 3

The Effects of Sample Processing on the Response of Lymphocytes in Cytotoxicity and Genotoxic Assays using Rat Blood Preparations

Table of Contents

Introduction

Blood cells can act as sentinels for cytotoxic and genotoxic exposures; it is possible that exposure to chemicals may cause detectable changes in circulating blood cells at the cellular, protein and gene levels, that correlate with detrimental changes that may be observed in other tissues in the body (Liew et al., 2006). Therefore, blood is a common biological sample used for biomonitoring studies (Angerer et al., 2007).

In larger biomonitoring studies, it is often necessary to store samples for later analysis due to the amount of time required for sample collection, processing and assaying. To avoid blood sample degradation, morphological and biochemical changes or a decrease in cell viability, fresh whole blood should be processed within 6 hours if kept at room temperature (Kristal et al., 2005). Storing blood samples at 4°C slows down changes, but does not prevent them. Therefore, even those samples stored at 4°C have to be processed within a maximum of 24 hours, and this reduces the number of assays that can be performed (Narayanan et al., 2001). For field-based collections, a large number of samples leads to an increase in the processing times which could result to sample deterioration (reduction of viability). It is not always feasible to use processed fresh blood samples such as peripheral blood mononuclear cells (PBMCs) or leukocytes that are isolated on the same day as the assays need to be performed. Time is the biggest limiting factor on the number of samples being collected and processed by a small number of operators and introduces inter-assay variability since processing cannot be done for a large number of samples for the one assay but the processing and collection as well as the assays have to be extended to multiple days (Narayanan et al., 2001). These limitations could be overcome by developing techniques for the preservation of blood that will allow processing of a large number of samples over time, without affecting cell viability, DNA stability or immune response.

The method of choice used to investigate the effects of environmental exposure on blood is the isolation of PBMCs as they are the most transcriptionally active cells in the blood (Fan and Hegde, 2005). As discussed in Chapter 2, PBMCs are the benchmark blood sample preparation method (Boyum, 1968), especially for biomonitoring endpoint studies (van Leeuwen et al., 2008). Isolation of PBMCs is the most common sample processing technique used to study lymphocytes in genotoxicity assays (Tuo et al.,

1996) for which endpoints such as the formation of micronuclei (Ranaldi et al., 1998), sister chromatid exchanges, chromosomal aberrations (Tompa et al., 1994) and DNA damage have been measured. The Comet assay has been used to detect DNA damage induced in various mammalian cells and, of particular relevance to this study, has been used to measure DNA damage in blood cells of mice exposed to benzene (Tuo et al., 1996). It permits direct visualization of DNA damage in cells by assessing single and double strand breaks in DNA and allows the evaluation of DNA damage in proliferating and non-proliferating cells. One of its many benefits is that it requires only a low quantity of cellular samples (McKelvey-Martin et al., 1993).

DNA damage can be caused by genotoxic compounds such as ethyl methanesulphonate (EMS) and cadmium chloride ($CdCl_2$). EMS is a direct monofunctional alkylating agent that causes DNA adducts leading to single strand breaks causing point mutations (Davies et al., 1993, Anderson et al., 1996). EMS is a standard positive control for genotoxicity studies including the Comet assay as it. Cadmium is a class 1 carcinogen (IARC, 1993) and one of the most toxic environmental and industrial pollutants causing DNA damage, elevating lipid peroxidation *in vitro* and *in vivo,* complexing with thiol groups of enzymes and increasing free-radical levels in different organs (Valverde et al., 2001). Furthermore, at low non-cytotoxic concentrations, cadmium inhibits unscheduled DNA synthesis after UV irradiation and partially inhibits the removal of UV-induced DNA lesions, suggesting an interference with DNA repair processes at relevant biological concentrations (Rojas et al., 1999). Whether these direct interactions are able to induce strand breakage, cross-linking, or conformational changes in DNA or affect DNA repair mechanisms *in vivo* is at present unclear.

Although they present many advantages, the isolation of PBMCs is a time and labour intensive process taking approximately 2 hours per sample (Shou et al., 2005). Therefore, other approaches to the processing of multiple samples need to be evaluated. Alternative cell preparations, including fresh leukocytes, fresh whole blood cultures (unprocessed), fresh processed (centrifuged) blood cultures and frozen blood have been described for the use in biomonitoring studies and can be obtained from blood samples collected in the field. The isolation of leukocytes (lymphocytes, monocytes and granulocytes) used for biomonitoring or genotoxic studies from blood is a faster (30 min per sample) and less labour intensive method than PBMC processing as red blood cells

(RBCs) are directly lysed (Renzi and Ginns, 1987, Ashmore et al., 1989) instead of using a density gradient separation which is necessary for PBMC isolation (O'Neil-Andersen and Lawrence, 2002). Another advantage of this technique is that when it is compared to fresh whole blood for *ex vivo* assays, the interference of red blood cells is avoided (Frenzilli et al., 1997). This permits the study of the interaction of all the major leukocyte subsets instead of just lymphocytes and monocytes as in the PBMC assay.

Fresh whole blood has been used in biomonitoring studies but to a lesser extent than PBMCs because it contains a mixed population of adherent and non-adherent leukocytes as well as autologous plasma and proteins (Connor, 2000, Remick et al., 2000). While it is a better representation of *in vivo* conditions and presents an advantage over the lymphocyte cultures supplemented with foetal bovine serum, the constituents such as granulocytes and blood proteins can influence the lymphocytes responses during *in vitro* experiments. The use of fresh whole blood requires only small samples and is a rapid and easy procedure without the need for PBMC isolation (Connor, 2000, Remick et al., 2000). For these reasons, fresh blood is frequently used for *in vitro* analysis in rodent studies for which large blood samples are not available. Additionally, whole blood culturing allows the study of lymphocytes, monocytes, granulocytes and RBCs and the interaction and crosstalk of these cells in the culture even though the endpoints only analyse lymphocytes. However, when performing *ex vivo* assays using biological samples there can be inherent problems with batch to batch variability.

Another approach for blood sample processing is the cryopreservation of the whole blood, which, compared with fresh PBMCs, offers the advantages of substantial cost savings in materials and labour and large scale storage of viable lymphocytes (Reimann et al., 2000, Hayes et al., 2002, Costantini et al., 2003, Stevens et al., 2007). The cryopreservation of whole blood is a very fast and much easier process than PBMC isolation and only involves the addition of 10% DMSO followed by control-rate freezing (Piperakis et al., 2000). It also offers a practical alternative to frozen PBMCs as the whole blood is frozen directly without requiring the additional lengthy step of the isolation of PBMCs.

In this study, various blood sample preparations, namely fresh leukocytes, unprocessed fresh whole blood, fresh processed blood, frozen blood and PBMCs were used. The objective was to compare and contrast the various sample preparation methods in

relation to viability and responses to common positive controls for genotoxic assays i.e. EMS & $CdCl_2$. Both basal and time effects were included in the analysis. The membrane integrity of lymphocytes was examined in culture using the propidium iodide (PI) exclusion method (Moore et al., 1998) and DNA damage was measured using the Comet assay (Woods et al., 1997).

Materials & Methods

Chemicals & Reagents

All chemicals used were of the highest grade available. RPMI 1640 medium with 2 mM L-glutamine, heat-inactivated foetal bovine serum (FBS), HEPES, phosphate-buffered saline (PBS), ethyl methanesulphonate (EMS), dimethyl sulphoxide (DMSO), propidium iodide (PI), low melting agarose (LMA), normal melting agarose (NMA), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), Tris-HCl, DNAse, sodium hydroxide (NaOH), 1% N-sodium lauryl sarcosinate, Triton X-100, Ficoll-density gradient medium (Histopaque 1083) and ethidium bromide were all purchased from Sigma-Aldrich (Ireland). Gentamycin was procured from GIBCO (UK). Sterile 96-well tissue culture plates and sodium heparin 9ml blood collection tubes were purchased from Sarstedt (Ireland). Cadmium chloride ($CdCl_2.5H_2O$) was obtained from Alkem (Ireland). Ammonium chloride RBC lysis solution buffer (Pharmlyse) was procured from BD Biosciences (UK). All biological samples and PBS buffers were kept on ice unless otherwise indicated.

Animals

Sprague-Dawley male rats, 1-year-old and weighing 250 to 400 g, were obtained from the Biological Services Unit of University College Cork, Ireland and had been purchased from Harlan, Bicester, U.K. The Rats were housed in groups of 6 under standard controlled conditions (21+/-1°C) on a 12 hour light/dark cycle (lights on at 8.00 a.m.) and received standard water and diet, provided *ad libitum*.

Blood Collection

Each rat was placed into a perspex anaesthetic chamber and anesthesia was induced with 4 % halothane vaporized in oxygen (3l per minute) followed by cervical dislocation. Afterwards, 5-7 ml of blood was extracted by cardiac puncture and transferred to 9 ml sodium heparin tubes. The blood samples were collected from a number of different rats and this blood was pooled together before being divided with some used for PBMC isolation, leukocyte isolation, some for the preparation of fresh whole blood by dilution, some was processed by centrifugation and the remaining blood was cryopreserved as described previously in Chapters 2 and shown in Figure 1.

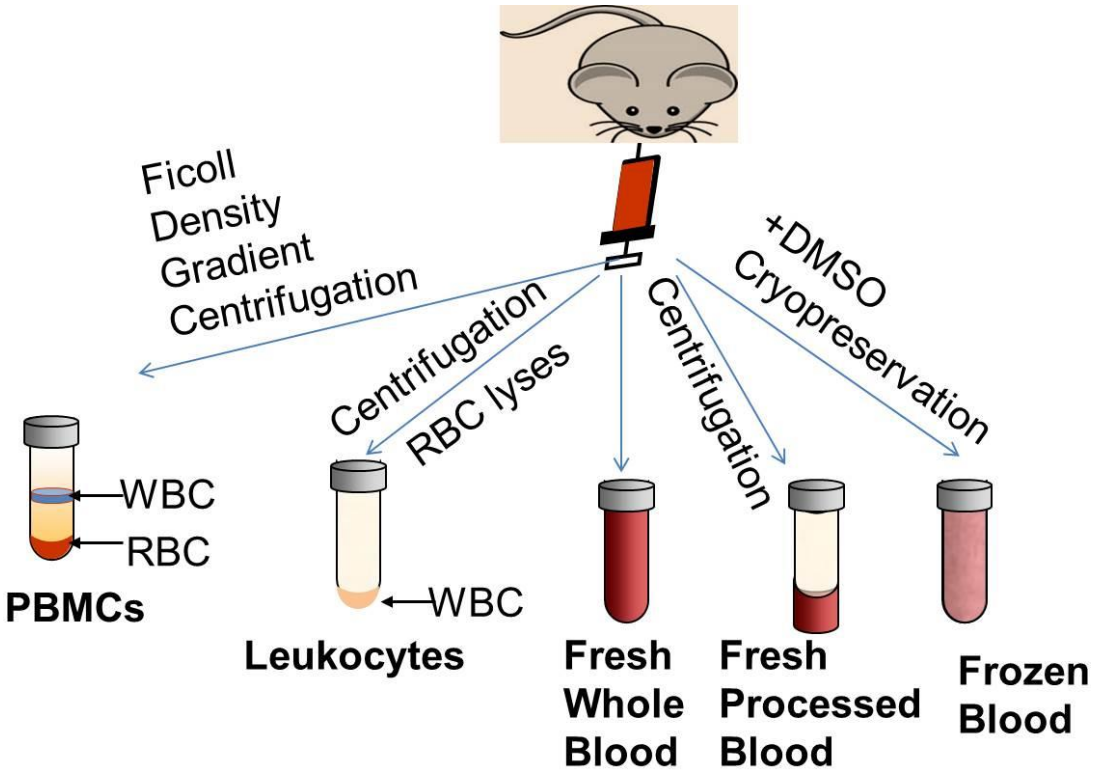


Figure 1. Schematic of the Different Blood Sample Processing Methods Used in this Chapter.

Sample Preparation

Isolation of PBMCs from Fresh Blood

Fresh rat PBMCs were isolated from heparinised blood by routine Ficoll-density gradient centrifugation as described by Boyum (1968) and as detailed previously in Chapter 2.

Isolation of Leukocytes from Fresh Blood

Whole blood (1ml) was transferred into a 50 ml centrifuge tube and diluted 1:20 with ammonium chloride RBC lysis solution buffer as previously described by O'Neil-Andersen et al., (2002). The sample was mixed thoroughly and incubated for 15 minutes at room temperature followed by 7 minutes centrifugation at 300 x g. The supernatant was decanted, leaving only a visible white blood cell (WBC) pellet. The cell pellet was resuspended in 5 ml complete culture medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, heat-inactivated 10 % foetal bovine serum (FBS) 10 mM HEPES and 20 µg/ml gentamycin). Cell viability and numbers were assessed using trypan blue stain and a haemocytometer. The cell suspension was diluted to a concentration of $1x10^{6}$ cells/ml and 500 µl of the leukocyte cell suspension was added per well in a 24-well plate. The plates were pre-incubated for 1 hour 30 minutes at 37°C prior to exposures to test chemicals as described below.

Fresh Whole Blood

1ml of fresh blood was diluted with 2ml of complete culture medium, mixed gently and put in culture as described by Connor et al, (2000). The ratio of blood to medium is based on the observation in earlier pilot studies that 1ml of blood contains $2x10^{6}$ lymphocytes and this dilution would provide $1x10^{6}$ cells /ml (data not shown).

Fresh Processed Blood

Fresh blood (1 ml) was added to 10 ml of complete culture medium and was transferred after dilution to tubes for 7 minutes centrifugation at 300 x g. Most of the supernatant was removed with aspiration. The remaining 1 ml pellet and interface were resuspended

in a ratio of 1:2 in 2 ml of complete culture medium. The ratio of blood to medium is based on the observation in earlier pilot studies that 1 ml of blood contains $2x10^6$ lymphocytes and this dilution would provide $1x10^6$ cells /ml.

Frozen Blood

150 µl (10% v/v) of DMSO (kept for 10 minutes at 4°C before use) was added drop wise to 1350 µl of fresh blood in a cryovial followed by gentle mixing. The cryovial was then placed in a slow- freeze container (Mr Frosty, Nalgene, UK) and stored in a −80°C freezer for 24 hours. This simplified method of controlled-rate freezing lowers the temperature of the samples by approximately 1°C per min to -80°C as previously described (Piperakis et al., 2000). The frozen samples were then stored in a liquid nitrogen dewar for 14 days before being thawed and assayed.

Thawing of Frozen Blood

Frozen samples were thawed in a 37°C water bath with continuous gentle agitation until no ice was visible and then placed on ice for 2 minutes according to the procedure adapted from the protocol described by Reimann and colleagues (Reimann et al., 2000). The thawing medium (RPMI 1640 medium supplemented with 20 % FBS, 0.2 µg/ml DNAse and 25 mM HEPES buffer) was heated to 37°C before being added to the cell suspension. 1 ml of thawed whole blood cell suspension was slowly diluted with thawing medium at room temperature. The slow-dilution protocol consisted of 0.1, 0.2, 0.4, 0.8 and 2 ml of thawing medium sequentially added at 1-minute intervals with further gentle agitation. Five minutes after the last addition of medium, the total volume was brought to 10 ml with thawing medium and centrifuged at 300 x g for 7 minutes. The supernatant was removed; the total volume was brought to 10 ml with complete medium and centrifuged again at 300 x g for 7 minutes. The cell pellet was resuspended in 1 ml of complete medium and at this stage a viability cell count was performed using trypan blue stain and a haemocytometer. The cell suspension was diluted until a cell concentration of $1x10^6$ cells/ml was achieved and 500 µl of the cell suspension was added per well in a 24-well plate. The plates were pre-incubated for 1 hour 30 minutes at 37°C prior to exposures as described below.

Cell Culture

All the blood sample preparations were cultured in complete culture medium with a volume of 500 µl/well in a 24-well plate and all treatments were performed in triplicate. Experiment/exposure was conducted at 37°C in a 5 % CO_2 humidified incubator for the duration of the culture (normally up to 24 hours) after which the lymphocytes were assayed.

Cadmium Chloride and Ethyl Methanesulphonate Stock Solutions and Exposures

For all sample preparations, 500 µl of the cell suspension was transferred to 24-well plates in triplicate. A 10mM stock solution of $CdCl_2$ was prepared in distilled water and then sterilized by passage through a 0.2 µm filter (Schleicher & Schuell, UK). This stock was diluted 1:1, 1:2 and 1:9 in RPMI medium to achieve 5 mM, 3.3 mM and 1 mM $CdCl_2$ stock solutions, respectively, as described previously (Carey, 2005). A volume of 50 µl of these stock solutions was added to the cells in a 24-well plate to achieve the final concentrations (500, 333 and 100 µM).

A 100 mM stock of EMS was prepared in RPMI medium and diluted 1:1, 1:3 and 1:9 to achieve 50 mM, 25 mM and 10 mM solutions. A volume of 50 µl of these solutions was added to the cells of a 24-well plate to achieve the desired final concentrations (5, 2.5 and 1 mM) (Mouchet et al., 2005).

Finally, 50 µl of RPMI medium was added to wells containing the cell suspension as controls. All additions of genotoxic compounds or the RPMI medium control were performed in triplicate wells. The plates were incubated for 3-24 hours at 37°C and then assayed as described below.

Propidium Iodide Exclusion Assay

The viability of lymphocytes in blood sample preparations was determined by propidium iodide (PI) exclusion as described by Moore et al., (1998) and as detailed in Chapter 2. As stated previously, the only cell types that were analysed in the blood sample preparations were lymphocytes as the monocytes and dendritic cells were still attached to the plastic tissue culture plates and the granulocytes were non-viable. This observation was independent of the matrix composition which does differ between sample preparations.

Comet Assay

The Comet assay for these experiments was adapted from a previous study (Woods et al., 1997). A triple layered agar sandwich was prepared on microscope slides. The slides were precoated by evenly spreading 100 µl of normal temperature-melting agarose (1 % NMA in PBS) and allowing it to solidify. Thereafter, another 100 µl of NMA was added, covered with a 22 x 22 mm cover slip and solidified on ice for 10 minutes. The cover slips were removed, 30µl of single cell suspension (1–5 x 10^6 cells/ml) was mixed with 70 µl low-melting point agarose (1.0 % LMA in PBS pH 7.4) and 70 µl of this mixture was quickly added onto the NMA layer before applying a cover slip and refrigerating at 4°C. When the gel had set, the cover slips were removed and the cells were lysed for 1 hour at 4ºC in the dark in an ice-cold salt buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1 % Na-lauryl sarcosinate). The pH was adjusted to 10 with NaOH, with 1 % (v/v) Triton X-100 and 10 % (v/v) DMSO added just before use. After lysis, the slides were gently placed into a horizontal electrophoresis tank and immersed in an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH > 12) for 25 minutes at 4ºC in the dark to allow the DNA to unwind. Without changing the alkaline electrophoresis solution, a 25 volt, 300 mA current was applied for 25 minutes to allow for electrophoresis to occur. The slides were removed from the electrophoresis chamber, placed on a flat tray and then neutralised three times with Tris buffer (0.4 M Tris-HCl, pH 7.4) at 5 minutes intervals. The slides were then washed with distilled water and stained using 2–3 drops of ethidium bromide (20 µg/ml) for 5 minutes. The slides were washed with distilled water and cover slips were placed over the gels and stored at 4ºC in the dark under damp conditions until visualised (usually within 24 hours) using an

epifluorescence microscope (Nikon EFD-3, Micron Optical Co. Ltd, County Wexford, Ireland). In total, 50 cells per slide were scored and the degree of DNA damage was evaluated using the % Tail DNA parameter. One slide was processed from each well and there were duplicate wells per concentration and a total of 3 independent experiments were performed for each blood sample preparation, chemical and exposure time. The analysis was performed using the imaging analysis software package Komet 4.0 (Kinetic Imaging Ltd, Liverpool, UK).

Some cells exhibited poly-lobated nuclei regardless of the degree of electrophoretic migration of the DNA and were identified as polymorphonuclear leukocytes (granulocytes), whereas others showed smaller nuclei and regular morphology and were considered to be mononuclear leukocytes (lymphocytes). Lymphocytes are the only leukocyte subset analysed by the Comet assay included in the results as they were the one leukocyte population that was present in all the sample preparations; the other cell types were excluded based on microscopic identification, which showed that these cells had polymorphological nuclei typical of granulocytes.

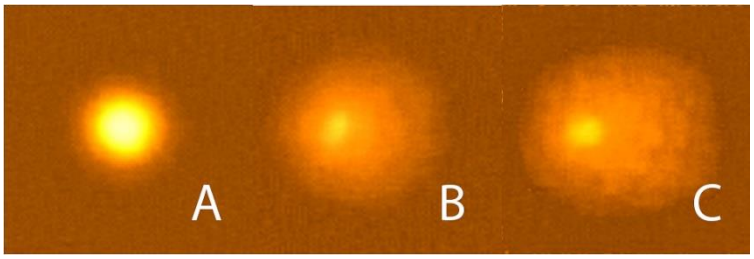


Figure 2. Micrographs of Comet Tail Length Displaying Varying degrees of DNA Damage

(A) Control untreated lymphocytes showing no DNA damage
(B) Moderate DNA damage in lymphocytes
(C) Severe DNA damage in lymphocytes

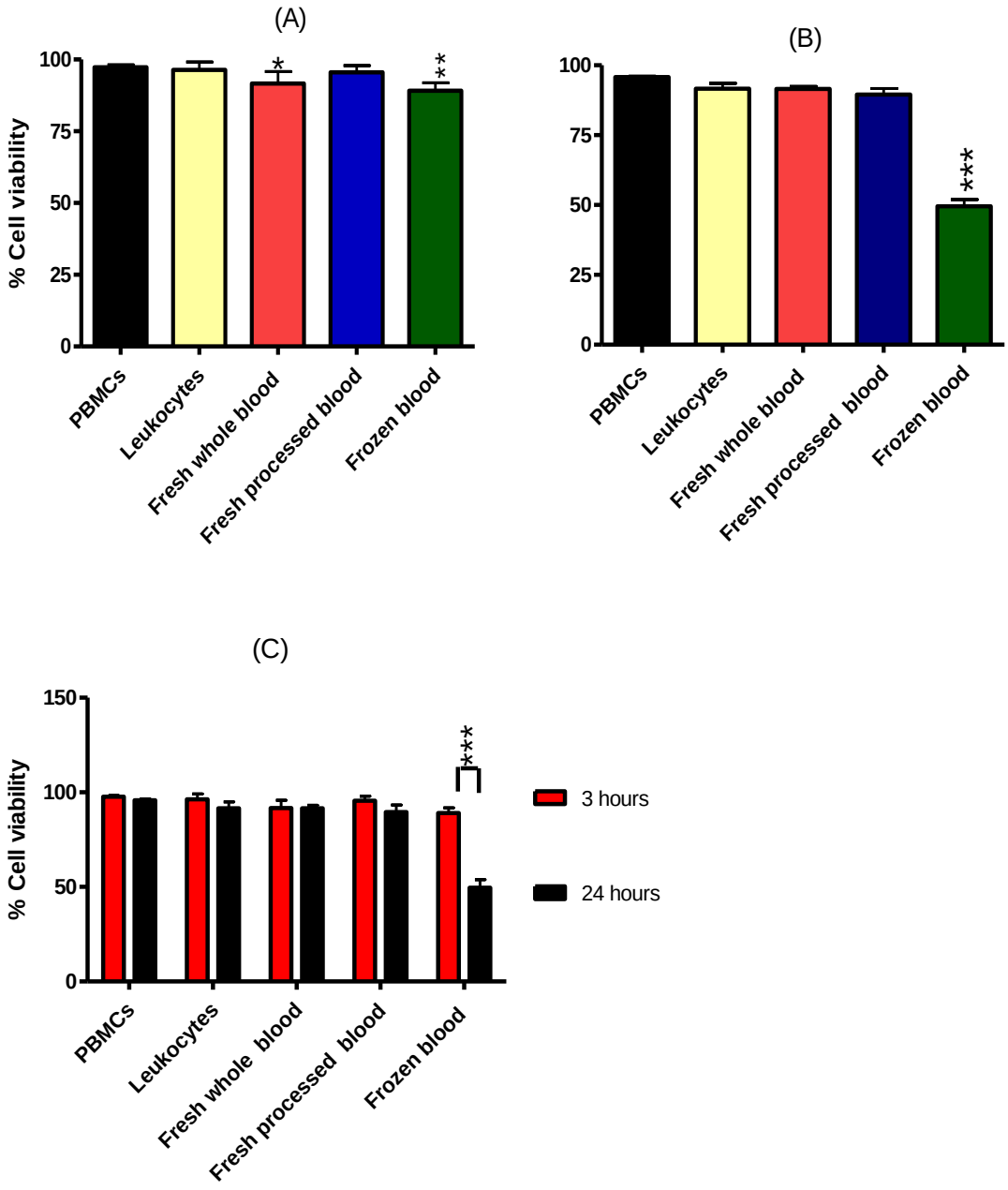
Statistical Analysis

Results are expressed as the means ± Standard Deviation (SD) for three independent experiments with 3 determinations per data point for each experiment. All statistical analysis used Sigma Stat software (version 2.03). Where data followed a normal distribution as assessed with a Kolmogorov-Smirmnov test, differences between groups were analysed using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons. Statistical correlation and linear regression analysis were performed using Prism 4.05 software. Statistical significance was set at $p<0.05$ and significant levels were defined as *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Effects of Different Sample Preparations on the Viability of Lymphocytes over 3 and 24 Hours in Culture.

The effects of sample processing on the viability of lymphocyte after 3 and 24 hours in culture are shown in Figures 3A and 3B, respectively. After 3 hours there was a significant 6 % difference in cell viability between fresh PBMCs and fresh whole blood ($p<0.05$) and between fresh PBMCs and frozen blood there was a difference of about 9 % ($p<0.01$). After 24 hours in culture, there was a significant and sizable difference in cell viability between fresh PBMCs and frozen blood samples with a drop of 46 % ($p<0.001$) in the latter. When comparing the viability of lymphocytes at 3 hours and 24 hours, there was a significant 40 % reduction in frozen blood overtime to 50 % ($p<0.001$) (Figure 3C).



Figures 3A, 3B and 3C. Effect of Sample Processing on Lymphocyte Viability after 3 Hours (A), 24 Hours (B) and both timepoints (C) in Culture. Data are represented as the percentage viability of rat lymphocytes cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations over culture periods of 3 hours (A), 24 hours (B) hours and both time points (C) assessed by PI staining. Data are represented as means +/- SD from three replicate wells in three independent experiments. In Figures 3A and 3B, statistical analysis is PBMCs vs. every other sample. In Figure 3C, statistical analysis is 3 hours vs. 24 hours for each fraction (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Effects of Cryopreservation on the Viability of Lymphocytes in Whole Blood Cultures over 24 Hours in Culture.

Because of the substantial decrease of viability of lymphocytes observed in frozen blood at 24 hours, a more detailed time-course study was undertaken (Figure 4)**.** There was no reduction in viability after 3 hours in culture. However, there was a progressive time-dependent decrease in cell viability of 10 % at 6 hours ($p<0.01$), 30 % at 13 hours ($p<0.001$), 40 % at 19 hours ($p<0.001$) and 46 % at 24 hours ($p<0.001$).

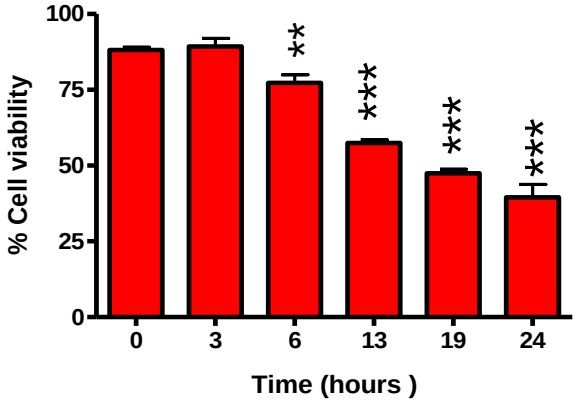


Figure 4. Cell Viability of Lymphocytes in Frozen Blood over 24 Hours in Culture. Data are represented as the percentage viability of rat lymphocytes cultured from frozen blood preparations over culture periods of 3, 6, 13, 19 and 24 hours assessed by PI staining. Data are represented as means +/- SD from three replicate wells in three independent experiments. 0 hour vs. all time-points * $p < 0.05$, ** $p < 0.01$ and *** $p < 0:001$.

The Effects of Different Sample Preparations on the DNA Damage of Lymphocytes over 24 Hours in Culture.

There was no statistical significant difference in DNA damage (as measured by increases in % Tail DNA values) detected by the Comet assay in the untreated sample preparations after 3 hours in culture as shown in Figure 5. In contrast, after 24 hours, the DNA damage was significantly higher in lymphocytes from frozen blood than in those in all other preparations, especially PBMCs ($p<0.05$). In agreement with this, there was an increase in DNA damage between 3 and 24 hours in culture for lymphocytes in frozen blood while there was no significant increase in any other sample preparation over 24 hours ($p<0.05$).

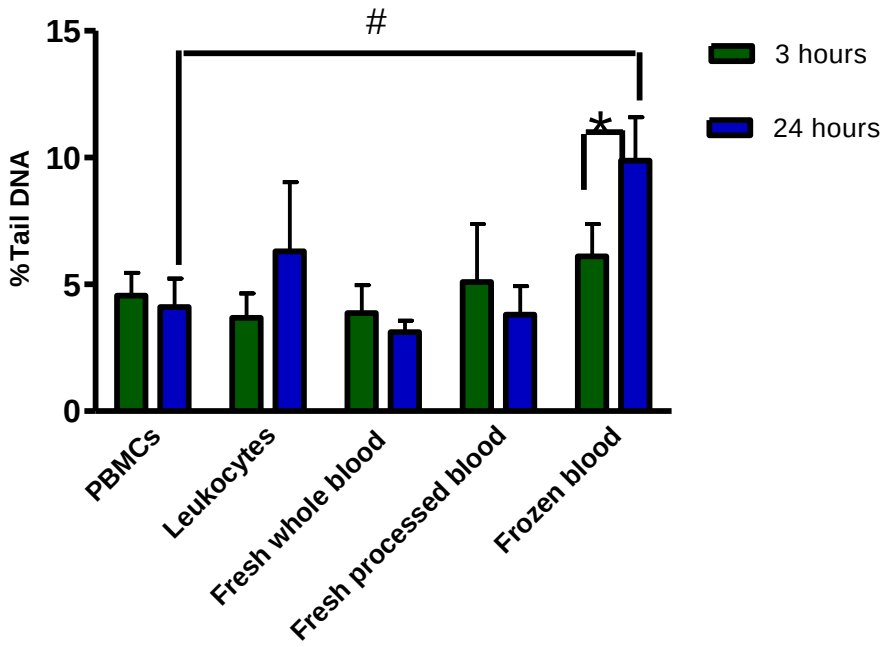
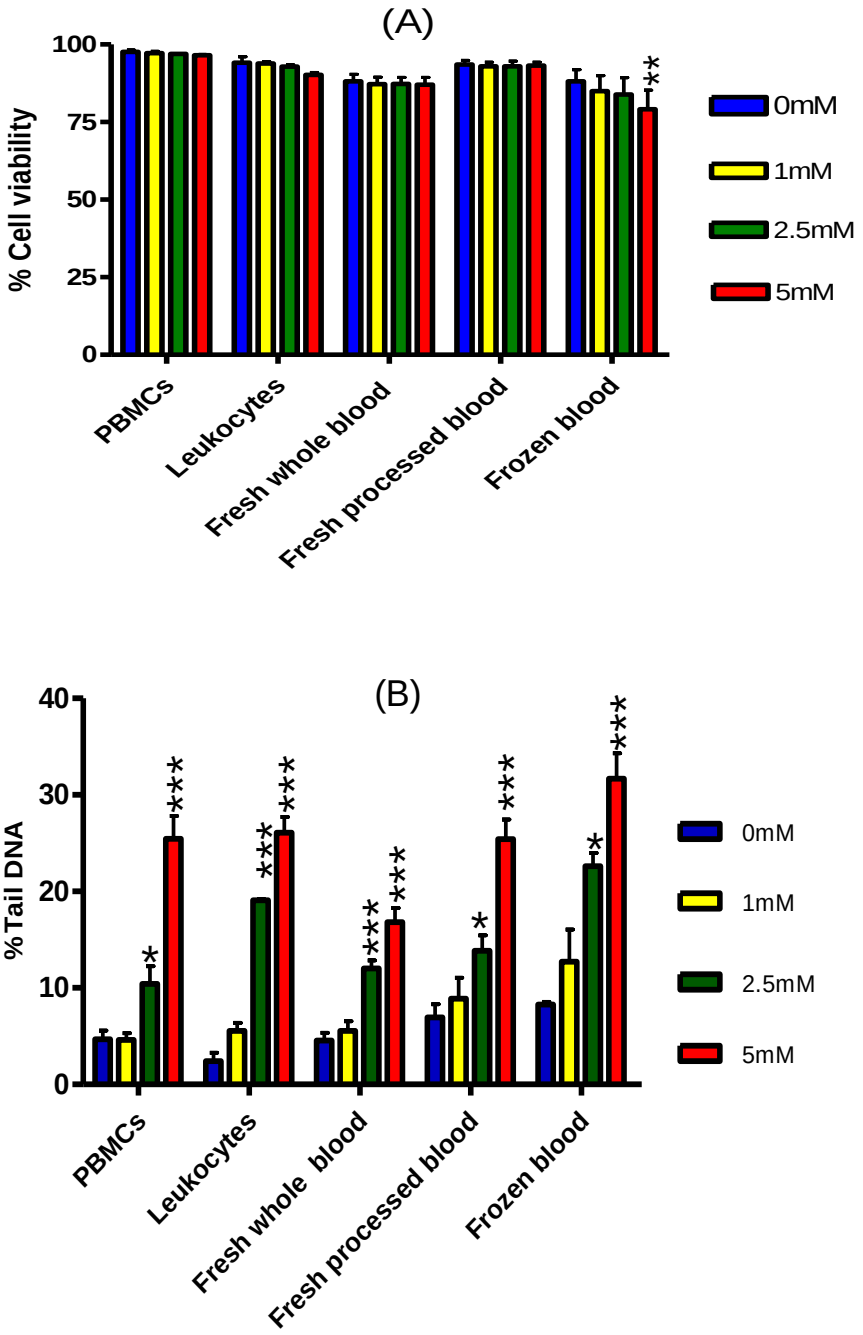


Figure 5. The Effect of Sample Processing on the DNA Damage of Lymphocytes over 24 Hours in Culture. Data are represented as DNA damage (as measured by increases in % Tail DNA values) of untreated rat lymphocytes cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations over culture periods of 3 and 24 hours assessed by the Comet Assay. Data are represented as means +/- SD from three replicate wells in three independent experiments. * represents a significant difference between the amount of DNA damage at 3 hours vs. 24 hours ($*p < 0.05$). # represents a significant difference between the amount of DNA damage in PBMCs after 24 hours vs. every other sample preparation at 24 hours ($\#p < 0.05$).

Response of Lymphocytes to Ethyl Methylsulphonate in Cytotoxicity and Genotoxicity Assays over 3 Hours in Culture.

The 3 hour time point was chosen for EMS exposure as this was the shortest incubation period possible whereby DNA damage could be induced with the concentrations used without inducing cytoxicity (data not shown). EMS had no cytotoxic effect in any sample preparations when the 1 mM and 2.5 mM doses were used when compared with the corresponding untreated controls (Figure 6A). At a dose of 5 mM, there was a significant (9 %) reduction ($p<0.01$) to 79 % in the case of frozen blood but no effect on the other samples.

The genotoxic effects of EMS are shown in Figure 6B. An increase in DNA damage (as measured by % Tail DNA) of lymphocytes in a dose-dependent manner was observed in all sample preparations. At 1mM concentration, there was only a slight and non-significant increase in DNA damage. At the concentration of 2.5 mM, the DNA damage increased significantly in all sample preparations compared to untreated controls: PBMCs ($p<0.05$), leukocytes ($p<0.001$), fresh whole blood ($p<0.001$), fresh processed blood ($p<0.05$) and frozen blood ($p<0.05$). At the highest concentration used, 5 mM, EMS caused a substantial increase in DNA damage in all sample preparations ($p<0.001$).



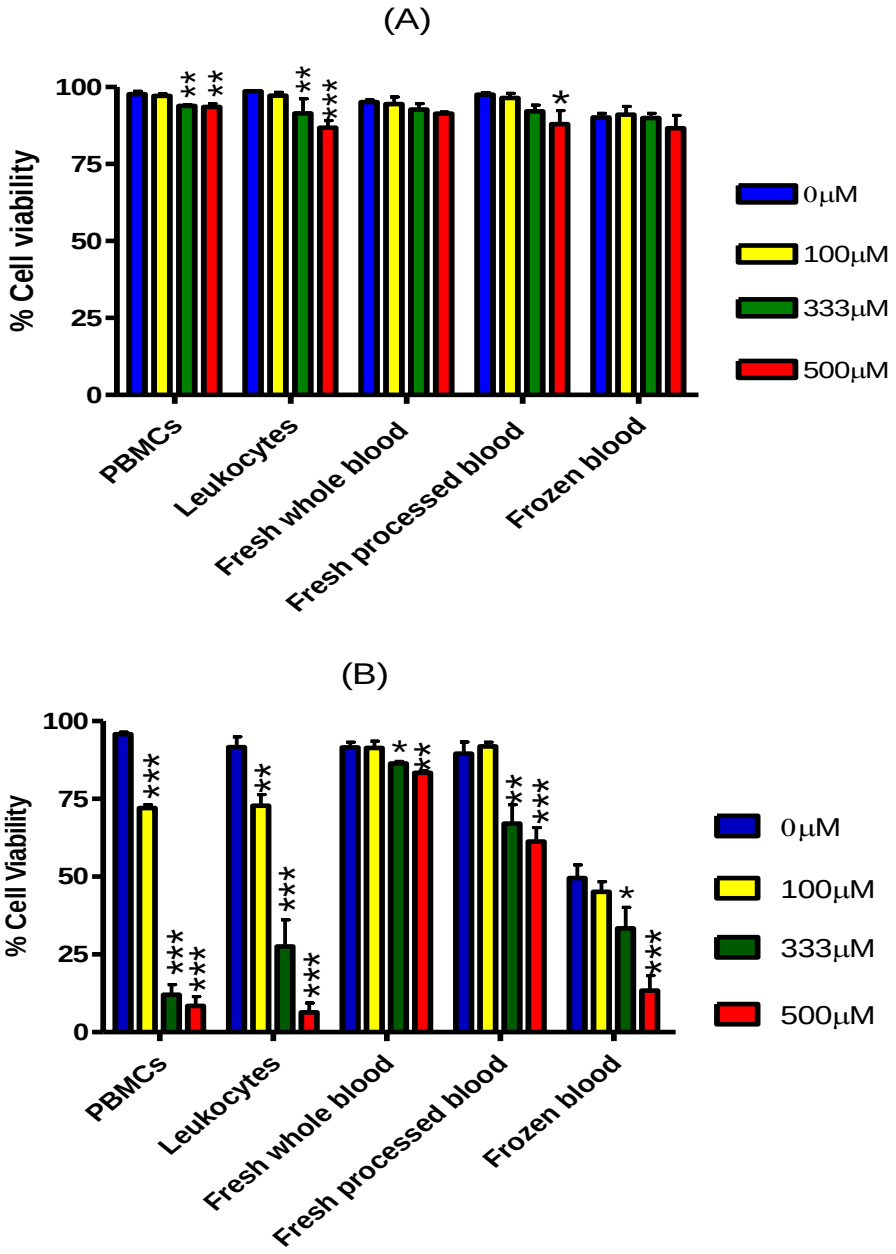
Figures 6A and 6B. The Effects of Short-Term (3 Hour) EMS Exposure on Cell Viability and DNA Damage in Lymphocytes. Data are represented as the viability (Figure 6A) and DNA damage (as measured by % Tail DNA)(Figure 6B) of rat blood lymphocytes after 3 hours exposure to EMS (1, 2.5 and 5 mM). Rat lymphocytes were cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations. Values represent the mean±SD from three replicate wells in three independent experiments. Control (0 mM) vs. all concentrations for each sample preparation * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$.

Response of Lymphocytes to Cadmium Chloride in Cytotoxicity and in Genotoxicity Assays over 3 and 24 Hours in Culture.

The objective of this work was to assess the genotoxic potential of $CdCl_2$ in short-term and longer-term exposure in the different sample preparations.

$CdCl_2$ caused a slight reduction in cell viability of lymphocytes compared to untreated controls in a dose-dependent manner in all sample preparations after 3 hours (Figure 7A). However, this reduction in cell viability was only significant for PBMCs ($p<0.01$) and isolated leukocytes ($p<0.01$) for the 333 μM $CdCl_2$ exposure. In addition, 500 μM $CdCl_2$, also caused a significant reduction in cell viability in PBMCs ($p<0.01$), isolated leukocytes ($p<0.01$) and fresh processed blood ($p<0.05$).

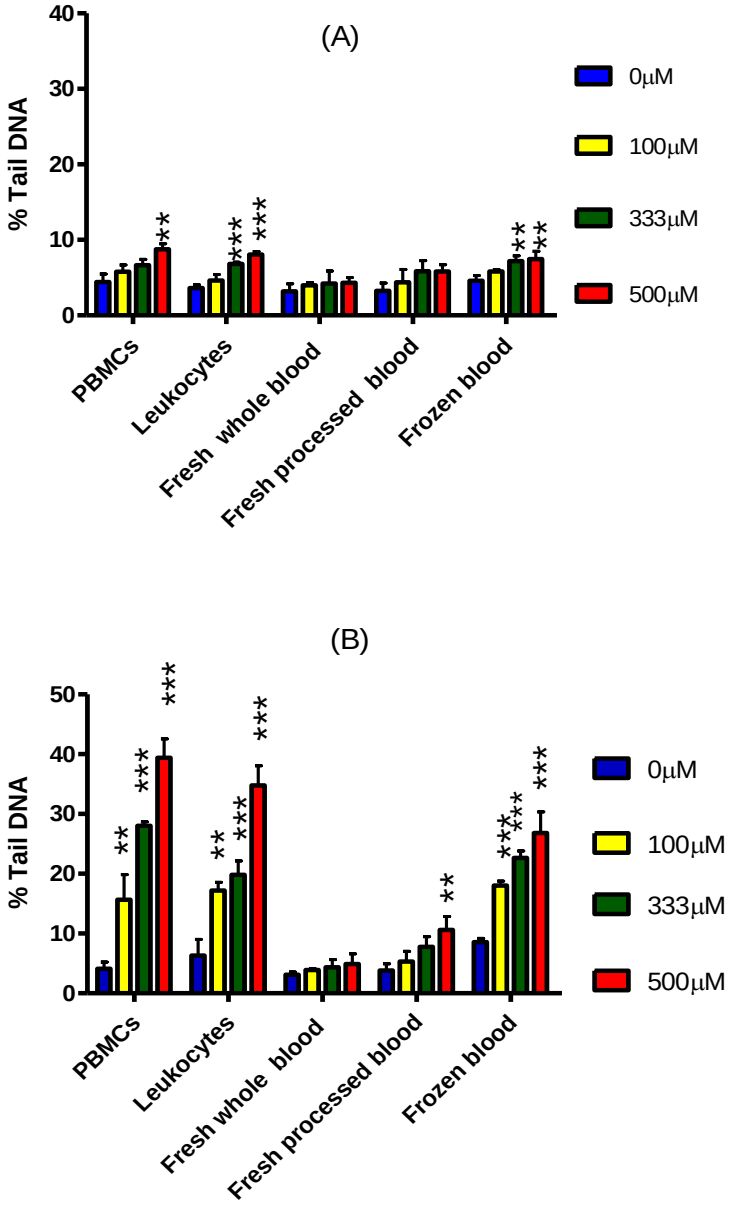
When exposure was extended to 24 hours, $CdCl_2$ substantially reduced the cell viability in lymphocytes compared to untreated controls in a dose-dependent manner in all sample preparations (Figure 7B). At 100 μM $CdCl_2$, this reduction was significant in 2 of the sample preparations: PBMCs ($p<0.001$) and isolated leukocytes ($p<0.01$). At the 333 μM $CdCl_2$ dose, the reduction in lymphocyte viability was significant in all sample preparations: PBMCs ($p<0.001$), isolated leukocytes ($p<0.001$), fresh whole blood, ($p<0.05$), fresh processed blood, ($p<0.01$) and frozen blood ($p<0.05$). The highest concentration (500 μM) had the most toxic effect and significantly reduced lymphocyte viability by 87 % in PBMCs ($p<0.001$), 85% in leukocytes ($p<0.001$) and 36 % in frozen blood ($p<0.001$). The 500μM dose also significantly reduced viability, although to a lesser extent, in fresh whole blood by 8 % ($p<0.01$) and in fresh processed blood by 28 % ($p<0.001$).



Figures 7A and 7B. The Effects of Short (3 Hours) and Long-Term (24 Hours) Exposure to $CdCl_2$ on Cell Viability in Lymphocytes. Data are represented as the percentage viability of rat lymphocytes cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations after 3 hours (Figure 7A) and 24 hours (Figure 7B) exposure to $CdCl_2$ (0, 100, 333 and 500 µM). Data are represented as means +/- SD from three replicate wells in three independent experiments. Control (0µM) vs. all concentrations for each sample preparation * $p < 0.05$, ** $p < 0.01$ and *** $p <$ 0:001.

With regard to the genotoxicity assessment, $CdCl_2$ increased the DNA damage (as measured % Tail DNA) in a dose-dependent manner at 3 hours (Figure 8A). There was a small effect observed at the 100 μM dose in all the sample preparations which did not contain RBCs (PBMCs, leucocytes and frozen blood). However, at 333 μM $CdCl_2$ exposure, isolated leukocytes ($p<0.001$) and frozen blood ($p<0.01$) had a significant amount of DNA damage. At the highest dose (500 μM), PBMCs ($p<0.01$), isolated leukocytes ($p<0.001$) and frozen blood ($p<0.01$) also showed a significant increase in DNA damage. At 3 hours, $CdCl_2$ exposure (all doses) did not have any significant effect on DNA damage in the lymphocytes of fresh processed and unprocessed blood.

Because the genotoxic effects of $CdCl_2$ were slight (although significant) at 3 hours and only occurred in some sample preparations, the incubation time was extended to 24 hours to assess if a more severe genotoxicity could be observed. At 24 hours, $CdCl_2$ increased DNA damage in a dose-dependent manner in all sample preparations (Figure 8B). The 100 μM dose induced a highly significant increase in DNA damage values in PBMCs ($p<0.01$), isolated leukocytes ($p<0.01$) and frozen blood ($p<0.001$). At both the 333 μM and 500 μM doses, $CdCl_2$ induced a highly significant increase in DNA damage values in PBMCs ($p<0.001$), isolated leukocytes ($p<0.001$) and frozen blood. $CdCl_2$ had no effect on DNA damage in fresh whole blood cells and only the 500 μM concentration had an effect when the blood was processed ($p<0.01$).



Figures 8A and 8B. The Effects of Short (3 Hours) and Long-Term (24 Hours) Exposure to $CdCl_2$ on DNA Damage in Lymphocytes. Data are represented as the DNA damage (% Tail DNA) of rat lymphocytes cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations after 3 hours (Figure 8A) and 24 hours (Figure 8B) exposure to $CdCl_2$ (0, 100, 333 and 500 μM). Data are represented as means +/- SD from three replicate wells in three independent experiments; Control (0 M) vs. all concentrations for each sample preparation * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Assessment of the Correlation between Cytoxicity and Genotoxicity of the Ethyl Methylsulphonate and Cadmium Chloride over 24 Hours in Culture.

Correlation and linear regression analysis were also conducted to assess if there was a statistical association between the DNA damage (as measured by increases in % Tail DNA values) and cell viability for the two genotoxic compounds. The increases in genotoxicity or DNA damage were directly related to the reduction in viability especially for $CdCl_{2,}$ exposures, as shown in Table 1. Indeed, the PBMC ($p<0.0058$), leukocyte ($p<0.0002$) and fresh processed blood ($p<0.0233$) preparations had a linear regression correlation between the DNA damage and cell viability at 3 hours exposure (Figure 9). When the exposure time was lengthened to 24 hours, the level of genotoxicity increased considerably from that present at 3 hours. PBMC ($p<0.0001$), leukocyte ($p<0.0001$), fresh processed blood ($p<0.0022$) and frozen blood ($p<0.0021$) preparations were determined to have a linear regression correlation between the DNA damage, and cell viability (Figure 9).

EMS exposure also had a statistical correlation between cytoxicity and genotoxicity for the cell preparations PBMCs ($p<0.0107$), leukocytes ($p<0.00228$) and frozen blood $p<0.0223$. There was a significant increase in DNA damage at the two highest concentrations, 2.5 mM and 5 mM, in the absence of significant cytotoxicity.

Fresh whole blood showed no correlation between the DNA damage and cell viability. When whole blood was centrifuged, the only correlation that was apparent was after short (3 hours) and long-term (24 hours) incubation with $CdCl_{2.}$ From these results, $CdCl_2$ has the strongest statistical association between DNA damage and cell viability (Figure 9).

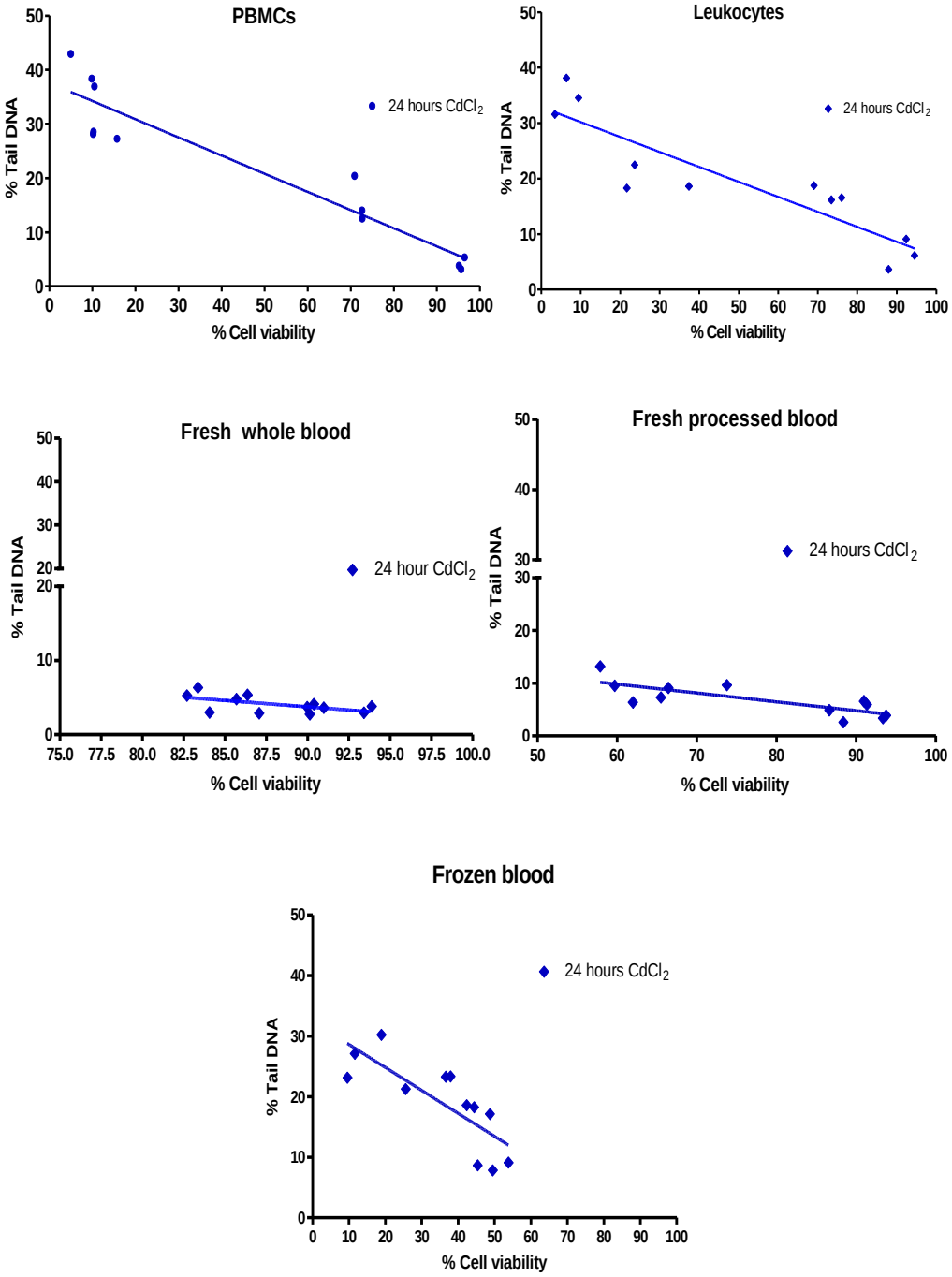


Figure 9. The Correlation between Cytoxicity and Genotoxicity Effects of $CdCl_2$ Exposure in Lymphocytes. Data are represented as DNA damage (as measured by increases in % Tail DNA values) versus the percentage of cell viability of rat lymphocytes cultured from PBMCs, leukocytes, fresh whole blood, fresh processed blood and frozen blood preparations after 24 hours exposure to $CdCl_2$ (0, 100, 333 and 500 µM) (Figure 9). Linear regression (P value) and R^2 goodness of fit were calculated for each exposure and cell preparation and are shown in Table 1.

Samples Preparations	Treatment	Time point (Hours)	Linear Regression P value	Goodness of Fit R squared
PBMCs	$CdCl_2$	3	0.0058	0.5495
Leukocytes	$CdCl_2$	3	0.0002	0.7558
Fresh whole blood	$CdCl_2$	3	0.2070	0.1540
Fresh processed blood	$CdCl_2$	3	0.0444	0.3456
Frozen blood	$CdCl_2$	3	0.2768	0.1168
PBMCs	$CdCl_2$	24	0.0001	0.8995
Leukocytes	$CdCl_2$	24	0.0001	0.8006
Fresh whole blood	$CdCl_2$	24	0.0551	0.3240
Fresh processed blood	$CdCl_2$	24	0.0022	0.62777
Frozen blood	$CdCl_2$	24	0.0021	0.6277
PBMCs	EMS	3	0.0107	0.4944
Leukocytes	EMS	3	0.0028	0.6073
Fresh whole blood	EMS	3	0.9268	0.00088
Fresh processed blood	EMS	3	0.5973	0.02891
Frozen blood	EMS	3	0.0233	0.4170

Table 1. Statistical Correlations between DNA Damage and the Cell Viability for the Different Sample Preparations after Exposure to Cadmium Chloride and Ethyl Methanesulphonate.

Discussion

For biomonitoring studies it is very important to have the right biological samples, i.e. those that are easy to get for fast processing, preferably non-invasive for repeated sampling (to avoid stress to the subjects) and in conditions which will not impact on the assays being performed (Okazaki et al., 2008). In biomonitoring studies, lymphocytes are the preferred sample for measurement of genotoxic effects, the immunological status and other effects (Faust et al., 2004, Linzalone, 2009). However, there is a processing time limit if the whole blood or PBMC samples are kept at 25°C or 4°C for a long period of time, and this makes cryopreservation of samples a very interesting alternative (Villavicencio, 2006). Because the preparation of frozen PBMCs can be a lengthy process and because the storage at room temperature reduces the viability of lymphocytes (as described in Chapter 2) frozen blood, fresh whole blood and fresh processed blood were investigated as possible alternatives.

In this Chapter, the effects that different blood separation and cell isolation techniques had on the viability of rat lymphocytes and their responses to genotoxic compounds were examined. The similarities and differences in responses of the sample methods were compared with regard to the presence of different cell types and blood components other than those contained in PBMCs. (The main comparison was between the gold standard method PBMCs vs. leukocytes (lymphocytes + granulocytes), fresh whole blood (lymphocytes+RBCs+granulocytes+blood plasma +proteins), fresh processed blood (lymphocytes+RBCs+granulocytes) and frozen blood (lymphocytes+break down products+granulocytes). The different degrees of homogeneity of the cell types and culture compositions generated by using the different fractionation and isolation techniques are shown in Table 2.

Blood Cell Type/ Separation Techniques					
Sample Preparations	**PBMCs**	**Leukocytes**	**Fresh Whole blood**	**Fresh processed blood**	**Frozen blood**
Separation Techniques	**Ficoll Density Gradient Centrifugation**	**RBC Lysis**	**Dilution**	**Centrifugation +Dilution**	**Cryopreservation Thawing+ Centrifugation+ +Dilution**
Lymphocytes	+	+	+	+	+
Erythrocytes	-	-	+	+	↓
RBC Breakdown Products	-	+	-	-	+
Granulocytes	-	+	+	+	↓
Foetal Bovine Serum	**10%**	**10%**	**10% (+13% Plasma)**	**8%**	**8%**

Table 2: Cell types and Blood Culture Components after Various Blood Separation and Fractionation Techniques. ↓ refers to substantial reduction of granulocytes and erythrocytes in the blood sample mixture following cryopreservation. +/- refers to the presence or absence of certain blood constituents after the different sample processing methods have been completed.

Frozen blood samples were processed as quickly as possible in order to minimize cell damage following the thawing step. In addition, the process was optimised to obtain samples of the highest lymphocyte viability possible (85% or above). This was achieved by the removal of DMSO by slow dilution and centrifugation and the addition of DNAase to remove the DNA from dead granulocytes to avoid the clumping of lymphocytes as demonstrated by Reimann et al., (2000) and Steven et al., (2007). The cell viability of 90% post thawing at 3 hours is consistent with the findings of Anderson et al., (1996), Cheng et al., (2001) and Stevens et al., (2007). However, the loss of lymphocyte viability was time-dependent and increased when measured at 6 hours and 24 hours.

The Comet assay is a very sensitive method which can detect very low levels of DNA breakage. Therefore, reliable sample processing is a critical issue so as not to increase the basal level of DNA damage through the processing steps (Collins et al., 1996). There was no significant difference in the viability and DNA damage of lymphocytes from frozen blood and those from the other sample preparations after 3 hours in culture. This is consistent with the findings of Duthie et al., (2002) who found that cryopreservation of lymphocytes did not increase DNA strand breakage above endogenous levels found in PBMCs. Similarly, lymphocytes obtained from blood samples stored at -80ºC for 60 days did not produce detectable DNA strand breaks (Visvardis et al., 1997, Tice et al., 2000, Chuang and Hu, 2004, Villavicencio, 2006). However, in these studies, the time for which the lymphocytes were cultured was less than 6 hours-the time at which DNA damage was observed to increase significantly in the present study. After 24 hours in culture, the % Tail DNA in frozen blood was increased compared to that from the other sample preparations. This observation is in agreement with Villavicencio et al., (2006) and Visvardis et al., (1997) who observed an increase in DNA strand breaks in frozen lymphocytes after culturing for longer than 4 hours. This increase in DNA damage could be attributed to the lysis of red blood cells and possibly an increase in early apoptosis after the freeze thaw process as observed in Chapter 2. The DNA fragmentation induced during the apoptotic process can be visualised in the Comet assay (Godard et al., 1999, Bagchi et al., 2000, Choucroun et al., 2001, Meintieres et al., 2001, Meintieres et al., 2003, Yasuhara et al., 2003).

Fresh whole blood had the lowest basal level of DNA damage compared to the other samples used in this study. This may reflect the fact that it involves no isolation of individual cell types. In a separate study, the use of whole blood cells for the Comet assay reduced the sampling time and prevented DNA damage during lymphocyte isolation (Speit and Hartmann, 2005). Also in the present study, fresh processed blood and freshly isolated leukocytes had a lower level of % Tail DNA than PBMCs. This is consistent with the finding that for PBMCs isolated using Ficoll-density separation centrifugation and measured by the Comet assay, there was an increase in oxidative DNA damage after isolation compared to non-separated leukocytes or whole blood (Vijayalaxmi et al., 1993, Collins, 1999, Narayanan et al., 2001).

Once the cell preparations were characterised for a 24 hour culture period, the effects of *in vitro* exposure of lymphocytes from different blood sample preparations to DNA-damaging agents, the alkylating ethyl methanesulphonate (EMS) and cadmium chloride ($CdCl_2$), were investigated.

EMS caused DNA damage in a dose–dependent manner in all sample preparations after a 3 hours exposure. This is consistent with the findings of De-Boeck et al, (2000) who demonstrated DNA damage in rat blood cells after exposure to direct-acting alkylating water-soluble mutagens for 3 hours. Other reports provide contrasting effects of EMS. Exposure to concentrations from 1-40 mM induced DNA damage at all doses (Wyatt et al., 2007). In another study, no significant DNA damage was observed when EMS concentrations of 1-5 mM were used for 30 minutes but at a concentration of 40 mM single strand breaks appeared in the DNA (Suggitt et al., 2003). However, even after exposure to 120 mM EMS, the DNA damage was fully repaired within 24 hours indicating that the EMS does not have a cytotoxic effect (Suggitt et al., 2003). The level of EMS-induced damage was similar for PBMCs and whole blood suggesting that, under the conditions applied, the proteins and other nucleophiles present in whole blood had no scavenging effect on the direct-acting mutagen. This is in agreement with a study that showed that whole blood exposed to EMS had huge increases in % Tail DNA (Speit et al., 2004). When fresh and frozen blood samples were exposed to EMS, the amount of DNA damage induced was comparable to that seen with PBMCs and leukocytes. This is in contrast to reports that the use of whole blood was unsuitable for measuring DNA damage using the Comet assay due to interferences from RBCs and

other blood components which can have an inhibitory effect in these assays (Gaetani et al., 1989, Scott et al., 1991).

At concentrations of 333 and 500 µM, $CdCl_2$ caused a significant reduction in viability when PBMCs, isolated leukocytes and fresh processed blood were exposed for 3 hours. When exposure was extended to 24 hours, all concentrations used impacted significantly on viability. Blasiak et al, (2000) showed that the viability of human lymphocytes was reduced when exposed to concentrations of 5 to 150 µM of $CdCl_2$ for 1 hour. In the present study, both whole and processed fresh blood samples had only a slight reduction in viability when exposed to 500 µM $CdCl_2$, the highest concentration used, for 24 hours while fresh whole blood was the least affected of all samples. This may be due to the presence of RBCs which are known for their binding potential to cadmium and also the higher quantity of plasma and blood proteins in the fresh whole blood.

A limited genototoxic effect was observed when PBMCs and leukocytes were exposed to $CdCl_2$ for 3 hours but it increased significantly when exposure was extended to 24 hours. Previous reports have shown that 0.16 -20 µM $CdCl_2$ caused an increase in DNA damage in peripheral blood lymphocytes (Cai and Zhuang, 1999) and 150 µM cadmium significantly increased DNA damage when 1-3 $x10^5$ cells per ml were exposed for 1 hour (Blasiak and Kowalik, 2001). In addition, *in vivo* exposure to $CdCl_2$ caused an increase in DNA damage in suckling Wistar rats and turbot fish (Kasuba et al., 2002, Kilemade et al., 2004). Consistent with the effects observed in the present study, $CdCl_2$ has been described as an indirect genotoxic compound (Bertin and Averbeck, 2006).

In the present study, lymphocytes from whole blood were resistant to $CdCl_2$-induced cytotoxicity and DNA damage but became very sensitive when exposed as PBMCs (isolated by using Ficoll-density separation centrifugation) or as leukocytes (prepared using red blood cell lysis buffer). Even at 24 hour exposure, $CdCl_2$ induced no DNA damage in lymphocytes prepared from fresh whole blood and had only a small effect when the blood was centrifuged to reduce the content of its plasma and blood proteins. This may be explained by the ability of the RBCs to bind cadmium in the absence of the scavenging effects of the plasma proteins. It is known that cadmium is detected in the RBCs component of the blood. The effects of $CdCl_2$ were dependent on sample

constitution, i.e. presence or absence of red blood cells and serum protein concentration and on exposure time. In contrast, in human whole blood exposed to $CdCl_2$, there was an increase in DNA damage in 2 out of 4 subjects (Rozgaj et al., 2002). The contrasting findings may reflect differences in the repair of induced DNA damage between rat blood and human blood (Rozgaj et al., 2002). The lack of genotoxic insult from $CdCl_2$ in the case of whole blood may be due to the fact that it becomes bound to plasma proteins such as albumin and transferin (Saljooghi and Fatemi, 2010a). This scavenging effect could have a major influence on the bioaccumulation of $CdCl_2$ (Saljooghi and Fatemi, 2010b). The scavenging effect of whole blood has been reported previously when whole blood and isolated lymphocytes were exposed to hydrogen peroxide (Andreoli et al., 1999, Chuang and Hu, 2004).

The criterion of true genotoxic effects proposed by Henderson et al., (1998) states that DNA strand breaks should only occur in the absence of cytotoxicity. Cytotoxins are able to induce an increase in DNA migration, but this observation only occurred when viability was $\leq 75\%$ (Henderson et al., 1998). The $CdCl_2$-induced Comet formation seen in this study may reflect indirect DNA damage which is likely to be due to a secondary consequence of physical or chemical damage to cell membranes or more simply an inhibition of the DNA repair process. The Comet assay can be used to discriminate efficiently between genotoxins and cytotoxins (Fairbairn et al., 1996). However, there are discrepancies regarding the genotoxic effects of $CdCl_2$ in a variety of systems where some studies proclaim the genotoxic effect of $CdCl_2$, whereas other studies claim that the DNA damage is secondary to cytotoxicity (Saplakoglu and Iscan, 1998, Rojas et al., 1999). The interpretation of the results from this study are in contrast to the those reached in some previous studies which reported that $CdCl_2$ induced a dose dependent DNA strand break in lymphoblastoid, fibroblasts and human leukocytes (Mikhailova et al., 1997, Cai and Zhuang, 1999, Bagchi et al., 2000, Blasiak et al., 2000, Blasiak and Kowalik, 2001, Mouron et al., 2004, Zhang, 2011). All the above studies concluded that $CdCl_2$ is a true genotoxic compound but the cell viability assessment was not performed. Cadmium has been shown to intercalate between DNA strands and disrupt transcription by destabilizing the helical structure of DNA (Webb, 1979). Therefore, the mechanism of cadmium genotoxicity i.e. increase in % Tail DNA of the exposed lymphocytes may be caused by the induction of DNA strand breaks which in turn can be attributed to direct binding to DNA bases, by enhancing reactive oxygen species production as well

as interference with the repair of DNA lesions (Waalkes and Poirier, 1984, Stohs and Bagchi, 1995, Stohs et al., 2001). The increase of % Tail DNA in lymphocytes after 24 hours exposure to $CdCl_2$ is similar to what was seen previously when the degradation of chromosomal DNA in CHO cells was linked to oxidative stress and apoptosis (Bagchi et al., 2000, Banfalvi et al., 2005). Cadmium has been demonstrated to induce DNA strand breaks but also apoptosis in various cell lines (Galan et al., 2000, Banfalvi et al., 2005, Fotakis et al., 2005).

Main Conclusions

In this Chapter, the merits and limitations of a number of sample preparations in short and long term culturing and their responsiveness to genotoxic compounds *ex vivo* were assessed. The different sample processing methods were shown to alter the responsiveness of lymphocytes to $CdCl_2$ but not to EMS. This shows the limitations of using peripheral blood from animals when using unknown genotoxic compounds, the importance of knowing the mode of action of each compound and also how the constituents of each culture will affect the potency of the genotoxic compounds. It was demonstrated that three hour incubations can be undertaken without considerable alterations in the viability of the lymphocytes after all sample processing methods described but when the culture times were extended for frozen blood this lead to a large decrease in viability. On the basis of the results using rat blood, one can conclude that direct cryopreservation of blood can be used as an alternative to PBMCs, especially for short term cultures and exposures. This is of prime interest due to the fact that there are time constraints in field-based biomonitoring studies so frozen blood looks a very promising technique for short term *ex-vivo* studies.

What is striking and important is that the direct cryopreservation of unprocessed blood does not affect responsiveness of lymphocytes to genotoxic compounds. For future studies, where the collection of field-based samples would require long term storage of the samples, the fresh whole and frozen blood protocol is very encouraging because these methodologies have additional advantages of simplicity, economy, and speed and involve less cell manipulation over these assays in gradient-isolation of blood cell subtypes. Future studies in this area should focus on the transfer of these methodologies

to a more relevant biomonitoring model, such as sheep, and fully characterise each blood processing protocol.

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Chapter 4

The Effect of Cryopreservation on the Viability of Lymphocytes in Sheep Blood Preparations

Table of Contents

Introduction

Animal environmental sentinels have been defined as any non-human organisms that can react to an environmental contaminant before it impacts humans (Stahl, 1997). The aim of this thesis was to investigate the effects of blood sample preparations and cryopreservation on cell viability and responses in *ex vivo* biomarker assays. The assessment of the exposure of selected animals to contaminants can be strengthened by using biomarkers which are common to animals and humans so that the effects of exposure can be translated to humans. For example, the measurement of metals and levels of selected proteins in blood are useful in the evaluation of the physical condition which can include the health/ disease status as well as the effect of chemical exposure of ruminants and other mammals (Duffy, 1996, Lagadic, 2000, Krausman, 2003, Duffy et al., 2005). The blood samples can be easily obtained from domestic animals that are relatively small and, therefore, easier to house and to handle. This leads to more economical methods of biomonitoring as they can be monitored repeatedly without requiring the destruction of the animal.

Sheep were chosen as the model for domestic animals in this study because of their relative abundance at varied locations in the Irish countryside and the availability of antibodies and other biochemical and cellular tools. In addition to being readily available, sheep are relatively small and, therefore easier, to house and handle and more economical than cattle. Although quantitative studies that describe the biochemical effects of environmental exposure of sheep are rare, they have been used to monitor the bioavailability and bioaccumulation of chemical contaminants and integrate exposures across their entire environment (van Kampen, 1969, Pistl, 2002, Mohammad, 2007 , Duffy, 2009).

The studies described in Chapter 3 demonstrated that carefully controlled cryopreservation was a suitable technique for the preservation of rat blood, although the viability of lymphocytes was significantly reduced after 6 hours in culture. The overall objective of this study was to determine whether cryopreservation could be successful for the long-term storage of whole blood in field-based collection (sheep blood) as well as in the laboratory setting (rat blood). In addition, it was decided to determine if any loss of viability (cell death) observed was caused by the generation of reactive oxygen

species (ROS) and involved apoptosis and/or necrosis (Wyllie, 1980, Corcoran et al., 1994).

ROS arise from cellular respiration which involves the reduction of molecular oxygen (O_2) to water in the mitochondrial electron transport chain (Yu, 1994). Upon subsequent addition of electrons to molecular oxygen, the superoxide anion radicals, hydroperoxyl radical, hydrogen peroxide and the hydroxyl radical are formed (Kelly et al., 1998). The hydroxyl radical is the potentially most potent oxidant encountered in biological systems and can react readily with a variety of molecules, such as lipids, DNA and proteins at close to diffusion limited rates (Yu, 1994, Lieu et al., 2001).

ROS such as superoxide, hydrogen peroxide and lipid hydroperoxides have roles in the regulation of intracellular calcium, kinases, transcription factors and apoptotic factors (D'Autreaux and Toledano, 2007). They also contribute to immune cell regulation as they are involved in the mechanism of respiratory burst (D'Autreaux and Toledano, 2007). However, when the levels of reactive species that include oxygen-centred and nitrogen-centred radicals produced outweigh the capacity of the combined cellular antioxidant defences, oxidative stress occurs and can lead to cell death manifested as apoptosis or necrosis (Bus et al., 1974, Cagen and Gibson, 1977, Burk, 1991, Kehrer, 1993, Halliwell, 1996, Berlett and Stadtman, 1997, Cheng et al., 1998, Cheng et al., 1999).

The major biochemical process of necrosis begins with the loss of ion homeostasis leading to an influx of water and extracellular ions. (Darzynkiewicz et al., 1997). Necrosis is a violent and non-discriminating process characterised by rapid cytoplasmic swelling, destruction of cell organelles (mitochondria and endoplasmic reticulum) and plasma membrane rupture which leads to expulsion of intracellular contents, including lysosomal enzymes and their release into the extracellular fluid or the surrounding medium (Wyllie, 1980, Darzynkiewicz et al., 1997, Halestrap et al., 2000). It has been described as a pathological process which occurs when cells are exposed to a serious physical or chemical insult, such as in hypoxia, ischemia, temperature fluctuations, disruption of membrane structure and exposure to toxins (Fink and Cookson, 2005).

In contrast, apoptosis is considered to be a form of “programmed cell death” and is a morphologically and biochemically distinct form of cell death compared to necrosis.

Apoptosis is an energy-requiring process that is characterised by DNA fragmentation, nuclear segmentation, cytoplasmic shrinkage and membrane blebbing. Alteration in membrane symmetry, such as translocation of phosphatidylserine from the cytoplasm to the extracellular side of membrane occurs (Kerr et al., 1972, Thompson, 1995). The change in the cell surface enables recognition by macrophages. This leads to phagocytosis whereby the macrophages rapidly and efficiently engulf apoptotic cells enabling them to be lysed in a non-inflammatory manner (Wyllie, 1980, Cohen et al., 1992, Darzynkiewicz et al., 1997, Leist and Nicotera, 1998). The induction of apoptosis has been assessed in sheep blood preparations to address the possibility that cryopreservation may mediate cell death induction and cause functional changes that have long been reported in immune cells after freeze–thawing (Riccio et al., 2002).

In this study, a range of sheep blood preparations, namely, fresh whole blood, fresh processed blood, frozen blood and PBMCs were obtained under field-conditions. The objectives were (i) to compare the preparations in terms of time efficiency of sample preparation and impact on cell viability in culture assays; (ii) to study the use of cryopreservation of whole blood as a method of storage and (iii) to investigate the production of reactive oxygen species (ROS), necrosis and apoptosis in the cryopreservation-induced cell death of lymphocytes present in sheep blood.

The production of ROS in lymphocytes (in the sheep blood) was determined by dicholorofluorescin oxidation (Zhu et al., 1994). The assay uses the redox fluorescent probe, 2', 7' dichloroflourescein diacetate (H_2DCFDA), which freely permeates cells and accumulates mostly in the cytosol, where it is deacetylated by esterases to dicholorofluorescin (H_2DCF). The H_2DCF becomes trapped within cells and is stable for a few hours (Bass et al., 1983, Zhu et al., 1994). H_2DCF is rapidly oxidised by ROS, especially hydroxyl radicals and lipid hydroperoxides, to a fluorescent form known as dichlorofluorescein (DCF). The green fluorescence at 530 nm is proportional to the level of ROS present.

The ability of the well-known water soluble antioxidant, ascorbic acid, to enhance the viability of the cryopreserved blood lymphocytes in culture was also assessed. Ascorbic acid is known to scavenge the superoxide anion radicals and hydroxyl radical and can also protect DNA against damage induced by ROS (Duthie et al., 1996).

The membrane integrity of lymphocytes in culture was used as an indicator of cell death and was measured using the propidium iodide (PI) exclusion method (Moore et al., 1998). Early apoptosis and late apoptosis / necrosis were measured using annexinV and PI staining (Fadok et al., 2000).

Materials & Methods

Chemicals & Reagents

All chemicals used were of the highest grade available: RPMI 1640 medium with 2 mM L-glutamine; heat-inactivated foetal bovine serum (FBS); HEPES; phosphate-buffered saline (PBS); dimethyl sulphoxide (DMSO); propidium iodide (PI); lymphocyte separation medium (Histopaque 1083) and ascorbic acid were purchased from Sigma-Aldrich, Ireland. The antibiotic gentamycin was procured from GIBCO, UK. Sterile 96-well tissue culture plates were purchased from Sarstedt (Ireland). Ammonium chloride RBC lysis buffer (Pharmlyse) annexinV-FITC apoptosis detection kits and sodium heparin and potassium EDTA blood collection tubes (10 ml) were procured from BD Biosciences, Ireland. The redox fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes Europe, the Netherlands.

Animals

Three two-year-old Cheviot-cross ewes, tagged 401, 440 and 642, were derived from a breeding stock at the Central Veterinary Laboratory (CVL) Dublin, Ireland and relocated to a farm 10 km from the laboratory at which the cell isolations and assays were performed. They were housed together in one pen and provided with hay *ad libitum* in racks and water in a large plastic container. All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Blood Collection

Jugular blood samples were extracted from the ewes after they were restrained in individual small pens. Blood (50-60 ml) was collected from each ewe in 9 ml sodium heparin tubes. The blood was then divided with some used for PBMC isolation, some for the preparation of fresh whole blood by dilution, some was processed by centrifugation and the remaining blood was cryopreserved as described previously in Chapters 2 and 3 and shown in Figure 1.

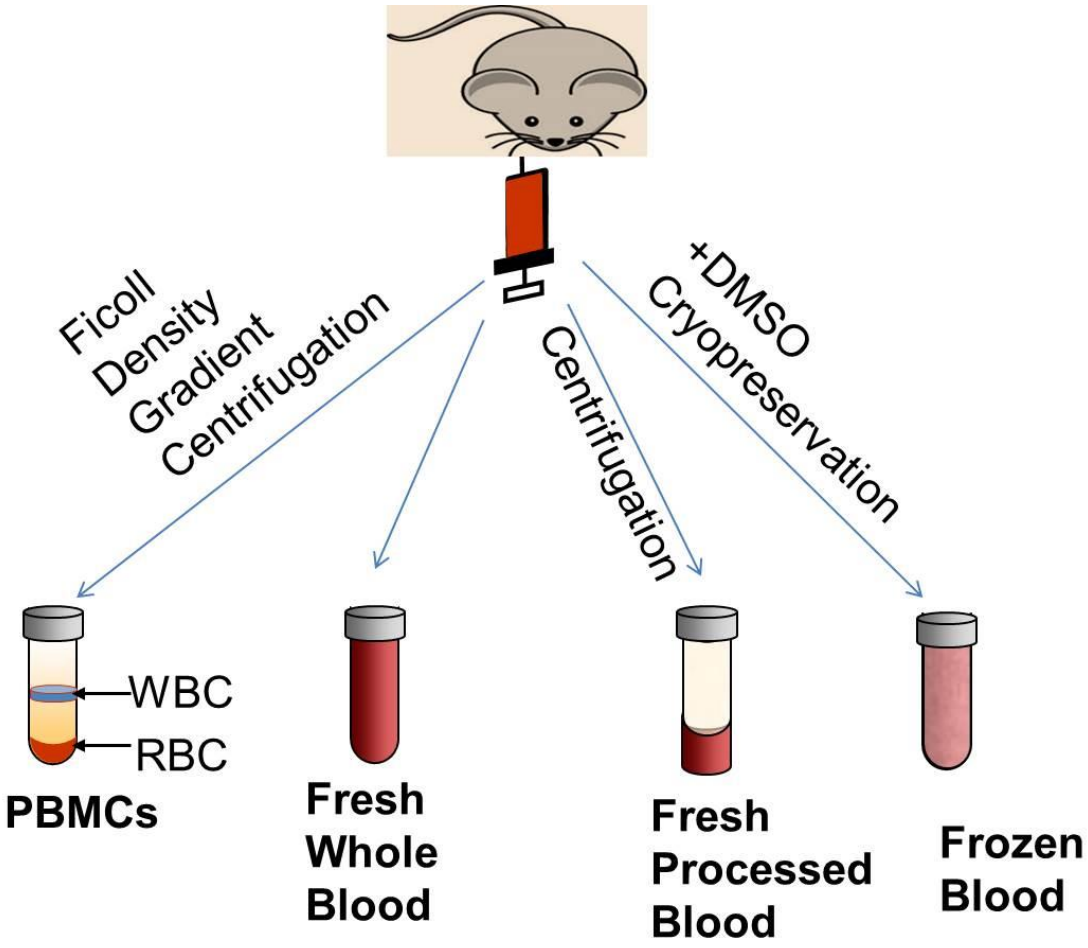


Figure 1. Schematic of the Different Blood Sample Processing Methods Used in this Chapter.

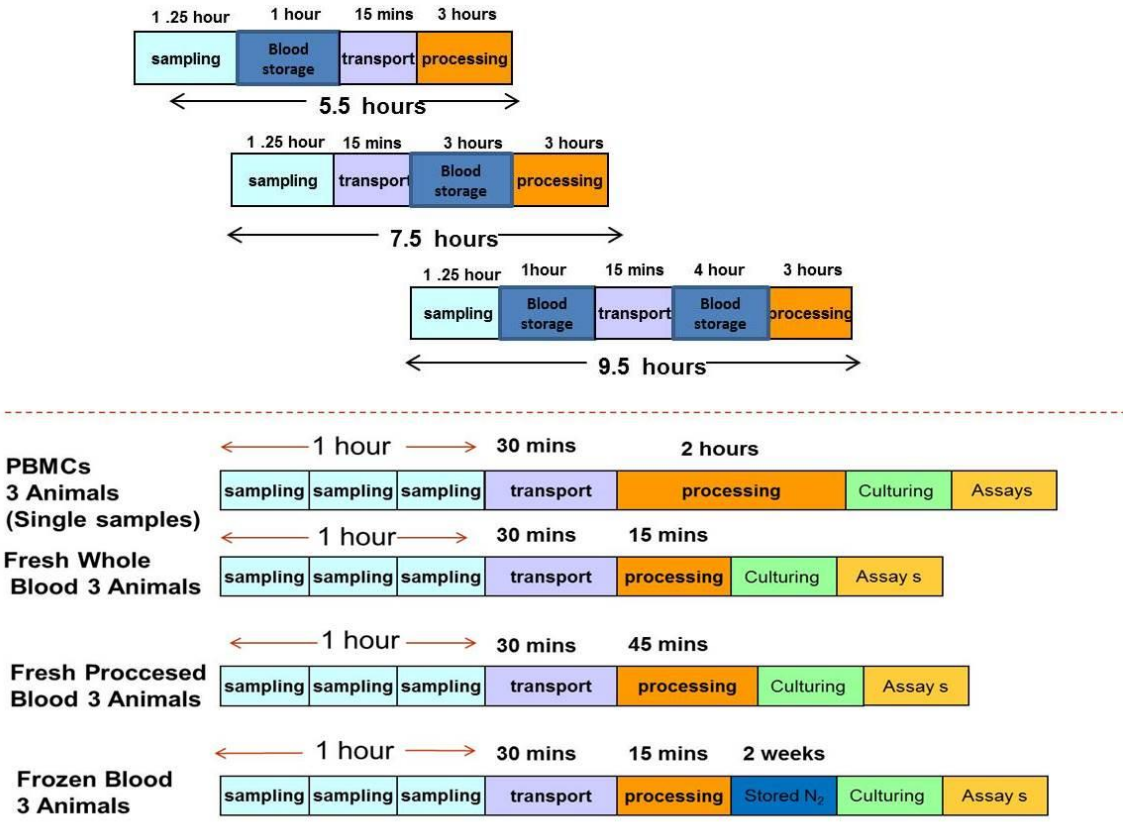


Figure 2. Schematic of the Schedule of Sampling, Transport, Storage and Processing when PBMCs were Isolated from Multiple Blood Samples (above the line) Compared to the Schedule of Sampling, Transport, Storage and Differential Processing of Blood samples (below the line) when Collected from Sheep in the Field.

Because the blood was collected under field-based conditions and multiple samples were taken from each sheep, the blood sampling draws were staggered in the order sheep 1, 2, and 3 (as shown previously in chapter 2). The veterinarian took samples in the field and a laboratory-based technician processed the blood and performed Ficoll-density gradient centrifugation to isolate PBMCs as described in Figure 1.

The time taken for the isolation and processing of PBMCs when multiple samples were taken from three sheep is depicted in Figure 2 (above the dotted line) and was explained in Chapter 2. In this scenario, it took up to 9.5 hours before all the samples were processed. In the experiments described in this chapter, only single blood samples were taken on each occasion from each sheep and then split between the 4 different sample

preparations. The samples were collected on three different sampling dates and treated as three independent experiments.

The duration for each sample processing method was recorded and together with the schedule of sampling, transport, and processing is shown in Figure 2.

The PBMCs in all samples were counted using trypan blue stain and a haemocytometer and whole blood was cryopreserved by placing it in cryovials in a slow-freezing-rate container (Mr Frosty, Nalgene, UK) and stored in a -80°C freezer for 24 hours. Finally, the cryovials containing the frozen blood were transferred to liquid nitrogen for storage. All the blood samples were procured, transported and processed within a 4 hour window.

Haematology and Serum Biochemistry

Haematological analyses were carried out on freshly collected blood with EDTA as anti-coagulant using an automated haematology analyzer (Sysmex E-820), which uses automatic windowing to differentiate between different cell subsets (courtesy of the Munster Regional Veterinary Laboratory (MRVL), Co Cork, Ireland). White Blood Cell (WBC) was determined by Wintrobe method (Coles, 1986). Differential counts of WBC (neutrophils, eosinophils, monocytes, basophils and lymphocytes) were determined by counting 100 WBC Giemsa's blood stain method (Coles, 1986). Serum biochemistry was determined in serum blood-tubes courtesy of the Munster Regional Veterinary Laboratory (MRVL), Co Cork, Ireland. Total protein determination was carried out by the Biuret method of Savory and Sunderman (1968). Albumin was determined by the method described by Johnson et al., (1999). Serum electrolytes (sodium, potassium, chloride) were determined by atomic absorption spectroscopy as reported by Adejumo and Onifade (2005).Serum minerals (calcium, and phosphorus) assayed using Sigma kits (Daly and Ertingshausen, 1972, Gindler and King, 1972, Harris, 1995; Moss and Henderson, 1999). Liver markers such as serum enzymes (AST, SDH, ALP, and CK) were determined according to Coles (1986). Renal markers such as urea were determined by the urease-Berthelot method (Coles, 1986) and creatinine was determined according to the method described by Henry (1974). Blood glucose was determined by Folin and Wu method (Allen, 1990). Clinical chemistry values were analyzed in serum on a selective discrete clinical chemistry analyzer (Hitachi 717).

Sample Preparation

Isolation of PBMCs

Fresh sheep PBMCs were isolated from heparinised blood by routine Ficoll-density gradient centrifugation as described by Boyum (1968) and as detailed previously in Chapter 2.

Fresh Whole Blood

Fresh blood (1 ml) was diluted with 2 ml of complete culture medium, mixed gently and put in culture as described by Connor et al, (2000) and as detailed previously in Chapter3.

Fresh Processed Blood

Fresh blood (1ml) was added to 10 ml of complete culture medium and centrifuged for 7 minutes at 300 x g. The supernatant was aspirated and the remaining 1 ml pellet and supernatant was diluted as detailed previously in Chapter 3.

Frozen Blood

DMSO (150 µl) was added to 1350 µl of fresh sheep blood in a cryovial placed in a slow- freeze container, stored in a −80 °C freezer for 24 hours then stored in liquid nitrogen dewars as detailed previously in Chapter 3.

Thawing of Frozen Blood

Frozen samples were thawed in 37°C water according to the procedure adapted from the protocol described by Reimann and colleagues (Reimann et al., 2000). Slow dilution with thawing medium was followed by centrifugation and resuspension as detailed previously in Chapter 3.

Cell Culture

All the blood sample preparations were cultured in complete culture medium with a volume of 500 µl/well in a 24-well plate and all treatments were performed in triplicate and kept at 37°C in a 5% CO_2 humidified incubator for the duration of the culture (normally up to 24 hours) after which the lymphocytes were assayed. In addition, frozen sheep blood was also cultured in triplicate wells and sampled at various time-points, 0, 3, 5 and 7 hours after initiation of culture for experiments using the annexin V-FITC and H_2DCFDA oxidation assays.

Propidium Iodide (PI) Exclusion Assay

The viability of lymphocytes in blood sample preparations was determined by propidium iodide (PI) exclusion as described by Moore et al., (1998) and as detailed in Chapter 2. As stated previously, the only cell types that were analysed in the blood sample preparations were lymphocytes as the monocytes and dendritic cells were still attached to the plastic tissue culture plate and the granulocytes were non-viable.

Annexin V-FITC Apoptosis Assay

Lymphocytes from frozen blood were prepared and stained with annexin V-FITC and propidium iodide (PI) as described by Moore et al., (1998) and as detailed previously in Chapter 2.

Determination of the Formation of Reactive Oxygen Species (H_2DCFDA Oxidation Assay)

Reactive oxygen species (ROS) production was assessed by oxidation of 2´, 7´-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, the Netherlands) to the fluorescent product, 2´,7´-dichlorofluorescein (DCF), which has a green fluorescence and is detected at 530 nm in the FL1 channel. For H_2DCFDA oxidation assays, $1x10^6$ cells were incubated in RPMI with 30 µM H_2DCFDA for 30 minutes at

37°C in a water bath on the gentle shaking setting. Thereafter, cells were centrifuged for 7 min at 300 x g, resuspended (washed) once with 10 ml PBS and resuspended in 500 µl PBS. The tubes were immediately placed on ice. Flow cytometric analysis was performed within 5 minutes.

Flow Cytometric Analysis

Flow cytometry was performed on a FACS Calibur instrument (Becton Dickinson, UK). A minimum of 10,000 cells, were analysed for each sample. Data were acquired and analysed using Cell Quest Pro software (Becton Dickinson, UK). The lymphocyte gate was set up in a forward scatter (FSC)/side scatter (SSC) dot plot. Lymphocytes were the only subset analysed for cell viability in the sheep blood as they were the only subset that was present in all the sample preparations.

Statistical Analysis

Results are presented as means ± SD where the mean was calculated from the 3 replicates per treatment and the means calculated from three independent experiments. All statistical analyses were conducted using SigmaStat software (version 2.03). Data were tested for normality using a Kolmogorov-Smirmnov test. Differences between groups were analysed using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons. Statistical significance was set at $p<0.05$ and significant levels were defined as *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Haematology and Serum Biochemistry Analysis

The blood differential leukocyte counts measured for each sheep are shown in Table 1 and were within the specifications as detailed by the MRVL for healthy sheep.

Test	Sheep ID			
	401	**440**	**642**	**Normal range**
Lymphocytes %	46	50	63	40–75
Neutrophils %	47.5	31	31	10–50
Band neutrophils %	1.5	0.7	1	0-2
Eosinophils %	3	1.5	1.5	0-10
Monocytes %	1	0	0	0-6
Basophils %	0	0.5	1.5	0-3
RBC x 10^{12}/L	12.64	13.58	12.85	9–15
WBC x 10^{9}/L	7.75	9.5	9	4–12
Lymphocytes x 10^{9}/L	3.77	5.38	5.695	2–9
Neutrophils x 10^{9}/L	3.04	3.19	2.69	0.7–6.0
Band Neutrophils x 10^{9}/L	0.11	0.07	0.08	0–0.1
Eosinophils x 10^{9}/L	0.275	0.12	0.15	0–1
Monocytes x 10^{9}/L	0.09	0.04	0.2	0–0.75
Basophils x 10^{9}/L	0	0.04	0.15	0–0.3

Table 1. Differential Blood Leukocyte Counts from the Three Sheep Used in this Study.

The serum biochemistry parameters measured for each sheep are shown in Table 2 and were within the specifications as detailed by the MRVL for healthy sheep.

Serum Biochemistry Parameter:	Sheep ID			
	401	440	642	Normal Range
Glucose	80 mg/dl	75 mg/dl	70 mg/dl	44–81 mg/dl
Blood Urea Nitrogen (BUN)	14 mg/dl	18 mg/dl	18 mg/dl	10–26 mg/ dl
Creatinine	1.4 mg/dl	1.8 mg/dl	1.5 mg/dl	0.9–2.0 mg/ dl
Calcium	11.9 mg/dl	11.9 mg/dl	12.2 mg/dl	9.3–13.7 mg/ dl
Phosphorus	7.7 mg/dl	6.6 mg/dl	8.8 mg/dl	4.0–9.3 mg/ dl
Total Protein	6.8 g/dl	6.4 g/dl	7.0 g/dl	5.9–7.8 g/ dl
Albumin	2.7 g/dl	2.7 g/dl	2.8 g/dl	2.7–3.7 g/ dl
Alkaline Phosphatase (ALP)	250 IU/L	227 IU/L	237 IU/L	97–266 IU/L
Sorbitol Dehydrogenase (SDH)	18 IU/L	14 IU/L	21 IU/L	3.5–21 IU/L
Aspartate Aminotransferase (AST)	180 IU/L	160 IU/L	150 IU/L	49–183 IU/L
Creatine Phosphokinase (CPK)	346 IU/L	220 IU/L	194 IU/L	107.7–351 IU/L
Chloride	102 mEq/L	105 mEq/L	92 mEq/L	91–113 mEq/L
Potassium	5.0 mEq/L	5.3 mEq/L	5.4 mEq/L	4.3–6.3 mEq/L
Sodium	149 mEq/L	142 mEq/L	145m Eq/L	132–160 mEq/L

Table 2. The Serum Biochemistry Profiles of the Blood of the Three Sheep Used in this Study.

Effects of Different Blood Sample Processing Preparations on the Viability of Lymphocytes over 24 Hours in Culture

The viabilities of lymphocytes in the various blood samples preparations (i.e. PBMCs. fresh whole, fresh processed and frozen blood) over 24 hours in culture are shown in Figure 3. All the sample preparations had a lymphocyte viability of over 85 % after 5 hours in culture. The greatest reduction in viability was observed for frozen blood. The time-dependent reduction became significant after 7 hours (13 % reduction to 75 %) ($p<0.001$) and was substantial (35 % reduction to 56 %) after 24 hours ($p<0.001$). The reductions in the viability of lymphocytes in the other samples after 24 hours culture were 14 % for fresh blood ($p<0.01$), 12 % for fresh processed blood ($p<0.01$) and 8 % for PBMCs ($p<0.01$).

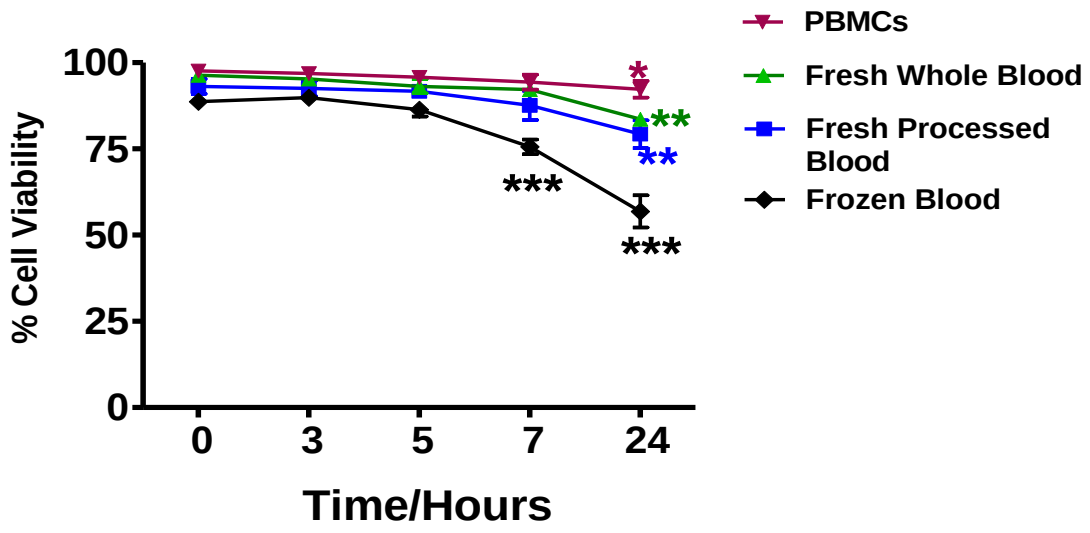
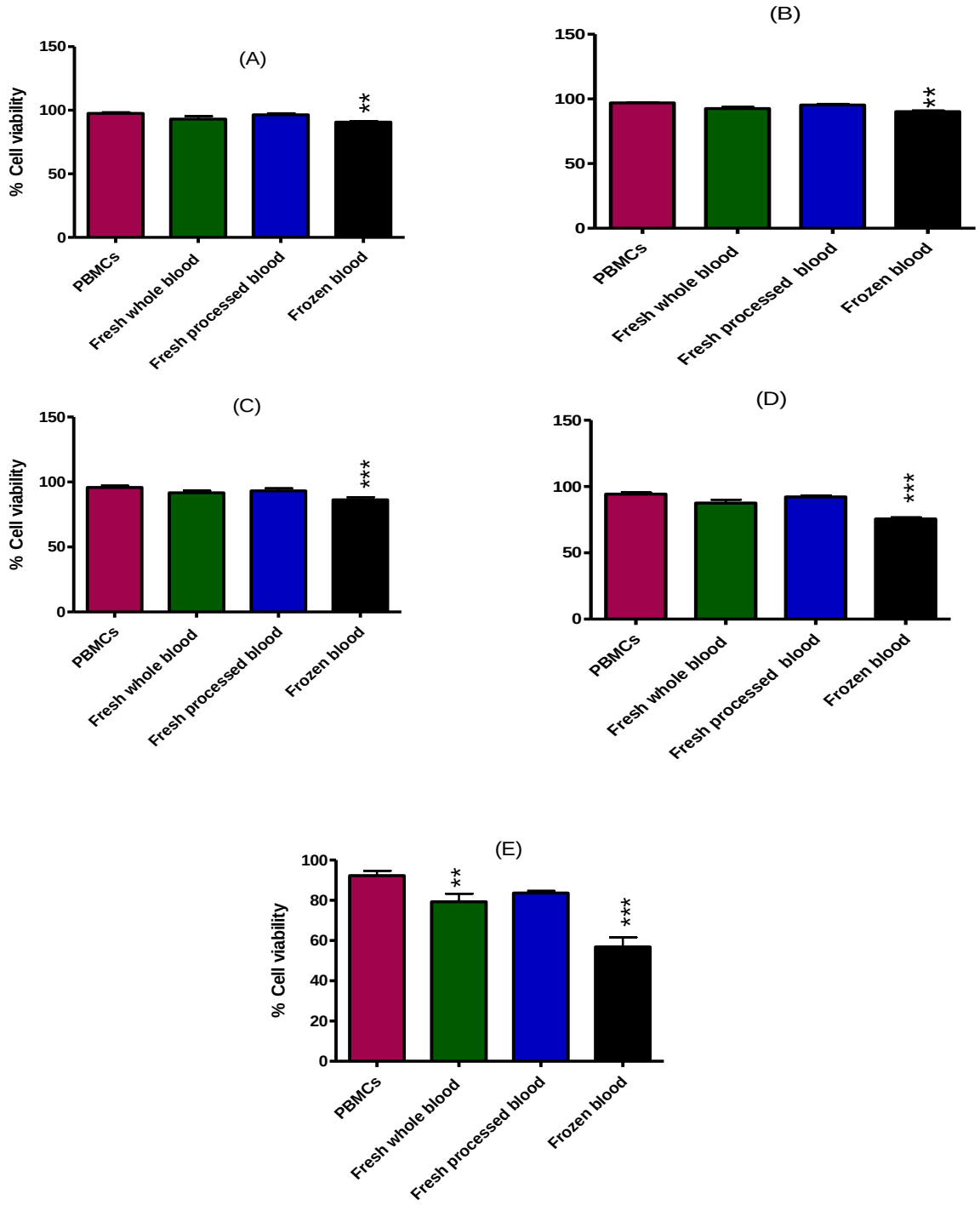


Figure 3. Effect of Sample Processing on Lymphocyte Viability over 24 Hours in Culture
Data are represented as the percentage viability of sheep lymphocytes cultured from PBMCs, fresh whole blood, fresh processed blood and frozen blood preparations over culture periods of 3, 5, 7 and 24hours as assessed by PI staining. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment. Statistical comparisons *represents 0 hrs vs. other time points (* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$).

Before culturing of blood sample preparations is initiated there was a 7 % ($p<0.01$) significant difference in cell viability between fresh PBMCs and frozen blood. After culturing of blood sample preparations there were significant differences in cell viability between fresh PBMCs and frozen blood of 7 % ($p<0.01$), 9.5 % ($p<0.001$), 19 % ($p<0.001$), 35 % ($p<0.001$) after 3, 5, 7 and 24 hours (Figure 4A, 4B, 4C, 4D, 4E). In addition, there was a significant difference in cell viability between fresh PBMCs and fresh blood of 13 % ($p<0.01$) after 24 hours (Figure 4E).



Figures 4A, 4B, 4C, 4D and 4E. Effect of Sample Processing on Lymphocyte Viability over 24 Hours in Culture

Data are represented as the percentage viability of sheep lymphocytes cultured from PBMCs, fresh whole blood, fresh processed blood and frozen blood preparations over culture periods of 0(4A), 3(4B) 5(4C), 7 (4D), and 24 (4E) hours as assessed by PI staining (same data as Figure 3). Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment. Statistical comparisons *represents PBMCs vs. other sample preparations (* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$).

The Levels of Total Cell Reactive Oxygen Species Production in Lymphocytes in Frozen Blood over 7 Hours in Culture.

The levels of reactive oxygen species (ROS), as assessed by H_2DCFDA oxidation, in lymphocytes from frozen blood over 7 hours in culture are shown in Figure 5. A significant increase in ROS levels was observed at 7 hours in culture ($p<0.05$) but not at the earlier time-points.

Frozen blood was selected for this analysis because of the substantial drop in viability of lymphocytes observed after 7 hours in culture compared to the other sample preparations. In addition, it was accepted that due to the time involved in processing a large number of samples, it was not feasible to include the fresh blood samples in this study.

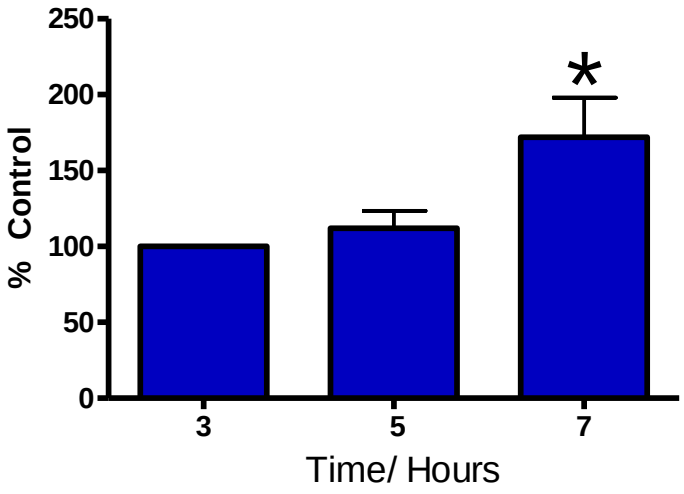


Figure 5. The Amount of Total Cell ROS Production in Lymphocytes in Frozen Blood over 7 Hours in Culture Data are represented as the ROS production of sheep lymphocytes cultured from frozen whole blood preparation over culture periods of 3, 5 and 7 hours as assessed by H_2DCFDA oxidation. Data are represented as the mean percentage increase in H_2DCFDA oxidation +/- SD for three independent experiments with 3 replicates per experiment (* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$). * Statistical analysis compares the percentage increase in H_2DCFDA oxidation at each time point to the 3 hour time point.

Effects of Ascorbic Acid on Total Cell Reactive Oxygen Species in Lymphocytes in Frozen Blood over 7 Hours in Culture.

The impact of ascorbic acid addition on the levels of reactive oxygen species (ROS) produced by lymphocytes in frozen blood over 7 hours in culture is shown in Figure 6. Ascorbic acid was chosen because it is a well-known water soluble antioxidant.

The addition of 100 µM ascorbic acid caused a significant reduction in the ROS levels detected at 7 hours in culture ($p<0.05$).

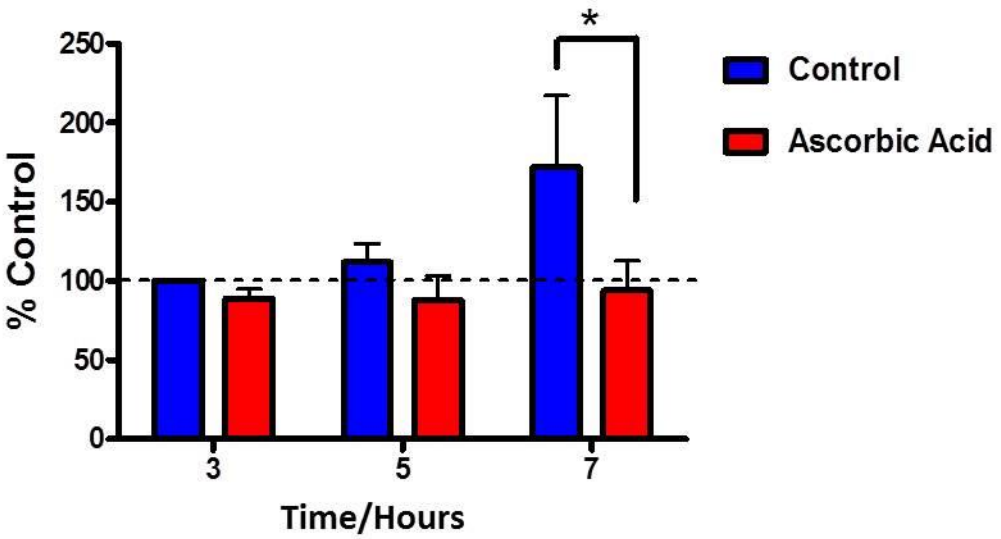


Figure 6. The Effect of Ascorbic Acid on Reactive Oxygen Species Production in Lymphocytes from Frozen Blood over7 Hour in Culture. Data are represented as the total cell ROS production of sheep lymphocytes cultured from frozen blood preparation over culture periods 3, 5 and 7 hours with and without ascorbic acid (100 µM) as assessed by H_2DCFDA oxidation. Data are represented as the mean percentage increase in H_2DCFDA oxidation +/- SD for three independent experiments with 3 replicates per experiment (* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$). * represents a significant difference between the mean relative fluorescence of a treatment group and its corresponding control at each particular time point.

Effects of Ascorbic acid on Lymphocyte Viability in Frozen Blood over 7 Hours in Culture.

The viability of lymphocytes in frozen blood, as assessed by PI staining, over 7 hours in culture in the presence and absence of ascorbic acid (100 µM) is shown in Figure 7. There was a significant reduction in the cell viability of lymphocytes after a 7 hour culture ($p<0.001$) in the absence but not in the presence of ascorbic acid. There was a 9 % drop in the levels of lymphocyte viability at 7 hours in culture compared to 4 % loss after the addition of ascorbic acid ($p<0.05$).

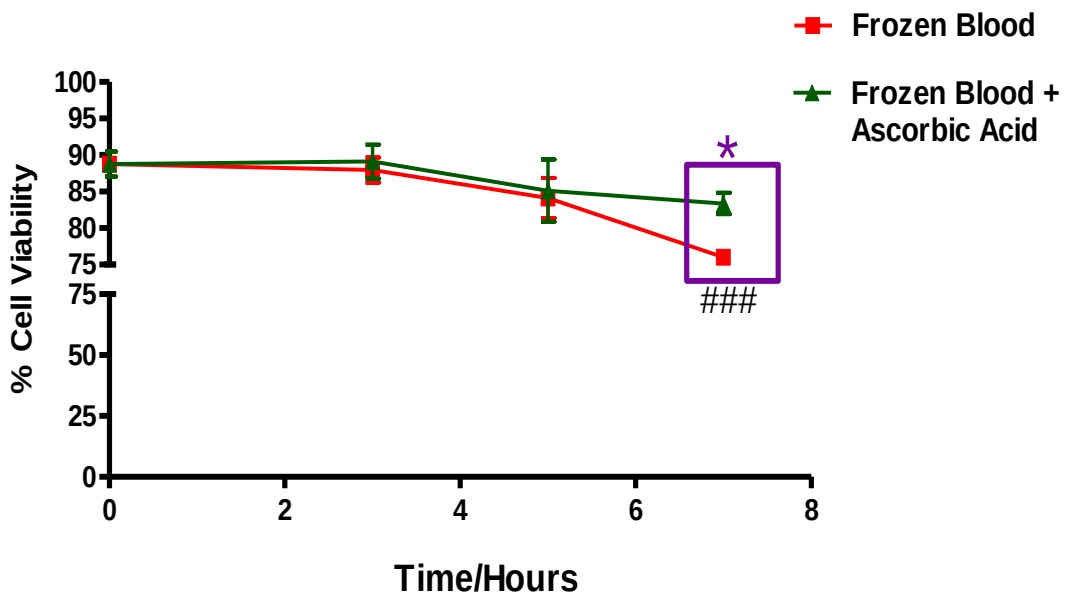


Figure 7. Effects of the Addition of Ascorbic Acid on the Viability of Lymphocytes in Frozen Blood over 7 hours in Culture. Data are represented as the percentage viability of sheep lymphocytes cultured from frozen whole blood preparation over culture periods of 3, 5 and 7 hours with and without ascorbic acid (100 µM) assessed by PI staining. Data are represented as means +/- SD for three independent experiments with 3 replicates per experiment. *represents a significant difference between the % viability of a treatment group and its corresponding control without ascorbic acid at each particular time point (* $p < 0.05$). # represents 0 hrs vs. other time points for both the lymphocytes with and without ascorbic acid (###$p < 0.001$).

The Levels of Apoptosis in Lymphocytes in Frozen Blood over 7 Hours in Culture.

The levels of apoptosis (AnV-FITC $^{+}$) and viability (PI^{-}) in lymphocytes from frozen blood over 7 hours in culture are shown in Figure 8.

The level of late apoptosis/necrosis (AnV-FITC $^{+}$/ PI^{+}) increased with time and became significant at 5 hours ($p<0.05$) and highly significant at 7 hours ($p<0.001$). There was a detectable level of early apoptosis (AnV-FITC $^{+}$/PI^{-}) at all-time points but it did not reach significance up to 7 hours in culture. There was a drop in number of viable cells (AnV-FITC $^{-}$/PI^{-}) at the 5 and 7 hour timepoints but it did not reach significance up to 7 hours in culture**.**

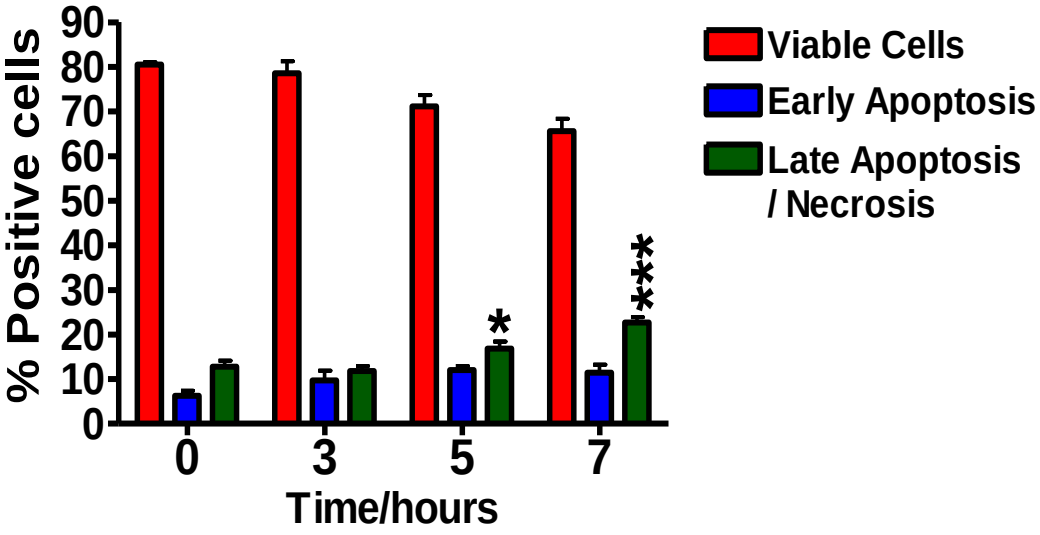


Figure 8. The Level of the Apoptotic Lymphocytes in Frozen blood over 7 Hours in Culture Data are represented as the percentage of viable, early and late apoptotic sheep lymphocytes cultured from frozen whole blood preparation over culture periods of 3, 5, 7 and 24 hours as assessed by annexin V-FITC/ PI staining. Data are represented as means +/- SD for three independent experiments with 3 replicas per experiment. Statistical comparisons *represents 0 hours vs. other timepoints. (* $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$).

Discussion

The results described in Chapter 3 demonstrated the potential of cryopreservation for the successful storage of rat blood collected under laboratory-based conditions. The viability of frozen rat lymphocytes was only marginally affected by the freeze thaw process after 3 hours in culture. However, there was a significant drop in viability after 6 hours in culture.

The objective of this study was to assess the suitability of cryopreservation for the storage of sheep blood collected under field-based conditions. A key determinant was the duration for which sheep lymphocytes could be cultured after the freezing and thawing steps without a substantial drop in their viability. In addition, the mechanisms by which cryopreservation induces cell death in lymphocytes in frozen sheep blood was investigated. In this regard, the levels of reactive oxygen species (ROS) present in lymphocytes following cryopreservation were measured as was the ability of the water-soluble antioxidant, ascorbic acid, to prevent their accumulation. The integrity of cell membranes and the extent of early apoptosis and late apoptosis/necrosis present in frozen/thawed lymphocytes were also determined.

The presence of different cell types and blood components in the sample preparations used, i.e. PBMCs, fresh whole blood, fresh processed blood and frozen blood are shown in Table 3.

Blood Cell Type/ Separation Techniques				
Sample Preparations	PBMCs	Fresh Whole Blood	Fresh Processed	Frozen Blood
Separation Techniques	Ficoll Density Gradient Centrifugation	Dilution	Centrifugation +Dilution	Cryopreservation +Thawing +Centrifugation +Dilution
Lymphocytes	+	+	+	+
Erythrocytes	-	+	+	↓
RBC Fragments	-	-	-	+
Granulocytes	-	+	+	↓
Foetal Bovine Serum	10%	10% (+13% Plasma)	8%	8%

Table 3: Cell types and Blood Culture Components after Various Blood Separation and Fractionation Techniques. ↓ refers to substantial reduction of granulocytes and erythrocytes in the blood sample mixture following cryopreservation. +/- refers to the presence or absence of certain blood constituents after the different sample processing methods have been completed.

When fresh whole blood, fresh processed blood and PBMCs were cultured for up to 7 hours, all samples retained a lymphocyte viability of greater than 85% while after 24 hours in culture the viability was still greater than 80%. The findings for fresh whole blood are in agreement with those of Fiebig and colleagues who reported a viability of 95% (Fiebig et al., 1997). It is speculated that whole blood provides an environment resembling *in vivo* conditions thus contributing to the high viability observed. Likewise, the high levels of viability observed with PBMCs (greater than 92% after 24 hours) are

in agreement with studies by Sarkar et al., (2003) , Disis et al., (2006), Garcia-Pineres et al., (2006) and Ruitenberg et al., (2006). The viability of lymphocytes in frozen sheep blood immediately after thawing was 90%. This was significantly reduced after 5 hours in culture and at 24 hours had dropped to 56%. These results are in agreement with other studies that showed that the viability in frozen blood was between 85 and 90% after thawing (Hayes et al., 2002, Schindler et al., 2004). In contrast, a starting lymphocyte viability of 70% has also been reported (Fiebig et al., 1997). This difference may be explained by the fact that Fiebig and colleagues thawed their frozen blood aliquots by allowing them to come to room temperature overtime in contrast to the quick thaw method at 37°C in the presence of 20% foetal bovine serum and DNAse used in the present study. The importance of the steps such as dilution, media additives and temperature profile during thawing in maintaining the viability of PBMCs after cryopreservation was shown by Disis et al., (2006). The results obtained in this study suggest that cryopreservation of whole blood samples collected in the field could be a promising technique for biomonitoring studies that use short-term culture times.

The loss in viability in the frozen sheep blood after thawing might be explained by the increased sensitivity of lymphocytes to undergo cell death after the loss of red blood cells due to lysis this could also be explained by the loss of antioxidants in the whole blood (Halliwell and Gutteridge, 1985). This haemolysis was defined by Wilmer et al., (1983) as the disruption of the integrity of the red cell membrane causing leaking of the cell contents into the surrounding media. The leaked contents contain the pigment haemoglobin as well as iron and iron-bound substances that have the ability to generate primary reactive oxygen species (ROS) and enhance the reactivity of other ROS generated by other pathways (Cooper et al., 2002). While in this study the impact of haemolysis would be reduced by the cell-washing step used after thawing, even relatively small amounts of ROS material released could have a marked effect on the lymphocyte population. It is noteworthy also that the lysis of red blood cells leads to a loss of protective antioxidant mechanisms such as superoxidase dismutase, catalase and glutathione peroxidase which are present in erythrocytes and this adds to the greater sensitivity of lymphocytes in frozen blood to ROS material (Halliwell and Gutteridge, 1985).

The use of the redox dye dichloroflourescein diacetate (DCFDA) following an extended culture whereby 2´,7´-dichlorofluorescein (DCF) fluorescence provides a measure of ROS production in the cell cytosol could possibly be an good tool to assess the effects of red blood cell lysis and freeze thawing on lymphocytes in frozen blood (Halliwell and Whiteman, 2004, Tarpey et al., 2004). This technique was used to demonstrate a significant increase in the levels of ROS in lymphocytes in frozen blood after 7 hours in culture in the present study. The addition of ascorbic acid reduced the levels of ROS detected and, therefore, prevented the oxidation of the DCFDA dye. Interestingly, co-incubation with ascorbic acid also prevented the loss in lymphocyte cell viability observed with frozen blood after 5 hours in culture. In this experiment, red blood cell lysis and ROS production were observed in addition to a drop in cell viability but a causative relationship has not been proved. The contribution of apoptosis to the reduction in lymphocyte viability when frozen blood was cultured was examined by using indices of early and late apoptosis/necrosis as assessed by annexin V and propidium iodide staining. While a detectable level (11% on average) of early apoptosis was observed there was no substantial drop in cell viability with time. However, cells will progress from early to late apoptosis. Fowke et al., (2000) showed that whole blood stored in liquid nitrogen had a greater level of apoptosis than matched PBMC samples stored at the same temperature. However, it is difficult to compare this finding with those of the present study because Fowke et al., (2000) used the Hoechst 33342 staining technique that does not separate lymphocytes, monocytes and granulocytes. Hoechst 33342 apoptosis analysis scores the nuclear morphology of cells using fluorescent microscopy. Granulocytes are completely non-viable after 24 hours in culture so this would give false-positive recognition of apoptotic lymphocytes when using the Hoechst 33342 staining (Pruett and Loftis, 1990). In contrast, the use of PI and annexin V staining by flow cytometry permitted the analysis of both lymphocytes and granulocytes in this study; however, lymphocytes were the only population analysed. This suggests that apoptosis was not the only cause of the cell death overtime and that maybe necrosis was also involved and both of these mechanisms of cell death can be caused by the production of ROS.

In the frozen blood the viability was significantly reduced after 5 hours in culture when assessed by the prodium iodide (PI) exclusion assay. However when measuring the number of viable cells (AnV-FITC $^{-}$/PI^{-}) using the annexin V/ PI assay there was a drop

in viable cells but this did not reach significance up to 7 hours in culture. These different observations can be explained by the presence of early apoptotic cells which are annexin-v positive but which are PI negative (Moore et al., 1998, Fadok et al., 2000).

The observed increase in ROS production probably occurred due to the presence of reactive hydroxyl radical species in the lymphocytes. A number of reactive species are capable of oxidising DCFDA to DCF. These include peroxyl, alkoyl, NO_2, NO, carbonate and •OH and $ONOO^-$ (Valkonen and Kuusi, 1997). However, neither $•O_2^-$ nor H_2O_2 can oxidise DCFDA in the absence of a cellular peroxidase so the actual oxidant involved is more likely to be a Fenton-type species rather than a peroxidase (LeBel et al., 1992, Zhu et al., 1994). When leukocytes were incubated in the presence of lysed red blood cells for 1 hour prior to analysis there was a significant increase in oxidative damage and DNA strand breaks (Villavicencio, 2006). The addition of deferoxiamine mesylate, the iron chelating agent used to bind iron during red blood cell lysis, prevents a Fenton type reaction from occurring (Villavicencio, 2006). Iron can participate in reactions leading to an increase in hydroxyl radical (Kasprzak, 2002). These radicals can be generated by the Fenton reaction through intermediates such as H_2O_2 and Fe^{2+} and can be scavenged by agents such as mannitol, urea and DMSO (Jimenez Del Rio and Velez-Pardo, 2004). A previous study showed that concentration dependent changes in DCF fluorescence were detected when lymphocytes were treated with ferrous ions (ferrous chloride) (Dix and Aikens, 1993).

There have been previous reports that show the ability of ascorbic acid to provide protection from hydroxyl radicals in mammalian cells. Huang and colleagues showed that exposure of Chinese hamster ovary cells (CHO) to nickel sulfide (Ni_3S_2) resulted in the generation of ROS, as measured by the increased formation of DCF. However, co-incubation with ascorbic acid and Ni_3S_2 led to scavenging of the ROS and prevented the oxidation of DCF-CA (Huang et al., 1993). In a later study involving rat lymphocytes, a 2 hour co-incubation of Ni_3S_2 with ascorbic acid resulted in a significant decrease in ROS-induced DNA-protein cross links (Chakrabarti et al., 2001). The addition of the dietary flavonoid, quercetin, has also been shown to act as a cryoprotectant when added to human lymphocytes prior to freezing (Duthie et al., 2002). One possible addition would be to include ascorbic acid in the thawing medium before the start of the

culturing of the frozen blood preparation. Ascorbic acid could not be added to the culture medium during the exposure as it would suppress the activity of the test compounds $CdCl_2$ and EMS. It has been shown previously that ascorbic acid is protective against cadmium-induced toxicity that may act via oxidative damage through the depletion of glutathione and binding to sulfhydryl groups of protein (Valko et al., 2005, Omonkhua, 2008)and EMS toxicity (Guha and Khuda-Bukhsh, 2002).

Main Conclusions

This study demonstrated that frozen blood is a promising sample preparation for field based biomonitoring studies when performed *in vitro* or *ex vivo* stimulations up to 3 to 5 hours. The viability results were consistent with those observed for laboratory-based rat blood studies in Chapter 3.

Cryopreservation of sheep blood led to an increase in the production of reactive oxygen species (ROS) after an incubation period of 7 hours.

The addition of ascorbic acid reduced the levels of ROS produced and protected against the decrease in cell viability. This suggests that ascorbic acid may be a useful additive in whole blood cryopreservation protocol, possibly in the thawing medium.

In the present study, it was shown that there were comparable levels of viability for lymphocytes in sheep frozen blood, fresh blood and PBMCs similar to what was shown for rat blood in Chapter 3. In addition, it has been shown that apoptosis does not appear to be the only cause of the cell death of lymphocytes in frozen blood in culture.

Future studies could assess the nature of reactions leading to ROS formation and the contribution of haemolysis and/or freezing to the increased ROS formation.

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Chapter 5

The Effects of Sample Processing on the Response of Lymphocytes in Cytotoxicity and Genotoxic Assays in Sheep Blood

Table of Contents

Introduction

With the realization that many environmental contaminants, especially heavy metals, e.g. cadmium and mercury, could cause severe negative effects to the health of humans and animals at low environmental concentrations, research attention turned to biologically-based monitoring, rather than contaminant (chemically-based) monitoring (Lam and Gray, 2001). The aim of this study was to develop biomarker assays for domestic animals rather than humans. Sheep were the environmental sentinels chosen even though they are generally not closer to industrialised areas than humans. They were chosen from among domestic animals for practical and economic reasons and because they are abundant at varied locations in the Irish countryside (Stahl Jr, 1997). Exposure specific biomarkers are common to sheep and humans and can be detected by antibodies and other biochemical and cellular tools which are commercially available. Blood is a common biological sample used for biomonitoring studies as the lymphocytes present are the most transcriptional active cells (Angerer et al., 2007). After exposure to chemicals, chemical specific or effect specific biomarkers can be monitored at the cellular, protein and gene level in lymphocytes (Liew et al., 2006).

As shown in Chapters 2, 3 and 4, the isolation of PBMCs is a time and labour intensive process. Therefore, alternative cell preparations, including fresh whole blood, fresh processed blood and frozen blood cultures were explored and these preparations were obtained from blood samples collected in the field. The studies described in Chapters 3 and 4 demonstrated that frozen blood is a promising sample preparation by giving results equivalent to fresh blood for both laboratory (rat blood) and field-sample collection (sheep blood) studies when *in vitro* or *ex vivo* stimulations were measured up to 3 hours in culture. One of the major limitations to having such a short possible culture time with frozen blood is that you can't do prolonged exposure with environmental contaminants.

The overall objective of this study was to determine if the findings from the toxicological studies on rat blood in a laboratory setting (Chapter 3) could be replicated using sheep blood collected in the field. In Chapter 3, the merits and limitations of a number of rat blood sample preparations in short and long-term culturing and their *ex vivo* responsiveness to the genotoxic compounds, ethyl methanesulphonate (EMS) and

cadmium chloride ($CdCl_2$), were assessed. The different rat blood sample processing methods were shown to alter the sensitivity of lymphocytes to $CdCl_2$ but not to EMS. It was demonstrated that incubations for 3 hours can be undertaken without considerable alterations in the viability of the lymphocytes after all sample processing methods described but when the culture times were extended beyond 6 hours for frozen blood a large decrease in viability was observed. On the basis of the results using rat blood, direct cryopreservation of blood can be used as an alternative to PBMCs or fresh whole blood, especially for short-term cultures and exposures up to 3 hours. It is of interest to determine if these findings could be replicated in the case of field-collected sheep blood samples that have associated time constraints.

As discussed in Chapter 3, the Comet assay has been used to detect the DNA damage induced in lymphocytes in various blood sample preparations. It permits direct visualization of DNA damage in cells by assessing single and double strand breaks in the DNA (McKelvey-Martin et al., 1993). DNA damage can be caused by genotoxic compounds such as EMS and $CdCl_2$ which are frequently used as positive controls for genotoxicity studies. In addition, it was decided to investigate the contribution of apoptosis or necrosis to the loss of viability induced by $CdCl_2$. Cadmium induced DNA damage can be caused by an increase in free radical species such as oxygen-centred radicals (Wyllie, 1980, Kehrer, 1993, Corcoran et al., 1994, Halliwell, 1996). In contrast, EMS is a direct monofunctional alkylating agent that causes DNA adducts leading to single strand breaks causing point mutations (Davies et al., 1993, Anderson et al., 1996).

In this study, various sheep blood preparations, namely, fresh whole blood, fresh processed blood, frozen blood and PBMCs were prepared in the laboratory after the blood was collected under field conditions. The objective was to compare and contrast the various sample preparation methods in relation to viability and responses to common positive controls for genotoxic assays i.e. EMS & $CdCl_2$. The membrane integrity of lymphocytes was examined in culture using the propidium iodide (PI) exclusion method (Moore et al., 1998) and DNA damage was measured using the Comet assay (Woods et al., 1997). The time effects of $CdCl_2$ exposure and the level/percentage of early apoptosis and late apoptosis/necrosis was assessed in lymphocytes in sheep blood. The final objective was to compare and contrast the frozen

blood collected in different anticoagulant-coated tubes in terms of viability and responses to EMS & $CdCl_2$.

Materials & Methods

Animals

Three two-year-old Cheviot-cross ewes, tagged 401, 440 and 642, were derived from a breeding stock at the Central Veterinary Laboratory (CVL) Dublin, Ireland and relocated to a farm 10 km from the laboratory at which the cell isolations and assays were performed. They were housed together in one pen and provided with hay *ad libitum* in racks and water in a large plastic container. All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Chemicals & Reagents

All chemicals used were of the highest grade available: RPMI 1640 medium with 2 mM L-glutamine, heat-inactivated foetal bovine serum (FBS), HEPES, phosphate-buffered saline (PBS), ethyl methanesulphonate (EMS), dimethyl sulphoxide (DMSO), propidium iodide (PI), low melting agarose (LMA), normal melting agarose (NMA), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), Tris-HCl; DNAse, sodium hydroxide (NaOH), 1% N-sodium lauryl sarcosinate, Triton X-100, Ficoll-density gradient medium (Histopaque 1083) and ethidium bromide were all purchased from Sigma-Aldrich (Ireland). The antibiotic gentamycin was procured from GIBCO (UK). Sterile 96-well tissue culture plates were purchased from Sarstedt (Ireland). Ammonium chloride RBC lysis buffer (Pharmlyse), annexinV-FITC apoptosis detection kits and sodium heparin and potassium EDTA blood collection tubes (9ml) were procured from BD Biosciences (Ireland). Sterile 24-well tissue culture plates were purchased from Sarstedt (Ireland). Cadmium chloride ($CdCl_2.5H_2O$) was obtained from Alkem (Ireland).

Blood Collection

Jugular blood samples were extracted from ewes after they were restrained in individual small pens. Blood (50-60 ml) was collected from each ewe in 9 ml sodium heparin (NaH) and potassium EDTA (KEDTA) tubes. The blood was then divided with some being used for PBMC isolation, some for the preparation of fresh whole blood by dilution, some processed by centrifugation and the remaining blood was cryopreserved as described previously in Chapters 2, 3 and 4 and shown in Figure 1.

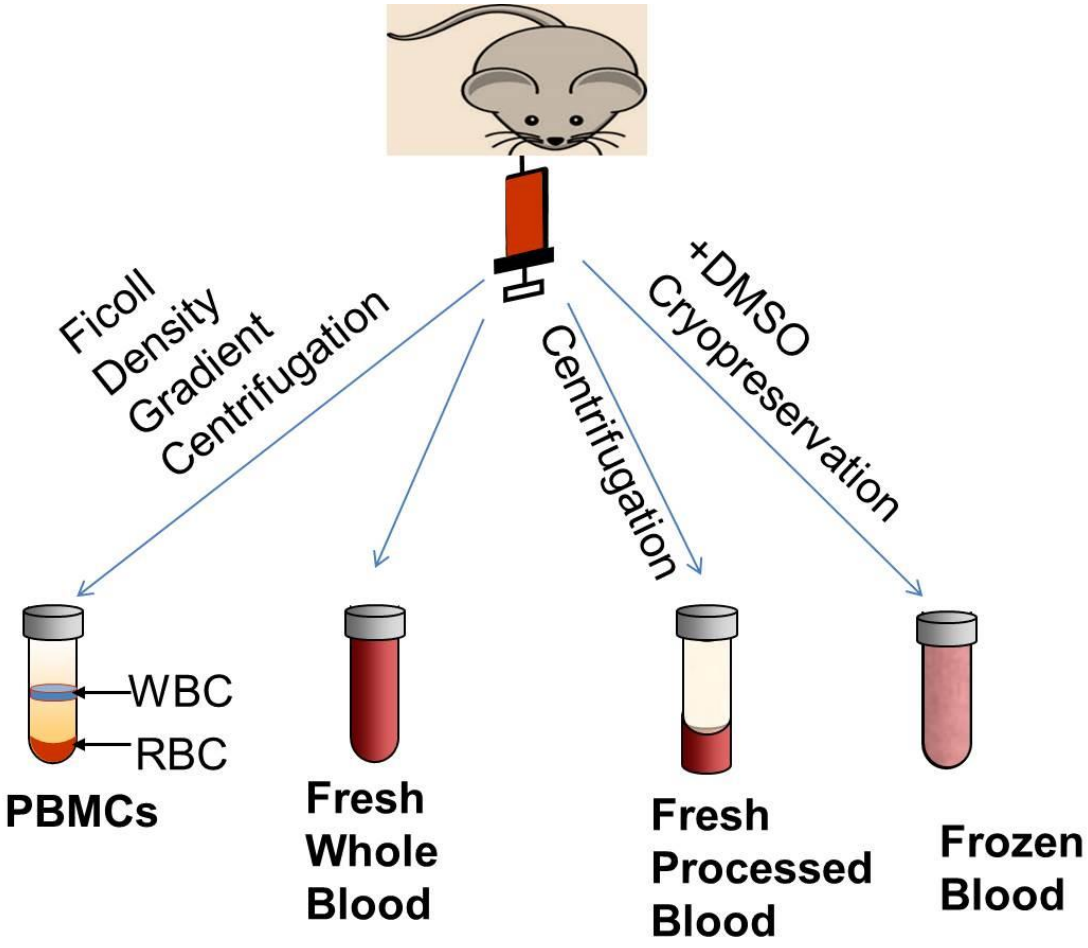


Figure 1. Schematic of the Different Blood Sample Processing Methods Used in this Chapter.

Sample Preparation

Isolation of PBMCs

Fresh sheep PBMCs were isolated from heparinised blood by routine Ficoll-density gradient centrifugation as described by Boyum (1968) and as detailed previously in Chapter 2.

Fresh Whole Blood

Fresh blood (1ml) was diluted with 2 ml of complete culture medium, mixed gently and put in culture as described by Connor et al, (2000) and as detailed previously in Chapter 3.

Fresh Processed Blood

Fresh blood (1 ml) was added to 10 ml of complete culture medium, centrifuged, the supernatant was aspirated and the remaining 1ml pellet and supernatant was diluted as detailed previously in Chapter 3.

Frozen Blood

DMSO (150 µl) was added to 1350 µl of fresh sheep blood in a cryovial, placed in a slow- freeze container, stored in a −80 °C freezer for 24 hours and then stored in a liquid nitrogen dewar as detailed previously in Chapter 3.

Thawing of Frozen Blood

Frozen samples were thawed in a 37°C water bath according to the procedure adapted from the protocol described by Reimann and colleagues (Reimann et al., 2000). Slow dilution with thawing medium was followed by centrifugation and resuspension as detailed previously in Chapter 3.

Cell Culture

All the blood sample preparations were cultured in complete culture medium with a volume of 500 µl/well in a 24-well plate and all treatments were performed in triplicate. Experiment/exposure was conducted at 37°C in a 5% CO_2 humidified incubator for the duration of the culture (3 hours) after which the lymphocytes were assayed. In addition, for time course experiments all sheep blood sample preparations were also cultured in triplicate wells and sampled at various time-points, 0, 3, 5 and 7 hours after initiation of culture.

Cadmium Chloride and Ethylene Methane Sulphonate Stock Solutions and Exposures

$CdCl_2$ was used at the final concentrations of 500, 333 and 100 µM as described previously and as detailed in Chapter 3.

EMS was used at the final concentrations of 5, 2.5 and 1 mM as described previously (Mouchet et al., 2005) and as detailed in Chapter 3.

Finally, 50 µl of RPMI medium was added to wells containing the cell suspension as a control. All additions of genotoxic compounds or the RPMI medium control were performed in triplicate wells. The plates were incubated for 3-7 hours at 37°C and then assayed as described below.

Propidium Iodide (PI) Exclusion Assay

The viability of lymphocytes in blood sample preparations was determined by propidium iodide (PI) exclusion as described by Moore et al., (1998) and as detailed in Chapter 2. As stated previously, the only cell types that were analysed in the blood sample preparations were lymphocytes as the monocytes and dendritic cells were still attached to the plastic tissue culture plates and the granulocytes were non-viable and

were excluded from the analysis by gating which was dependent on forward scatter and side scatter.

Annexin V-FITC Apoptosis Assay

Lymphocytes from frozen blood were prepared and stained with annexin V-FITC and propidium iodide (PI) as described by Moore et al., (1998) and as detailed in Chapter 2.

Comet Assay

The DNA damage to lymphocytes in blood sample preparations was determined by the Comet assay as adapted from a previous study (Woods et al., 1997) and as detailed in Chapter 3. As stated previously, the only cell types that were analysed in the blood sample preparations were lymphocytes.

Statistical Analysis

Results are expressed as the means ± Standard Deviation (SD) for three independent experiments. All statistical analysis used Sigma Stat software (version 2.03). Where data followed a normal distribution as assessed with a Kolmogorov-Smirmnov test, differences between groups were analysed using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons. Statistical significance was set at $p<0.05$ and significant levels were defined as *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

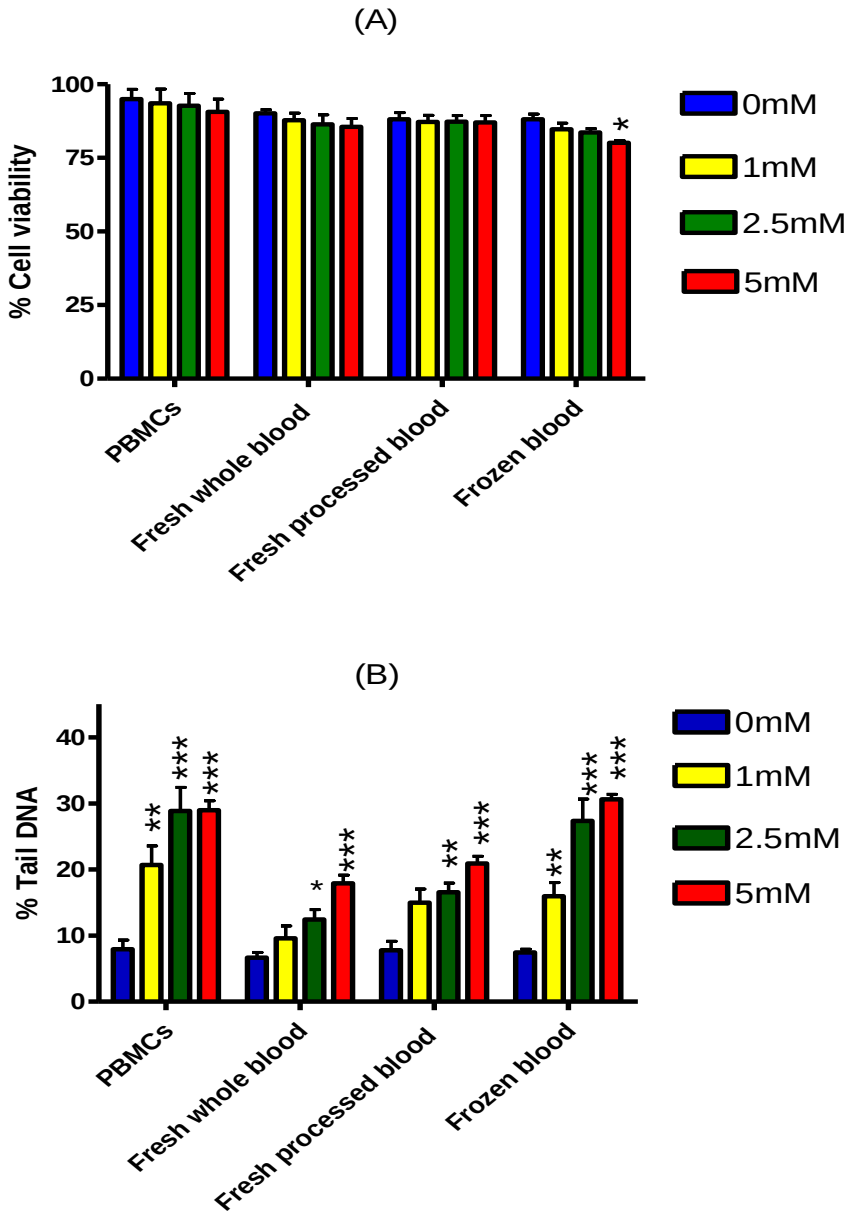
Results

Response of Lymphocytes to Ethyl Methylsulphonate in Cytotoxicity and Genotoxicity Assays over 3 Hours in Culture.

In the case of untreated controls, there was a significant reduction (6%) in cell viability to in both fresh whole blood and frozen blood when compared to PBMCs ($p<0.05$) (data not shown).

At each of the doses used (1 mM, 2.5 mM and 5 mM), EMS reduced the viability of lymphocytes in all sample preparations when the incubation period was greater than 3 hours (data not shown). For this reason, the effects of EMS after 3 hours were studied in detail and the effects are shown in Figure 2A. EMS had no cytotoxic effect in any sample preparations when the 1 mM and 2.5 mM doses were used. At a dose of 5 mM, there was a significant (6 %) reduction ($p<0.05$) to 80 % in the case of frozen blood but no effect on the other samples (Figure 2A).

The genotoxic effects of EMS are shown in Figure 2B. An increase of DNA damage in all sample preparations of lymphocytes in a dose-dependent manner was observed. At 1 mM concentration there was only a slight increase in DNA damage and it was significant for the PBMCs ($p<0.01$) and frozen blood sample preparations ($p<0.01$). At the concentration of 2.5 mM, the DNA damage increased significantly in all sample preparations compared to untreated controls: PBMCs ($p<0.001$), fresh whole blood ($p<0.05$), fresh processed blood ($p<0.01$) and frozen blood ($p<0.001$). At the highest concentration used, 5 mM, EMS caused a substantial increase in DNA damage in all sample preparations ($p<0.001$).

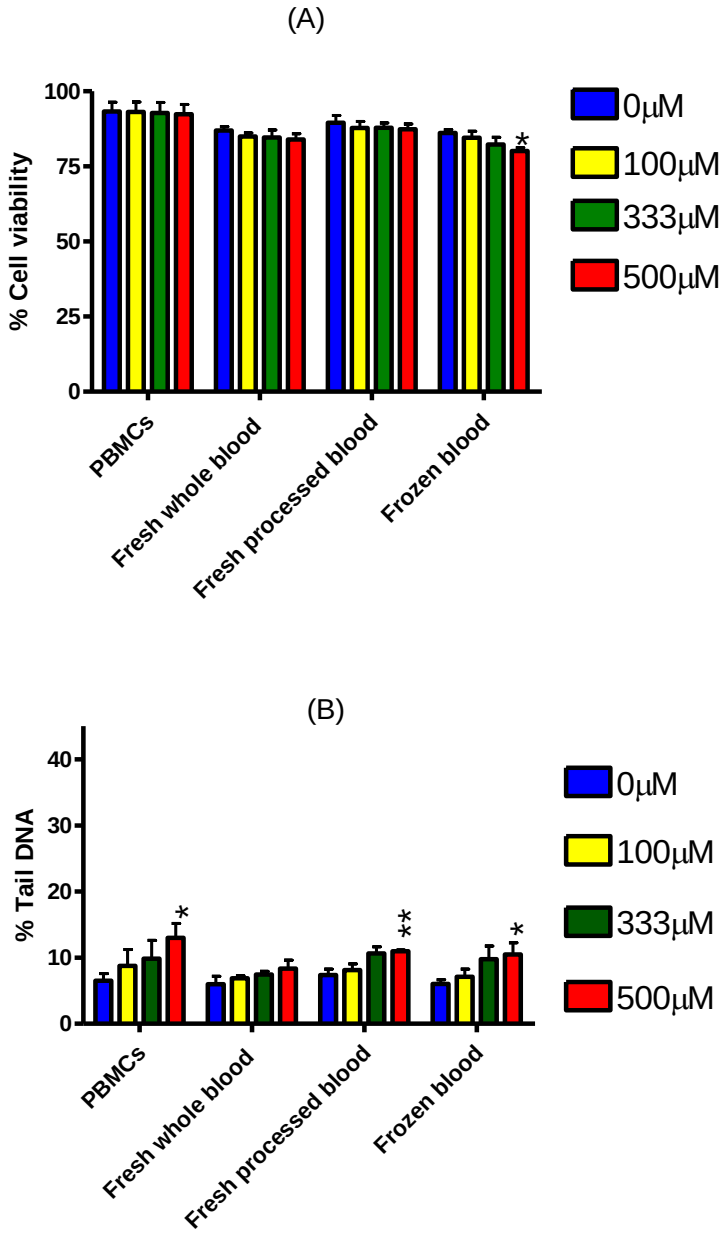


Figures 2A and 2B. The Effects of Short (3 Hour) EMS Exposure on Cell Viability and DNA Damage in Lymphocytes. Data are represented as the viability (Figure 2A) and DNA damage (as measured by % Tail DNA) (Figure 2B) of sheep blood lymphocytes after 3 hours exposure to EMS (1, 2.5 and 5 mM). Sheep lymphocytes were cultured from PBMCs, fresh whole blood, fresh processed blood and frozen blood preparations. Values represent the mean±SD from three replicate wells in three independent experiments. Control (0 mM) vs. all concentrations for each sample preparation * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Response of Lymphocytes to Cadmium Chloride in Cytotoxicity and in Genotoxicity Assays over 3 Hours in Culture.

$CdCl_2$ caused a slight reduction in cell viability compared to untreated controls in a dose-dependent manner in all sample preparations after 3 hours in culture (Figure 3A). However, this reduction in cell viability of lymphocytes was only significant for frozen blood ($p<0.05$) for the 500 μM $CdCl_2$ exposure.

With regard to the genotoxicity assessment, $CdCl_2$ increased the DNA damage in a dose-dependent manner at 3 hours (Figure 3B). There was a small effect observed at the 333 μM dose in all the sample preparations which did not contain red blood cells (PBMCs and frozen blood). At the highest dose (500 μM), PBMCs ($p<0.05$), fresh processed blood ($p<0.05$) and frozen blood ($p<0.05$) also showed a significant increase in DNA damage. None of the $CdCl_2$ doses used had a significant effect on DNA damage in the lymphocytes of fresh unprocessed blood.

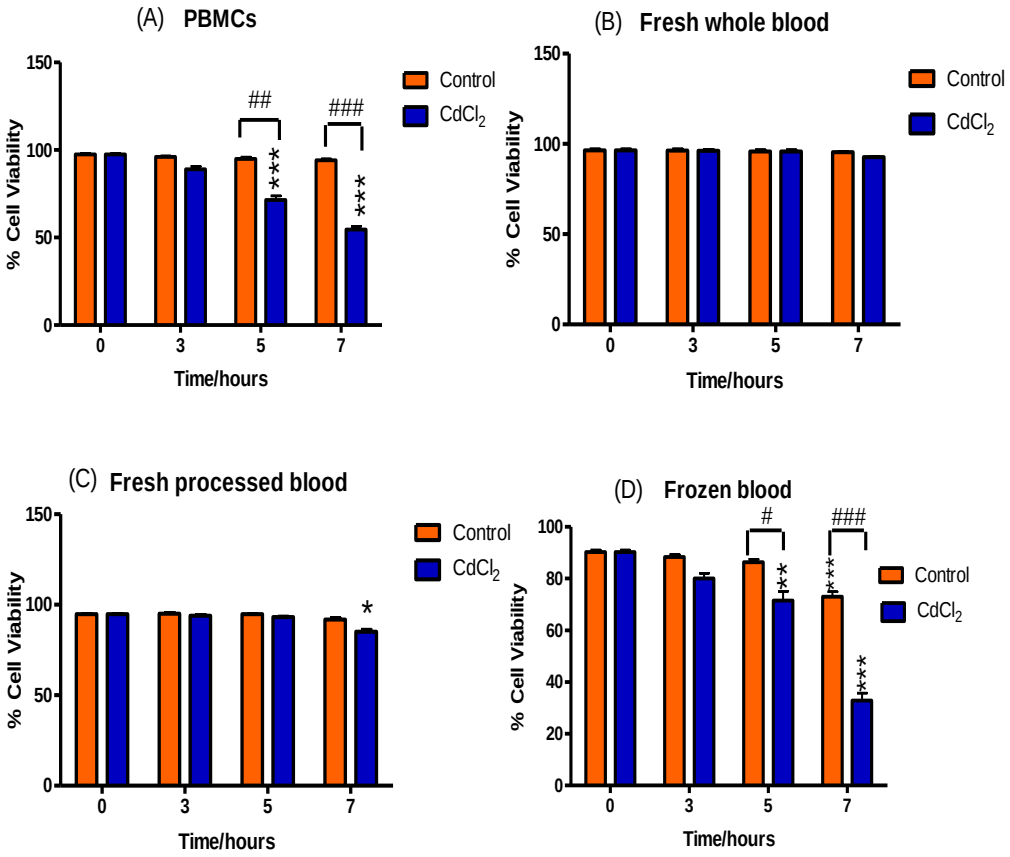


Figures 3A and 3B. The Effects of Short (3 Hours) Cadmium Chloride Exposure on Cell Viability and DNA Damage in Lymphocytes. Data are represented as the viability (Figure 3A) and DNA damage (as measured by % Tail DNA) (Figure 3B) of sheep blood lymphocytes after 3 hours exposure to $CdCl_2$ (0, 100, 333 and 500 µM). Sheep lymphocytes were cultured from PBMCs, fresh whole blood, fresh processed blood and frozen blood preparations. Values represent the mean±SD from three replicate wells in three independent experiments. Control (0 µM) vs. all concentrations for each sample preparation * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$.

Response of Lymphocytes to Cadmium Chloride (333 μM) in a Cytotoxicity Assay over 7 Hours in Culture.

The 7 hour culture period was chosen because at 3 hours there was only a slight increase in DNA damage but when exposure was increased to 24 hours both genotoxcity and cytotoxicity effects were observed (results described in Chapter 3 for rat blood). The 333 μM dose of $CdCl_2$ was chosen because there was an increase in DNA damage in PBMCs after 3 hours without a significant drop in viability (Figures 3A and 3B).

The levels of viability in lymphocytes from different sample preparation after exposure to 333 μM $CdCl_2$ over 7 hours in culture are shown in Figures 4A, 4B, 4C, and 4D. However, the reduction in cell viability after 333 μM $CdCl_2$ exposure was only significant for PBMCs at 5 ($p<0.01$) and 7 ($p<0.001$) hours and for frozen blood at 5 ($p<0.05$) and 7 ($p<0.001$) hours compared to untreated controls. The loss in viability for PBMCs after 333 μM $CdCl_2$ exposure increased with time and became highly significant at 5 hours ($p<0.001$) and at 7 hours ($p<0.001$)(Figure 4A). Similarly, the loss in viability after 333 μM $CdCl_2$ exposure for frozen blood increased with time and became significant at 5 hours ($p<0.01$) and highly significant at 7 hours ($p<0.001$)(Figure 4D).



Figures 4A, 4B, 4C and 4D. The Effects of 7 Hours Cadmium Chloride Exposure on Cell Viability in Lymphocytes. Data represented as the viability of sheep blood lymphocytes over exposure periods of 3, 5, 7 hours to $CdCl_2$ (0 and 333 µM). Sheep lymphocytes were cultured from PBMCs (Figure 4A), fresh whole blood (Figure 4B), fresh processed blood (Figure 4C) and frozen blood (Figure 4D) preparations. Values represent the mean±SD from three replicate wells in three independent experiments for three different sheep.* represent the statistical analysis of Control (0 hours) vs. all time points for each sample preparation * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$. $^{\#}$ represent the statistical analysis of Control (0 µM) vs. 333 µM $CdCl_2$ at each time point for each preparation$^{\#}$ $p< 0.05$; $^{\#\#}$ $p< 0.01$; $^{\#\#\#}$ $p< 0.001$.

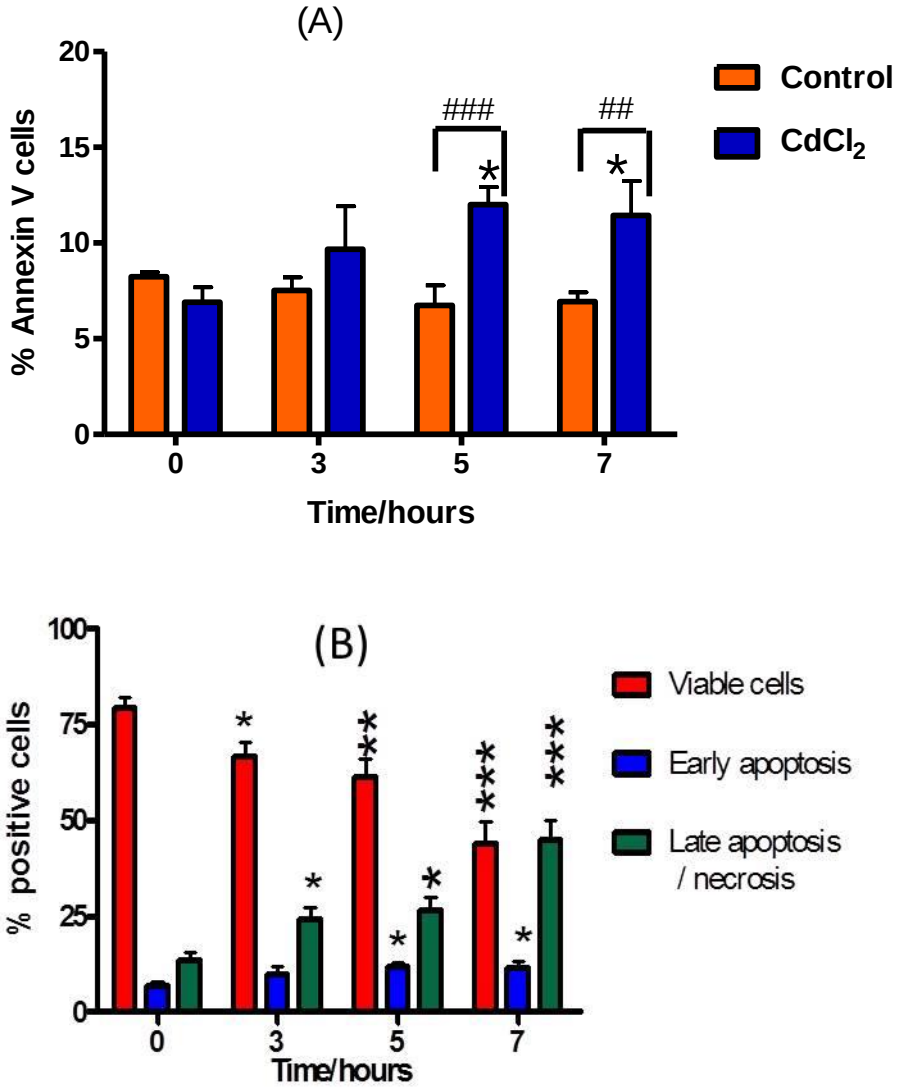
The Levels of Apoptosis Induction in Lymphocytes in Frozen Blood following Exposure to Cadmium Chloride (333 μM) over 7 Hours in Culture

The levels of apoptosis as measured by annexin V-FITC (AnV-FITC$^+$) and viability (PI$^-$) in lymphocytes from frozen blood after exposure with and without 333 μM $CdCl_2$ over 7 hours in culture are shown in Figures 5A.

Frozen blood was the sample preparation chosen for these assays because of the significant and substantial drop in viability observed after 7 hours exposure to 333 μM $CdCl_2$ (Figure 4D) was considered worthy of further investigation. In addition, it was realised that it was not practical to assess all the sample preparations immediately after collection. There was a detectable increase in early apoptosis (AnV-FITC $^+$/ PI$^-$) at all-time points but it did not rise significantly compared to the time zero control over the 7 hours in culture. In contrast, there was a detectable increase in early apoptosis (AnV-FITC $^+$/ PI$^-$) at all time points and this reached significance at the 5 ($p<0.05$) and 7 ($p<0.05$) hour time points (Figure 5A).

The levels of apoptosis (AnV-FITC $^+$) and viability (PI$^-$) in lymphocytes from frozen blood over 7 hours after exposure with 333 μM $CdCl_2$ in culture are shown in Figure 5B.

The level of late apoptosis/necrosis (AnV-FITC $^+$/ PI$^+$) increased with time and became significant at 3 hours ($p<0.05$), at 5 hours ($p<0.05$) and highly significant at 7 hours ($p<0.001$). There was a drop in the number of viable cells (AnV-FITC $^-$/PI$^-$) which increased with time and became significant at 3 hours ($p<0.05$), at 5 hours ($p<0.01$) and highly significant at 7 hours at which time the viability was 45% ($p<0.001$) (Figure 5B).



Figures 5A and 5B. The Level of the Apoptotic Lymphocytes in Frozen Blood Following Exposure to Cadmium Chloride over 7 Hours in Culture.

(Figures 5A) Data are represented as the percentage of early apoptotic sheep lymphocytes cultured from frozen whole blood preparations after exposure with 333 µM $CdCl_2$ or medium alone over culture periods of 0, 3, 5 and 7 hours as assessed by annexin V-FITC/ PI staining. Values represent the mean±SD from three replicate wells in three independent experiments for three different sheep. *represent the statistical analysis of Control (0 hours) vs. all time points * p< 0.05; ** p< 0.01; *** p< 0.001. $^{\#}$ represent the statistical analysis of Control (0 µM) vs. 333 µM $CdCl_2$ at each time point for each preparation$^{\#}$ p< 0.05; $^{\#\#}$ p< 0.01; $^{\#\#\#}$ p< 0.001.

(Figures 5B) Data are represented as the percentage of viable, early and late apoptotic sheep lymphocytes cultured from frozen blood preparation after exposure with 333 µM $CdCl_2$ over culture periods of 0, 3, 5, and 7 hours (same data as used in Figure 5A). * represent the statistical analysis of Control (0 hours) vs. all time points * p< 0.05; ** p< 0.01; *** p< 0.001.

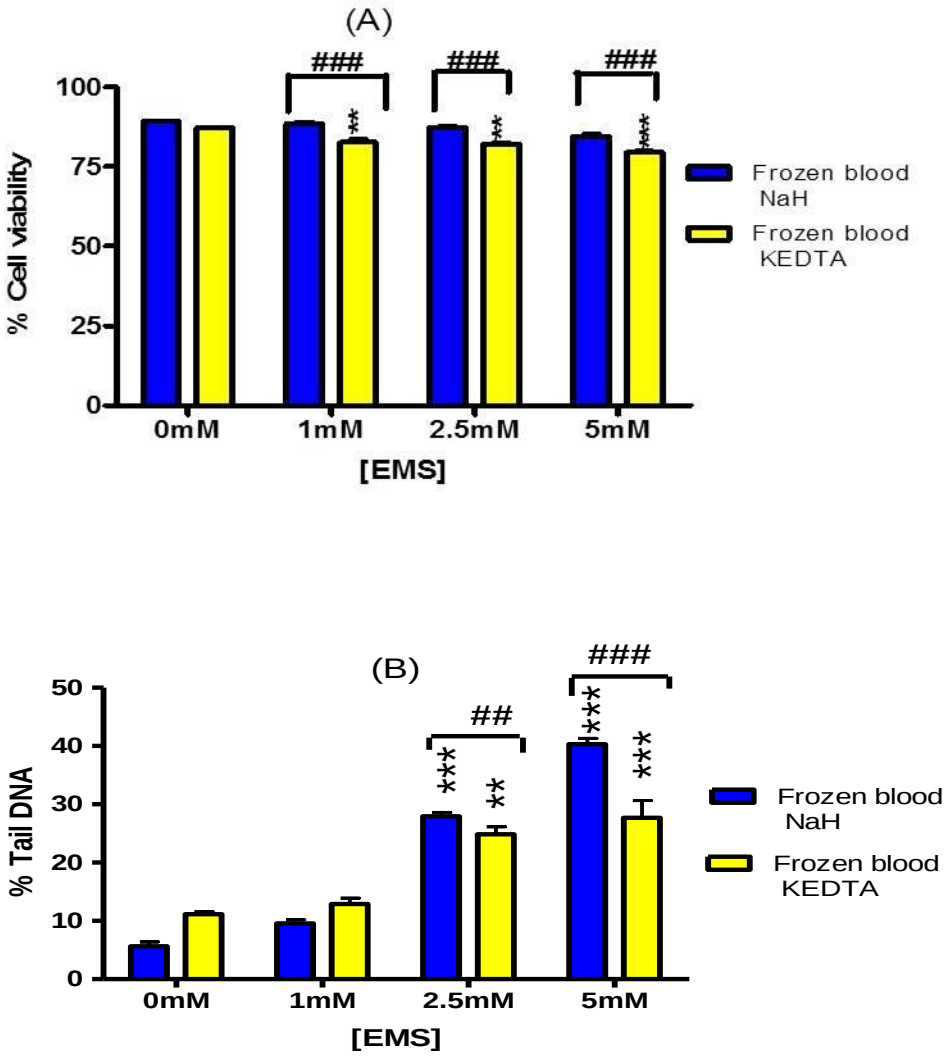
Effect that Storage in Different Anticoagulants had on the Response of Lymphocytes in Frozen Blood to Ethyl Methylsulphonate in Cytotoxicity and Genotoxicity Assays over 3 Hours in Culture.

There was no significant difference in viability for untreated controls for the frozen blood stored either with the anticoagulants sodium heparin or potassium EDTA (Figure 6A).

EMS had no cytotoxic effect in frozen blood stored in sodium heparin tubes when 1, 2.5 and 5 mM doses were used (Figure 6A). However, when potassium EDTA tubes were used there was a significant 5% reduction at both 1 mM and 2.5 mM doses ($p<0.01$), and an 8% reduction at the 5 mM dose of EMS ($p<0.001$), at which the final viability was 79%.

When the effects of EMS on the viability of lymphocytes in frozen blood collected in the two anticoagulants were compared directly, there was a significant reduction in the case of potassium EDTA i.e. 6 % at 1 mM EMS ($p<0.001$), 5 % reduction at 2.5 mM ($p<0.001$) and 5 % reduction at the 5 mM dose of EMS ($p<0.001$) (Figure 6A).

The genotoxic effects of EMS are shown in Figure 6B for frozen blood stored in different anticoagulants were increase of DNA damage in all sample preparations of lymphocytes in a dose-dependent manner was observed. At 1 mM concentration there was only a slight increase in DNA damage and it was not significant for either anticoagulant. At the concentration of 2.5 mM, the DNA damage increased significantly in both sodium heparin ($p<0.001$) and potassium EDTA ($p<0.01$) tubes. At the highest concentration used, 5 mM EMS caused a substantial increase in DNA damage in both frozen blood sample preparations ($p<0.001$). There was no significant difference in the level of DNA damage for untreated controls in frozen blood stored in different anticoagulant tubes. When the effect of EMS exposure in samples collected in potassium EDTA tubes were compared to those in sodium heparin tubes, there was a significantly greater DNA damage at 2.5 mM ($p<0.01$) and at 5 mM ($p<0.001$) in the later.



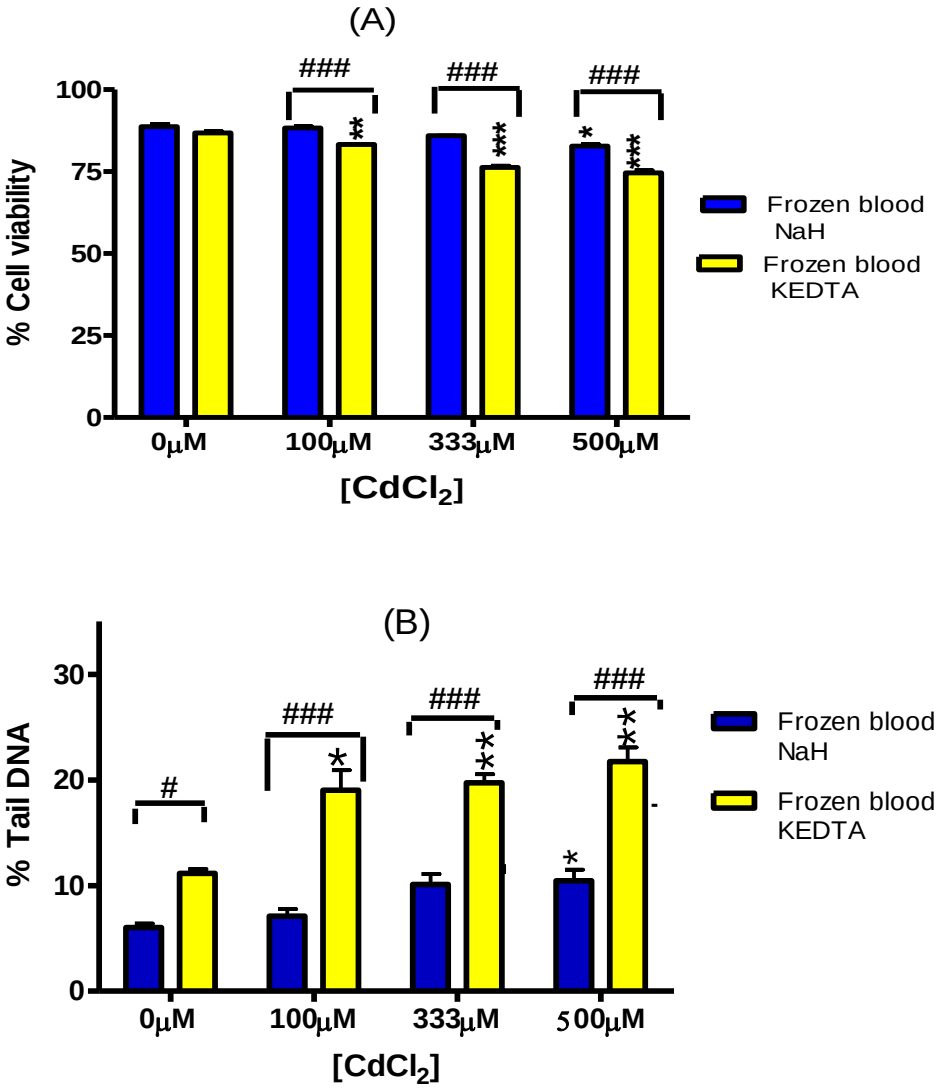
Figures 6A and 6B. The Effects of Short (3 Hour) EMS Exposure on Cell Viability and DNA Stability in Lymphocytes Stored in Different Anticoagulants. Data are represented as the viability (Figure 6A) and % Tail DNA (Figure 6B) of sheep blood lymphocytes stored in either sodium heparin (NaH) or potassium EDTA (KEDTA) tubes after 3 hours exposure to EMS (1, 2.5 and 5 mM). Sheep lymphocytes were cultured from frozen blood preparation. Values represent the mean±SD from three replicate wells in three independent experiments. *represent the statistical analysis of Control (0 hours) vs. all concentrations for each anticoagulant storage condition used * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$. $^{\#}$ represent the statistical analysis of NaH vs. KEDTA for each all concentrations $^{\#}$ $p< 0.05$; $^{\#\#}$ $p< 0.01$; $^{\#\#\#}$ $p< 0.001$.

Effect that Storage in Different Anticoagulants had on the Response of Lymphocytes in Frozen Blood to Cadmium Chloride in Cytotoxicity and Genotoxicity Assays over 3 Hours in Culture.

There was no significant difference in viability for untreated controls for the frozen blood stored either as anticoagulants sodium heparin or potassium EDTA (Figure 7A).

$CdCl_2$ had no cytotoxic effect in frozen blood stored in sodium heparin tubes following exposure to the 100 and 300µM doses. However, at a dose of 500µM, the reduction in cell viability of lymphocytes (5%) was significant ($p<0.05$) (Figure 7A). When potassium EDTA tubes were used there was a significant reduction in viability of 4% at 100µM ($p<0.01$), 10% at 333µM ($p<0.001$) and 12% at 500µM $CdCl_2$ ($p<0.001$) at which the final viability was 74%. When the effects of $CdCl_2$ on the viability of lymphocytes in frozen blood collected in the two anticoagulants were compared directly, there was a significant reduction in the case of potassium EDTA i.e. 4% at 100µM ($p<0.001$), 8% at 333µM ($p<0.001$) and 7% reduction at 500 µM ($p<0.001$) (Figure 7A).

The genotoxic effects of $CdCl_2$ are shown in Figure 7B for frozen blood stored in different anticoagulants. An increase of DNA damage in all sample preparations of lymphocytes in a dose-dependent manner was observed. At the 100µM dose, there was an increase in DNA damage but it was only significant for frozen blood collected in potassium EDTA tubes ($p<0.05$). At the concentration of 333µM, the DNA damage increased significantly in potassium EDTA ($p<0.01$) but not in in sodium heparin tubes. The highest concentration used, 500 µM, caused a substantial increase in DNA damage in both frozen blood sample preparations collected in sodium heparin ($p<0.05$) and potassium EDTA ($p<0.01$) tubes. There was no significant difference in the level of DNA damage for untreated controls when the frozen blood was stored in different anticoagulants tubes. When the effect of $CdCl_2$ exposure in samples collected in potassium EDTA tubes were compared to those in sodium heparin tubes, the DNA damage was significantly higher in potassium EDTA tubes at all concentrations ($p<0.001$).



Figures 7A and 7B. The Effects of Short (3 Hour) Cadmium Chloride Exposure on Cell Viability and DNA Stability in Lymphocytes Stored in Different Anticoagulants Data are represented as the viability (Figure 7A) and % Tail DNA (Figure 7B) of sheep blood lymphocytes stored in either sodium heparin (NaH) or potassium EDTA (KEDTA) tubes after 3 hours exposure to $CdCl_2$ (500, 333 and 100 μM). Sheep lymphocytes were cultured from a frozen blood preparations. Values represent the mean±SD from three replicate wells in three independent experiments. *represent the statistical analysis of Control (0 hours) vs. all concentrations for each anticoagulant storage condition used * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$. $^{\#}$ represent the statistical analysis of NaH vs. KEDTA for each all concentrations $^{\#}$ $p< 0.05$; $^{\#\#}$ $p< 0.01$; $^{\#\#\#}$ $p< 0.001$.

Discussion

When collecting samples from sheep to be used in field-based biomonitoring, jugular venipuncture proved to be very suitable to obtain blood samples which contain lymphocytes, the preferred cell type for measurement of genotoxic, immunological and metabolic effects (Faust et al., 2004, Okazaki et al., 2008, Linzalone, 2009). In Ireland, the exposure to environmental chemical contaminants from factories or abandoned mines has been touted as a possible cause for the decrease in the health of particular farm animals in Askeaton Co. Limerick, Lucan Co. Dublin and in the Silvermines area in Co. Tipperary (Department of Agriculture, Food and Rural Development, 2000, EPA, 2002, Li et al., 2011, Qin et al., 2011). The heavy metal, cadmium, which is a class 1 carcinogen (IARC, 1993) and one of the most toxic environmental and industrial pollutants is used as a relevant positive control in this study. Exposure could be through ingestion of food and drinking water, inhalation of particulates from ambient air, or ingestion of contaminated soil or dust. Cadmium toxicity normally acts through elevating lipid peroxidation, complexing with thiol groups of enzymes and increasing free-radical levels in different organs eventually leading to DNA damage in cells (Valverde et al., 2001). In the case of acute exposure of humans to cadmium, the concentration levels are measured in blood and these procedures could be transferred to domestic animals. Subchronic exposure to cadmium in humans is monitored by measuring cadmium levels in urine but this approach is more challenging in domestic animals which excrete indiscriminately. While the methods used in this study may be useful in this regard, the overall aim is to develop biomarkers that are effect rather than compound specific.

The results described in Chapter 3 and Chapter 4 demonstrated the potential of cryopreservation for the successful storage of rat blood collected under laboratory-based conditions as well as sheep blood collected under field-based conditions. The viability of lymphocytes in both frozen rat and sheep bloods was only marginally affected after 3 hours in culture. However, there was a significant drop in lymphocyte viability in frozen blood after 7 hours in culture. Therefore, the objective of this study was to assess the effects that different blood separation and cell isolation techniques have on the viability

of sheep lymphocytes and their responses to genotoxic compounds. Also, the suitability of cryopreservation for the storage of sheep blood collected under field-based conditions for this *ex vivo* toxicity testing was examined. A key determinant was the duration for which sheep lymphocytes could be cultured after the freezing and thawing steps without a reduction in their viability and whether the use of a different anticoagulant, potassium EDTA, could improve the viability of the lymphocytes and their sensitivity to genotoxic compounds. In addition, the contribution of both apoptosis and necrosis to cadmium chloride induced cell death in lymphocytes present in frozen sheep blood was investigated.

Fresh whole and processed bloods and PBMCs processed from sheep blood collected in the field retained a lymphocyte viability of greater than 85% after culturing for up to 7 hours. This is in agreement with our findings from Chapter 3 when rat blood in a laboratory setting was used. The findings for fresh whole blood are in agreement with those of Fiebig and colleagues who also reported a viability of greater than 85% (Fiebig et al., 1997). It is speculated that whole blood provides an environment resembling *in vivo* conditions, thus contributing to the high viability observed. Fresh whole and fresh processed sheep blood had the lowest basal levels of DNA damage of all the sample preparations and this is in agreement with what was reported for rat blood in Chapter 3.

Frozen blood samples were processed as quickly as possible in order to minimize cell damage following the thawing step. The viability of lymphocytes in frozen sheep blood immediately after thawing was 90 %. This is in agreement with what was observed in Chapters 3 and 4 and with other studies that showed that the viability in frozen blood was between 85 and 90 % straight after thawing (Hayes et al., 2002, Schindler et al., 2004). When frozen blood was cultured for 3 hours, the viability of 90 % was consistent with the findings of Anderson et al, (1996), Cheng et al, (2001) and Stevens et al, (2007). Lymphocyte viability in frozen sheep was still above 80% at 5 hours but after 7 hours in culture it had dropped to 73 %. This drop in viability had already been observed in frozen sheep blood in Chapter 4 and was explained in part by the induction of reactive oxygen species (ROS) at 7 hours following the freeze-thaw lysis of red blood cells and also by cryopreservation-induced apoptosis.

There was no significant difference in the DNA stability of lymphocytes from frozen sheep blood and those from the other sample preparations after 3 hours in culture. This

is consistent with the findings from Chapter 3 and other studies where lymphocytes obtained from blood samples were stored at -80 ºC (Visvardis et al., 1997, Tice et al., 2000, Chuang and Hu, 2004, Villavicencio, 2006). The results described in this study demonstrated the potential of cryopreservation for the successful storage of sheep blood collected under field-based conditions for use in *ex vivo* exposures up to 5 hours in culture.

Once the lymphocytes from the different sheep sample preparations were characterised, the effects of *ex vivo* exposure to DNA-damaging agents, the EMS and $CdCl_2$ were investigated. EMS caused DNA damage in a dose–dependent manner in all sheep blood sample preparations after 3 hours exposure. This is consistent with the findings from Chapter 3 where the all doses increased the level of DNA damage in lymphocytes in the different rat blood sample preparations after 3 hours. These results agree with the findings of De-Boeck et al, (2000) and Wyatt et al., (2007) who demonstrated that DNA damage was induced in lymphocytes in rat blood after exposure to direct-acting alkylating water-soluble mutagens for 3 hours using concentrations from 1-40 mM. The levels of EMS-induced damage were similar for PBMCs, fresh whole and frozen sheep blood suggesting that, under the conditions applied, the proteins and other nucleophiles present in sheep whole blood had no scavenging effect on the direct-acting mutagen.

At a concentration of 500 μM, $CdCl_2$ caused a significant reduction in viability when frozen sheep blood was exposed for 3 hours. When exposure was extended to 7 hours, the 333 μM dose significantly reduced viability of all the sheep samples preparations except fresh whole blood. Both PBMCs and frozen blood samples had a significant reduction in viability when exposed to 333 μM and 500 μM, for 5 and 7 hours while both whole and processed fresh blood samples had only a slight and non-significant reduction in viability. This may be due to the presence of RBCs which are known for their binding potential to cadmium and also the higher quantity of plasma and blood proteins in the fresh whole blood. Similar results were observed in Chapter 3, whereby $CdCl_2$ at doses of 333 and 500 μM caused a minor, although significant, reduction in viability when PBMCs and fresh processed blood were exposed for 3 hours. When exposure was extended to 24 hours, all concentrations used led to a much greater reduction in viability. Blasiak et al, (2000) showed that the viability of human lymphocytes was greatly reduced when exposed to concentrations of 5 to 150 μM of

$CdCl_2$ for 1 hour.

A limited genototoxic effect was observed in all the sheep blood sample preparations when they were exposed to $CdCl_2$ for 3 hours. There was a significant increase in DNA damage in PBMCs, fresh processed blood and frozen blood at the concentration of 500 μM but there was no effect on fresh whole blood. These findings are similar to what was seen in Chapter 3 where there was a small increase in DNA damage in all rat blood sample preparations after 3 hours but this increase in DNA damage became substantial at 24 hour in all sample preparation except fresh whole blood. Previous reports which are not fully in agreement with these findings have shown that 0.16-20 μM $CdCl_2$ increases DNA damage in peripheral blood lymphocytes (Cai and Zhuang, 1999) and 150 μM cadmium significantly increased DNA damage when lymphocytes were exposed (Blasiak and Kowalik, 2001). The effects of $CdCl_2$ are dependent on sample constitution, i.e. the presence or absence of red blood cells and serum protein concentration and on exposure time. The lack of genotoxic insult from $CdCl_2$, in the case of whole blood, may be due to the fact that cadmium becomes bound to plasma proteins such as albumin and transferin (Saljooghi and Fatemi, 2010a). This scavenging effect could have a major influence on the bioavailability of $CdCl_2$ (Saljooghi and Fatemi, 2010b).

This study was designed to bridge the gap between toxicological testing based in the laboratory and field-based sampling followed by laboratory-based analysis. In Chapter 3, blood sample handling and blood sample preparations were optimised using rat blood under controlled conditions. In Chapter 4, blood was collected from sheep in the field and again sample handling and blood sample preparations were optimised. The length of time that blood could be left before processing, the number of samples that could be processed, and the length of the time for which the samples could be cultured without deterioration were also recorded. In this study, the experiments of Chapter 3 and 4 were successfully repeated for sheep blood collected in the field and toxicity tests using genotoxic compounds were undertaken. With the combined knowledge of both Chapters 3 and 4 and this Chapter, our methods are now robust and reproducible enough to increase the number and type of endpoints looked at in sheep blood.

The contributions of two different causes by which $CdCl_2$ induces cell death in lymphocytes in frozen sheep blood was assessed by determining the integrity of cell membranes and the extent of early apoptosis and late apoptosis/necrosis present in lymphocytes. While a detectable level (6-8% on average) of early apoptosis was observed, there is no evidence that it preceded the substantial drop in cell viability in frozen sheep blood. This suggests that apoptosis is not the only cause of the cell death and that maybe necrosis could also be involved. This is in agreement with the findings from Chapter 4 which found a similar low level of early apoptosis in frozen rat blood. Exposure to $CdCl_2$ for up to 7 hours caused a significant increase in early apoptotic lymphocytes at 5 and 7 hours and there was also a significant increase in late apoptosis at 3 and 5 hours and this increase became substantial at 7 hours. This is in agreement with other studies as cadmium is well recognised for its ability to induce apoptosis in cells such as hepatic (Habeebu et al., 1998, Shimoda et al., 2001, Yu et al., 2001, Aydin et al., 2003, Tzirogiannis et al., 2003, Lemarie et al., 2004, Oh et al., 2006, Oh and Lim, 2006) renal (Tanimoto et al., 1993, Yan et al., 1997, Ishido et al., 1998, Ishido et al., 2002, Lee et al., 2006) neural (Zhao et al., 2000, Choi et al., 2002, Watjen et al., 2002, Lopez et al., 2003, Watjen and Beyersmann, 2004, Huang et al., 2006, Silva et al., 2006) reproductive (Xu et al., 1996, Zhou et al., 1999, Achanzar et al., 2002, Goyer et al., 2004, Sen Gupta et al., 2004) lung (Hart et al., 1999, Waalkes and Diwan, 1999, Lag et al., 2002, Lu et al., 2002, Shih et al., 2003, Huang et al., 2006), connective (Biagioli et al., 2001, Kim et al., 2002, Shih et al., 2004) , myeloid primarily monocytic (Bagchi et al., 2000b, Galan et al., 2000a, Galan et al., 2000c, Li et al., 2000, Galan et al., 2001a, Galan et al., 2001b, de la Fuente et al., 2002, Sancho et al., 2003, Jeon et al., 2004, Miguel et al., 2005, Sancho et al., 2006) and lymphoid (el Azzouzi et al., 1994, Tsangaris and Tzortzatou-Stathopoulou, 1998, Bagchi et al., 2000b, Fujimaki et al., 2000, Iryo et al., 2000, Li et al., 2000, Dong et al., 2001, Feng et al., 2001, Shen et al., 2001, de la Fuente et al., 2002, Takagi et al., 2002, Colombo et al., 2004, Pathak and Khandelwal, 2006b, a, Qu et al., 2006) cells. However, there is considerable variation in the sensitivity of each of these cell types to the apoptotic effects of cadmium. This may be attributable to differences in metallothionein levels in these cells, with several studies reporting pro-and anti-apoptotic functions for these high affinity cadmium-binding proteins (Habeebu et al., 2000, McAleer and Tuan, 2001, Shimoda et al., 2001, Lu et al., 2002, Shimoda et al., 2003). The contribution of apoptosis to the observed increase in DNA damage, as observed in the Comet assay, could be another explanation for the

increase of % tail DNA in lymphocytes after 3 hours exposure to $CdCl_2$ and the increase in early apoptotic cells seen at 5 and 7 hours exposure. Cadmium has been demonstrated to induce DNA strand breaks but also apoptosis in various cell lines (Bagchi et al., 2000a, Galan et al., 2000b, Banfalvi et al., 2005, Fotakis et al., 2005).

In an effort to improve the survival of lymphocytes in frozen blood, an alternative anticoagulant, potassium EDTA, was compared with sodium heparin which was used for the all previous studies in Chapters 2, 3, and 4. However, there was a significant reduction in the DNA stability of lymphocytes from frozen blood after 3 hours in culture when potassium EDTA was used as the anticoagulant. These results may be considered surprising as potassium EDTA stored blood has been used previously in the Comet assay and is used regularly for the collection of blood in clinical and veterinary studies. When potassium EDTA was used as the anticoagulant, frozen blood exhibited a reduction in cell viability and an increase in DNA damage in a dose–dependent manner after 3 hours exposure to the genotoxic compounds EMS and $CdCl_2$.

When lymphocytes prepared using potassium EDTA were compared to those prepared using sodium heparin, the DNA damage induced by EMS was similar but the former were much more sensitive to $CdCl_2$ induced cytotoxicity and DNA damage. This might be due to the role of EDTA as a calcium chelator where the cadmium-activation of MAP kinase and Akt survival pathways result in part from an elevation of intracellular calcium concentration (Misra et al., 2002, Kim and Sharma, 2004) and the lack of extracellular calcium might be affected by the use of EDTA.

Main Conclusions

In this Chapter, the merits and limitations of a number of sample preparations of sheep blood collected in the field after short term culturing and their response to genotoxic compounds *in vitro* were assessed. The different sample processing methods were shown to alter the sensitivity of lymphocytes to $CdCl_2$ but not to EMS. This is in complete agreement with the results described in Chapter 3 where rat blood was exposed to genotoxic compounds after being collected in a laboratory setting.

Three hour incubations can be undertaken on sheep blood without considerable alterations in the viability of the lymphocytes for sample processing methods described but when the culture times were extended up to 7 hours for frozen blood this lead to a significant decrease in viability. On the basis of the results using sheep blood, one can conclude that direct cryopreservation of blood can be used as an alternative to PBMCs, especially for short term cultures and exposures but it really does depend on the anticoagulant used as potassium EDTA stored frozen blood samples had a significantly higher basal level of DNA damage than those stored in sodium heparin tubes. These results are of prime interest due to the fact that there are time constraints in field-based biomonitoring studies and therefore, frozen blood looks a very promising technique for short-term *ex-vivo* studies.

What is striking and important is that the direct cryopreservation of unprocessed blood does not affect the responsiveness of lymphocytes to genotoxic compounds for up to 3 hours exposure. When collecting field-based sheep blood samples which require long-term storage, the fresh whole blood and frozen blood processing protocols are very encouraging because these methodologies have additional advantages of simplicity, economy, and speed and involve less cell manipulation than the isolation of PBMCs. This study demonstrated that frozen blood is a promising sample preparation for field-based biomonitoring studies when performed *in vitro* or *ex vivo* stimulations with genotoxic compounds up to 3 hours. There was only minor a loss in cell viability up to 7 hours in culture which is similar to what was observed for laboratory-based rat blood studies in Chapter 3. When in frozen blood lymphocytes were exposed to $CdCl_2$, the contribution of apoptosis to the loss in viability was identified.

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Chapter 6

Effect of Cryopreservation on Leukocyte Subpopulations and Immune Function Parameters in Sheep Blood Preparations

Table of Contents

Introduction

The studies described in Chapter 5 demonstrated that frozen sheep blood is a promising sample preparation for field-based biomonitoring studies when *ex vivo* exposures up to 3 hours in culture are performed. In addition to giving results equivalent to fresh blood in both the Comet and PI exclusion assays, cryopreservation has a number of advantages. Firstly, it is convenient, since samples can be collected over a selected time period and stored for later analysis. Secondly, it helps to overcome problems associated with inter-assay variation or with comparison of samples from different occasions or different groups of animals, since all samples can be analysed together.

The overall objective of the study in this Chapter was to extend the previous findings and to determine the effects that cryopreservation has on whole blood, collected under field-based conditions, when used for immune-monitoring of sheep. Available experimental animal data indicate that the immune system is a sensitive target for chemical-induced toxicity, especially for the chlorinated compounds, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Burleson et al., 1996), polychlorinated biphenyl (PCB) (Tryphonas et al., 1989, Brown et al., 1994, Summerfield et al., 2009) and heavy metals (Yurkow and Makhijani, 1998, Marth et al., 2001) and is, therefore, a useful target for environmental risk assessment studies with domestic animals such as sheep.

Leukocytes are a major component of the mammalian immune systems and consist of granulocytes (polymorphonuclear neutrophils and basophils), lymphocytes and monocytes which are all functionally unique. Peripheral lymphocytes circulate in the blood and aid in defending the body against infections and the intrusion of foreign materials. The two main classes of lymphocytes are T and B lymphocytes, which differ in functions and molecule secretions (Owen, 2012).

Leukocytes, their subpopulations and their stage of differentiation are typically identified by different glycoprotein antigens expressed on their cell surfaces and these antigens are named systematically by assigning them a cluster of differentiation (CD) numbers which are recognized by specific antibodies. Flow cytometric

immunophenotyping is the gold standard for distinguishing different leukocyte subpopulations from each other by measuring the combinations of CD antigens on the surface of the cells and has been used routinely in diagnostic analysis in clinical medicine since its first commercialization in the 1950s, especially in diagnosing immunologic and hematologic disorders (Muller-Steinhardt et al., 2009, Connelley et al., 2011).

Among the many CD antigens on sheep leucocytes, CD11a and CD5 are especially relevant to this study because of their specificity for selected leukocyte subpopulations. There are antibodies available commercially which recognise ovine CD11a and CD5 and that have been used for the detection of these surface markers by flow cytometry (Mackay et al., 1985, Dhabhar et al., 1995, Gonzalez-Amaro and Sanchez-Madrid, 1999, Halliday et al., 2005).
CD11a, which is part of the lymphocyte function-associated antigen-1 complex (LFA-1, $\alpha_L\beta_2$, CD11a/CD18), is expressed on 100% of leukocytes in sheep blood (Gonzalez-Amaro and Sanchez-Madrid, 1999). Granulocytes are identified as showing high levels of CD11a expression ($CD11a^{High}$) and lymphocytes are detected as showing low levels of CD11a expression ($CD11a^{Low}$)(Mackay et al., 1990). CD5 is expressed on 98% of T lymphocytes (Mackay et al., 1985, Dhabhar et al., 1995) and is also expressed by B-1 lymphocytes (a relatively small subset -5% of B lymphocytes) in mammals including sheep (Tornberg and Holmberg, 1995, Chevallier et al., 1998).

The measurement of the surface immunoglobulin (Ig) can be used as an alternative to the use of CD antigens to identify ovine B lymphocytes. IgM is expressed on 100% of sheep B lymphocytes in the peripheral blood and is the major immunoglobulin produced in the primary immune response (Thompson and Hoffmann, 1971, Langezaal et al., 2002, Halliday et al., 2005).

Exposure to viruses and chemicals may alter leukocyte subpopulations which can adversely affect the immune function. This can be detected by changes in the expression of CD antigens on the cell surface. Therefore, by measuring leukocyte subpopulations one gets a broad picture of immune competence at the cellular level. Cadmium chloride is an example of a chemical that is able to cause damage both to the humoral immune response and the cell-mediated immunity (Dan et al., 2000, Langezaal et al., 2001). In this study, leukocyte subpopulations were identified by the expression of CD11 for

granulocytes vs. lymphocytes, the IgM cell surface antigens for B lymphocytes and CD5 for T lymphocytes.

When looking at the effects of sub-chronic exposure of environmental chemicals, it may be necessary to look at biomarkers at the protein and gene levels to detect the subtle changes that have both biological and toxicological significance (Duramad and Holland, 2011). Cytokines are small secreted soluble proteins that act as secondary messengers and signal cells to mediate and regulate immunity and haematopoiesis (Cher and Mosmann, 1987). Their excretion can be affected by sub-chronic exposure (Iavicoli et al., 2008). Clinical studies show that many cytokines play a role in autoimmunity, allergy and chronic inflammation. Measurements of cytokine levels are useful for understanding the pathogenesis of these conditions and as diagnostic and prognostic indicators in each of these conditions. Quantification of cytokines following activation of immune cells can also generate useful information regarding the mechanism of action of environmental chemical contaminants which are possible triggers of T helper 1 (Th1) autoimmune diseases, T helper 2 (Th2) allergic reactions and chronic inflammation (Schmidt, 2011).

Signature cytokines produced by Th1 and Th2 cells, as well as cytokines linked to the acute phase response which triggers chronic inflammation, are of particular importance and include:

IFN-γ which is a signature cytokine produced by Th1 lymphocytes that promotes cell-mediated immunity both by increasing the presentation of viral peptides by antigen presenting cells and by controlling the production of pro-inflammatory Th1 cytokines such as TNF-α and IL-12p70, making it a master regulator of autoimmunity (Wu et al., 1994, Foster et al., 2007).

Interleukin-4 (IL-4) which is a key pleiotropic cytokine produced by Th2 lymphocytes and mast cells. It induces Th2 lymphocyte differentiation and growth (Gross et al., 1993) as well as the activation and growth of B lymphocytes and IgG to IgE class switching, all of which are prominent in allergic diseases (Snapper et al., 1988).

IL-6 which is a pro-inflammatory cytokine which has a wide range of functions including stimulation of B and T lymphocytes and hepatocytes as well as the induction of B lymphocyte hybridoma proliferation. While IL-6 is produced by many cell types, including B and T lymphocytes, monocytes are thought to be the major source in the body (Aarden et al., 1987, Van Snick, 1990). IL-6 initiates the acute phase response

which is linked to the pathological conditions initially caused by chronic inflammation (Hirano et al., 1988).

While peripheral blood lymphocytes produce a range of cytokines, a Th1 cytokine (IFN-γ), a Th2 cytokine (IL-4) and a pro-inflammatory cytokine (IL-6) were chosen for this study because there are antibodies commercially available which can recognise or be cross reactive to these ovine cytokines and which can be measured using intracellular staining and detected by flow cytometry (Weynants et al., 1998, McWaters et al., 2000, Pedersen et al., 2002).

Because of their roles in the regulation of the immune response, the cytokines IFN-γ, IL-4, and IL-6, are useful biomarkers for immunotoxicity in *in vitro* bioassays. Pool et al., (2003) used IL-6, to screen for inflammatory activity in whole blood. The measurement of IFN-γ and IL-4 can provide direct or indirect information on both immunostimulation and immunosuppression by chemical exposure. For instance Langezaal et al., (2001 and 2002), showed that exposure of whole blood to a wide range of immunomodulatory chemicals caused changes in IFN-γ and IL-4 release.

In the case of immune function-dependent assays such as intracellular cytokine production or cytokine secretion, *in vitro* stimulation is usually required to induce specific immune functions. When measuring immune biomarkers in field–based immune monitoring, *ex vivo* stimulation of peripheral blood lymphocytes followed by measurement of intracellular cytokine production is extremely sensitive and relevant. In addition, measurement of intracellular cytokine production does not have the inherent problems associated with the measurement of secreted cytokines i.e. the rapid uptake of secreted cytokines by nearby immune cells. To avoid this uptake, samples must be analysed within 2-3 hours after blood collection to account for cytokine absorption kinetics (Pala et al., 2000). This may be difficult in biomonitoring studies where samples are collected in the field and then transported to a laboratory for analysis.

The flow cytometric-based method of intracellular lymphocyte cytokine detection has proved to be a great advantage in immunology. This is because of the opportunity of counterstaining with antibodies to cell surface CD antigens, thus allowing accurate identification of the lymphocytes that produce the specific cytokines. When measuring the intracellular production of pro-inflammatory cytokines in lymphocytes, the cells are

stimulated by phorbol 12-myrisate 13-acetate (PMA) plus the calcium ionophore, ionomycin, followed by inhibition of cytokine release using Brefeldin A (BFA) (Andersson et al., 1990, North et al., 1996). PMA and ionomycin are commonly used as stimulants and this combination activates protein kinase C (PKC) and calcium ion influx, respectively, and these in turn induce cytokine expression of cells previously activated by physiological stimuli. This is also used as a positive control to show the potential expression by cells that respond weakly to other stimuli (Maino et al., 1995, North et al., 1996, Jason and Larned, 1997, Sewell et al., 1997, Mascher et al., 1999, Rostaing et al., 1999, Mendes et al., 2000, Baran et al., 2001). The blocking of cytokine secretion and intracellular accumulation is achieved by treatment with BFA (Sander et al., 1991, Jung et al., 1993, Dinter and Berger, 1998).

The aim of the present study was to immunologically characterize frozen sheep blood. The different immune endpoints, leukocyte subpopulation analysis and intracellular cytokine production following *in vitro* stimulation, were assessed to quantify the distribution of leukocytes subsets circulating in the blood and the levels of cytokines that these cells produce. The objective of the short term exposure of frozen blood to common positive controls for genotoxic assays i.e. EMS & $CdCl_2$, was to determine which leukocyte subset was the most sensitive in relation to viability and responses. The different immune endpoints were assessed with the prospect of using them as immune markers in field-based monitoring.

Materials & Methods

Animals

Three two-year-old Cheviot-cross ewes, tagged 401, 440 and 642, were derived from a breeding stock at the Central Veterinary Laboratory (CVL), Dublin, Ireland. They were housed together in one pen and provided with hay *ad libitum* in racks and water in a large plastic container. The studies were conducted at a farm located 10 km from the laboratory at which the cell isolations, culturing and assays were performed. All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Chemicals & Reagents

All chemicals used were of the highest grade available: RPMI 1640 medium with 2mM L-glutamine, heat-inactivated foetal bovine serum (FBS), HEPES, phosphate-buffered saline (PBS), dimethyl sulphoxide (DMSO), ethyl methanesulphonate (EMS), propidium iodide (PI), lymphocyte separation medium (Histopaque 1083), phorbol 12-myrisate 13-acetate (PMA), ionomycin; Brefeldin A (BFA), formaldehyde, bovine serum albumin (BSA), sodium azide and glucose were purchased from Sigma-Aldrich (Ireland). The antibiotic, gentamycin, was procured from GIBCO (UK). Sterile 24-well tissue culture plates were purchased from Sarstedt (Ireland). Ammonium chloride RBC lysis buffer (Pharmlyse), sodium heparin and potassium EDTA blood collection tubes (9ml) were procured from BD Biosciences (Ireland). Leucoperm (A and B) and all flow cytometry antibodies used were obtained from Serotec (UK): monoclonal mouse anti-bovine CD5: FITC antibody, clone CC15 (IgG1), mouse IgG1:FITC (IgG1 isotype control), monoclonal mouse anti-ovine CD11a:FITC clone 72.87 (IgG2a) ; mouse IgG2a:FITC (IgG2a isotype control), monoclonal mouse anti-ovine IgM:FITC clone 25.69 (IgG1) ; monoclonal mouse anti-bovine interleukin-4:RPE clone CC303 (IgG2a), mouse IgG2a:RPE (IgG2a isotype control), monoclonal mouse anti-ovine interleukin-6 clone 4B6(IgG1), mouse IgG1 (IgG1 isotype control), polyclonal rabbit $F(ab')_2$ anti-

mouse IgG:FITC and monoclonal mouse anti-bovine interferon gamma:FITC clone CC302 (IgG1). Cadmium chloride ($CdCl_2.5H_2O$) was obtained from Alkem (Ireland).

Blood Collection

Jugular blood samples were extracted from the ewes after they were restrained in individual small pens. Blood (50-60 ml) was collected from each ewe in 9 ml sodium heparin and potassium EDTA tubes. The blood was then divided with some being used for PBMC isolation, some for the preparation of fresh whole blood by dilution and the remainder was cryopreserved as described previously in Chapters 2, 3 and 4 and shown in Figure 1.

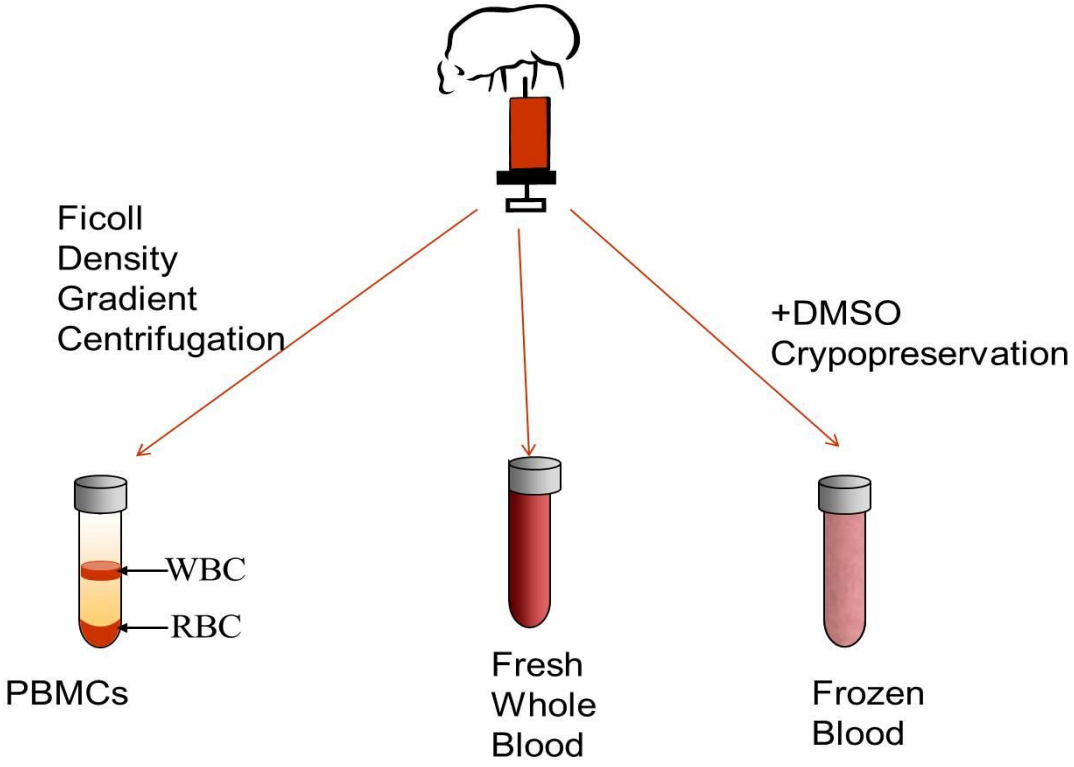


Figure 1. Schematic of the Different Blood Sample Processing Methods Used in this Chapter.

Haematology and Serum Biochemistry

Blood differential leukocyte counts were determined in potassium-EDTA-blood tubes using an automated haematology analyser (Sysmex F-820, courtesy of the Munster Regional Veterinary Laboratory, Co Cork, Ireland). Serum biochemistry was assessed to determine the health status of each sheep before proceeding with the processing of samples. Both methods are described in detail in Chapter 4.

Sample Preparation

Isolation of PBMCs

Fresh sheep PBMCs were isolated from heparinised blood by routine Ficoll-density gradient centrifugation as described by Boyum (1968) and as detailed previously in Chapter 2.

Fresh Whole Blood

Fresh whole blood (1ml) was diluted with 2 ml of complete culture medium, mixed gently and put in culture as described by Connor et al, (2000) and as detailed previously in Chapter 3.

Frozen Blood

After 150 µl of DMSO was added to 1350 µl of fresh sheep blood, the cryovials were placed in a slow-freeze container, stored in a −80°C freezer for 24 hours and then stored in a liquid nitrogen dewar as detailed previously in Chapter 3.

Thawing of Frozen Blood

Frozen samples were thawed in a 37°C water bath according to the procedure adapted from the protocol described by Reimann and colleagues (Reimann et al., 2000). Slow dilution with thawing medium was followed by centrifugation and suspension as detailed previously in Chapter 3.

Cell Culture

The cells from each sample preparation were cultured at 37°C in a 5 % CO_2 humidified incubator in RPMI 1640 medium supplemented with 2 mM L-glutamine, heat inactivated 10 % foetal bovine serum (FBS), 10 mM HEPES and 20 µg /ml gentamycin. 200 µl of the appropriate cell suspension was added to wells in 96-well plates which were incubated for 1 hour 30 minutes at 37°C prior to exposures.

Cell Surface Marker Analysis

The CD5 and CD11a antibodies used were conjugated to the fluorescent dye, fluorescein isothiocynate (FITC). These antibodies and isotype controls were each diluted in PBS/1% (w/v) BSA/20 mM glucose and stored at 4°C before use. The cells from each sample preparation were cultured for 3 hours in a 24-well plate as described previously and then 200 µl of the cell suspension was removed and transferred in a flow tube. Tubes were centrifuged for 7 minutes at 400 x g and the supernatant discarded and 100 µl of PBS/1% (w/v) BSA/20 mM glucose was added to each tube. 10 µl of each antibody solution or isotype controls was added to the appropriate flow tubes which were incubated in the dark for 30 minutes at room temperature. The cells were washed with 2 ml of PBS/BSA added to the flow tubes and they were centrifuged for 7 minutes at 300xg and the supernatant discarded. The cells were then resuspended in 0.1 % (v/v) formalin in PBS/1% (w/v) BSA and stored at 4°C in the dark until flow cytometry analysis was performed within 24 hours of staining.

The immunofluorescent staining of IgM expression on B lymphocytes required an additional procedure to remove serum immunoglobulin which would otherwise interfere

and block staining with immunoglobulin-specific antibodies. Blood (1ml) was mixed well with 13 ml of PBS/BSA (pre-warmed to 37ºC in a 15 ml tube) and centrifuged at 400 x g for 7 minutes. The supernatant was carefully aspirated and the pellet resuspended in the residual supernatant. The process was repeated (two washes in total) before continuing with staining with the FITC conjugated anti-IgM antibody following the procedure described above. All these FITC conjugated detection antibodies were used in tandem with the viability dye, propidium iodide.

Propidium Iodide (PI) Exclusion Assay

The viability of lymphocytes in blood sample preparations was determined by the propidium iodide (PI) exclusion assay as described by Moore et al., (1998) and as detailed in Chapter 2. As stated previously, the only cell types that were analysed in the blood sample preparations were lymphocytes and granulocytes as the monocytes and dendritic cells were still attached to the plastic tissue culture plates.

Lymphocyte Cytokine Induction and Intracellular Cytokine Staining

Sample preparations, 1ml of PBMCs, fresh and frozen whole blood containing 1×10^6 lymphocytes were incubated with 10 μg/ml Brefeldin A (BFA), 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin at 37°C in 5% CO_2 for 4 hours to stimulate the lymphocytes for cytokine production.

Spontaneous cytokine production in unstimulated controls was assessed in an equivalent manner without the PMA and ionomycin treatments but with 10 μg/ml BFA only. After exposures, the red blood cells in fresh and frozen blood samples were lysed using an ammonium chloride lysing agent, Pharmlyse, (1X) where the 10X concentrate was diluted 1:10 with distilled water and warmed to room temperature prior to use. 1X lysing solution (1.0 ml) was added to each tube containing up to 200 μl of a fresh whole blood or frozen blood. Each tube was gently vortexed immediately after adding the lysing solution. The tubes were then incubated at room temperature, protected from light, for 15 minutes. The cell samples were then centrifuged at 400 x g for 7 minutes and then the supernatant was carefully aspirated without disturbing the pellet. PBS (2 ml) containing 1% heat-inactivated fetal bovine serum and 0.1% sodium azide was

added and the tubes centrifuged at 400 X g for 7 minute and then the supernatant, was carefully aspirated without the disturbing pellet. After culture, harvesting and washing, the cells were resuspended in 250 µl of medium. The cell suspension (100 µl) was fixed and permeabilized using Leucoperm according to the manufacturer's protocol: Leucoperm A solution (50 µl) was added to each tube, to fix the external epitopes and after a wash and centrifugation at 400 x g for 7 minutes, 50 µl of Leucoperm B solution was added for permeabilization along with 10 µl of the anti-cytokine monoclonal antibodies to IFN-γ, IL-4 and IL-6. The tubes were then incubated in the dark for 30 minutes at room temperature. After washing with PBS containing 1% (w/v) BSA and 0.1% (w/v) sodium azide, cells were resuspended in 1% formaldehyde in PBS and stored at 4°C in the dark until measurement. All samples were analysed by flow cytometer within 24 hours of staining the cells.

Minor changes were made to the protocol for the intracellular staining of IL-4 due to the low quantity produced. In order to increase the chances of detection, the samples were double stained with both the anti-IL-4: RPE (R-phycoerythrin) and the anti-CD5-FITC antibodies. This allowed the selection of the $CD5^{+}$(T lymphocytes) subpopulation through gating during flow cytometric analysis. After culture, harvesting and washing, 100 µl of the blood sample was added to a flow tube and 10 µl of the anti-CD5-FITC antibody was added and incubated for 15 min. The preparation was washed again and centrifuged for 7 minutes at 400 x g. The rest of the procedure was the same as that used earlier for the intracellular staining of cytokines.

Due to the lack of a commercially available fluorochrome-conjugated IL-6 antibody, the protocol for IL-6 staining was changed to an indirect immunofluorescence staining in which the unconjugated monoclonal antibody recognizing ovine IL-6 was visualised using a FITC-conjugated secondary antibody. After the first incubation step with anti-ovine IL-6 , cells were washed and incubated with 10 µL of the $F(ab)_2$ rabbit anti-mouse IgG:FITC (secondary antibody) in PBS/1% (w/v) BSA/0.1% sodium azide heat inactivated rabbit serum for 30 minutes at room temperature in order to minimise non-specific binding of the secondary antibodies. The rest of the procedure was similar to the standard intracellular staining protocol used above.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FACS Calibur instrument (Becton Dickinson, UK). Readings were taken on a minimum of 10,000 leukocytes and the percentage of positive cells within two gated populations, ‘‘Granulocyte gating’’ and ‘‘Lymphocyte gating’’ was determined for each staining. Lymphocyte and granulocyte gating were determined by analysing the forward scatter (FSC) and side scatter (SSC) profile of the whole blood on a dot plot as shown in Figure 2A. Objective gating was used based on those cell populations identified from the Cell Quest Pro manual (Becton Dickinson, UK). The criteria for gating included lymphocytes which were both positive and negative for PI staining. These gates were further verified using the differential staining of the CD11a antibody as shown in Figures 2C and 2D, which has either low or high expression depending on the leukocyte population. The same gates were used for all sheep samples. Data were acquired and analysed using Cell Quest Pro software (Becton Dickinson, UK). For certain samples, the mean fluorescence intensity (MFI) was also determined, as a measure of changes in the mean expression of the cytokine molecules on the cells.

Statistical Analysis

Results are presented as means ± SD from 3 independent experiments. All statistical analyses were performed using SigmaStat software (version 2.03). Data were tested for normality using a Kolmogorov-Smirmnov test. Differences between groups were analysed using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons. Statistical correlation and linear regression analysis were performed using Prism 4.05 software. Statistical significance was set at $p<0.05$ and significant levels were defined as *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Haematology and Serum Biochemistry Analysis

The blood differential leukocyte counts measured for each sheep are shown in Table 1 and were within the specifications as detailed by the MRVL for healthy sheep.

Test	Sheep ID			
	401	**440**	**642**	**Normal range**
Lymphocytes %	60	75	66	40–75
neutrophils %	31	16	24	10–50
Band neutron %	1.0	0.0	0	0-2
Eosinophils %	6	3	3	0-10
Monocytes %	2	0	4	0-6
Basophils %	0	1	3	0-3
RBC x 10^{12}/L	12.23	13.08	12.13	9–15
WBC x 10^{9}/L	9.2	7.75	9.9	4–12
Lymphocytes x 10^{9}/L	5.52	6.32	5.53	2–9
Neutrophils x 10^{9}/L	2.65	1.28	2.36	0.7–6.0
Band Neutrophils x 10^{9}/L	0.09	0	0	0–0.1
Eosinophils x 10^{9}/L	0.55	0.24	0.30	0–1
Monocytes x 10^{9}/L	0.18	0	0.4	0–0.75
Basophils x 10^{9}/L	0	0.1	0.30	0–0.3

Table 1. Differential Blood Leukocyte Counts from the Three Sheep Used in this Study.

The serum biochemistry parameters measured for each sheep are shown in Table 2 and were within the specifications as detailed by the MRVL for healthy sheep.

Serum Biochemistry Parameter:	Sheep ID			
	401	440	642	Normal Range
Glucose	80 mg/dl	75 mg/dl	70 mg/dl	44–81 mg/dl
Blood Urea Nitrogen (BUN)	14 mg/dl	18 mg/dl	18 mg/dl	10–26 mg/ dl
Creatinine	1.4 mg/dl	1.8 mg/dl	1.5 mg/dl	0.9–2.0 mg/ dl
Calcium	12.2 mg/dl	13.5 mg/dl	11.6 mg/dl	9.3–13.7 mg/ dl
Phosphorus	7.7 mg/dl	6.6 mg/dl	8.8 mg/dl	4.0–9.3 mg/ dl
Total Protein	6.8 g/dl	6.4 g/dl	7.0 g/dl	5.9–7.8 g/ dl
Albumin	2.7 g/dl	2.7 g/dl	2.8 g/dl	2.7–3.7 g/ dl
Alkaline Phosphatase (ALP)	223 IU/L	213 IU/L	264 IU/L	97–266 IU/L
Sorbitol Dehydrogenase (SDH)	13 IU/L	13 IU/L	21I U/L	3.5–21 IU/L
Aspartate Aminotransferase (AST)	180 IU/L	160 IU/L	150 IU/L	49–183 IU/L
Creatine Phosphokinase (CPK)	323 IU/L	213 IU/L	205 IU/L	107.7–351 IU/L
Chloride	102 mEq/L	105 mEq/L	92 mEq/L	91–113 mEq/L
Potassium	5.5 mEq/L	5.8 mEq/L	5.2 mEq/L	4.3–6.3 mEq/L
Sodium	134 mEq/L	148 mEq/L	139m Eq/L	132–160 mEq/L

Table 2. The Serum Biochemistry Profiles of the Blood of the Three Sheep Used in this Study.

Immunophenotyping Profiles of Leukocyte Subpopulations in Blood.

The cell surface markers, CD5, CD11a and IgM, were measured by flow cytometry to assess the distribution of leukocyte subpopulations in three different sheep. Lymphocytes (L) and granulocytes (G) were differentiated by gating on individual populations forward scatter (FSC) vs. side scatter (SSC) to separate the leukocyte subpopulations in terms of size, shape and granularity as shown in Figure 2A. All the samples were gated with the same criteria for all animals. The distribution of gated peripheral blood granulocytes (Figure 2C) and lymphocytes (Figure 2D) were also identified using the differential expression of CD11a which are consistently used as the criteria to classify the cells as shown in Figures 2C and 2D. In the lymphocytes gate, CD11a ($CD11a^{Low}$) expression was much lower than the CD11a expression ($CD11a^{High}$) in the granulocyte gate.

The percentage of gated peripheral blood T lymphocytes and B lymphocytes were identified using CD5 expression (Figure 2E) and IgM expression (Figure 2 F) in lymphocytes (analysed as gated using FSC/SCC): 59-65% were CD5 positive and 23-30% were IgM positive. All samples were also stained using the corresponding isotype control antibody for each cytokine antibody. The isotype control staining for each antibody served two purposes. Firstly, it confirmed the specificity of the primary antibody. Secondly, it showed the background fluorescence resulting from mouse Fc receptor mediated binding and other non-specific cellular protein interactions. The appropriate staining procedures were verified by using the isotype control antibodies and a typical example of the negative control staining pattern is shown in Figure 2B. This negative control pattern can correct for non-specific staining and inform whether gates are in the right position.

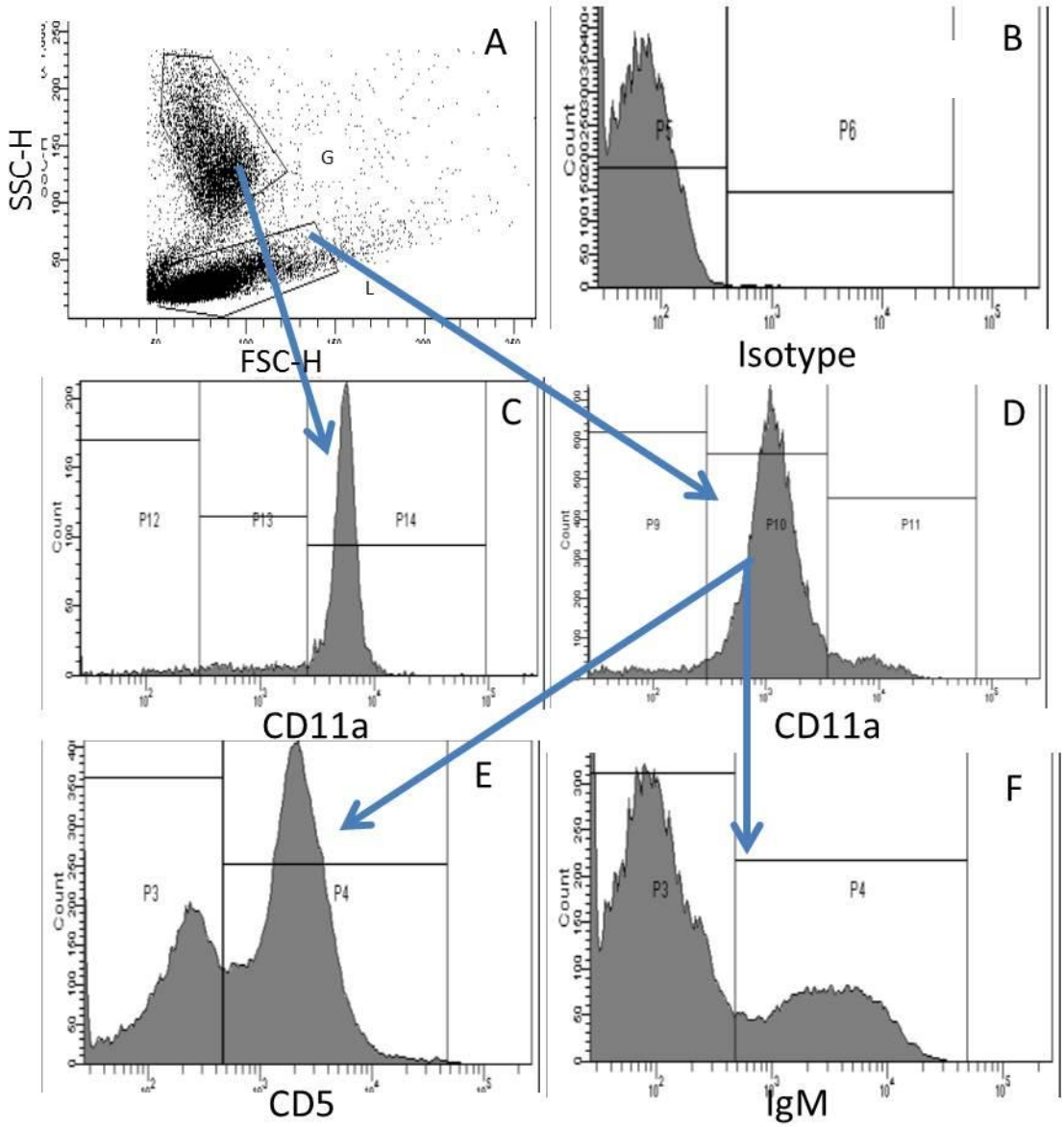


Figure 2. Flow Cytometry Profiles of Different Leukocyte Subpopulations from Sheep using Different Parameters. A dot plot of whole sheep blood is differentiating between lymphocytes (L) and granulocytes (G) using forward scatter (FSC) vs. side scatter (SSC) as shown in Figure 2A. Fluorescence (CD11a) vs. frequency histogram shows the distribution of gated peripheral blood granulocytes (Figure 2C) and lymphocytes (Figure 2D) using mouse anti-ovine CD11a:FITC (IgG2a) as shown in Figures 2C and 2D. Fluorescence (CD5 or IgM) vs. frequency histogram shows distribution of gated peripheral blood T and B lymphocytes, respectively, using mouse anti-bovine CD5:FITC(IgG1) (Figure 2E) and mouse anti-ovine IgM:FITC(IgG1) (Figure 2F) antibodies as shown in Figures 2E and 2F. A fluorescence (isotype) vs. frequency histogram showing the staining pattern of mouse IgG2a:FITC (isotype control for the anti-Ovine CD11a:FITC) is shown in Figure 2B and is a typical result for all the isotype controls.

Comparison of the Two Methods, Automated Haematology Analyser and CD11a Cell Surface Marker Expression, used to Measure the Leukocyte Subpopulations in Fresh Whole Blood

There was no significant difference in the relative proportions of the leukocyte subpopulations, lymphocytes and granulocytes, in fresh whole blood when either an automated haematology analyser or CD11a cell surface marker expression, as measured by flow cytometer, was used to measure them (Figure 3).

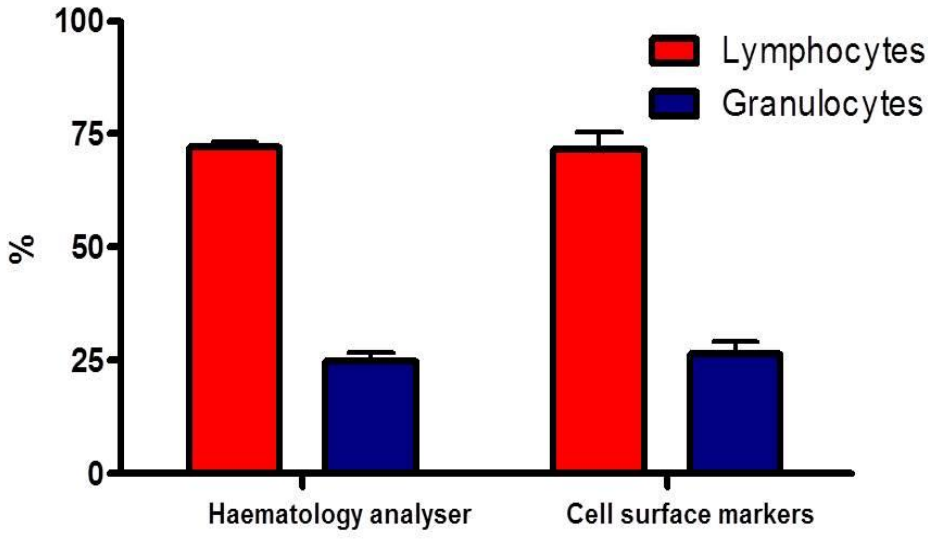


Figure 3. Comparison of the Two Methods, Automated Haematology Analyser and CD11a Cell Surface Marker Expression, used to Measure the Leukocyte Subpopulations in Fresh Whole Blood: Data compare the standard automated haematology analyser method and cell surface marker expression anti CD11a to measure the proportion of lymphocytes and granulocytes in fresh whole sheep blood. All values are expressed as percentages of total leukocytes. Values represent the mean ± SD of one representative experiment from three independent experiments from 3 different sheep. * Represents a significant difference between the automated haematology analyser and CD marker analysed subpopulations $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

The Effect of Sample Processing and Cryopreservation on Leukocyte and Lymphocyte Subpopulations in Blood.

The freezing and thawing procedure significantly reduced the percentage of granulocytes in frozen whole blood ($p<0.01$) as measured in whole blood by the reduction of % $CD11^{High}$ cells. This reduction was confirmed using the flow cytometer parameters FSC (forward scatter) and SSC (side scatter). Thus freezing and thawing procedure altered the distribution of lymphocytes and granulocytes within the leukocyte population (Figure 4A) but this cell distribution is not further altered further by culturing for 3 hours (Figure 4A).

The relative proportions of T and B lymphocytes as measured by the percentage of CD5 cells and IgM cells were the same in fresh whole blood and frozen blood and were not altered by culturing for 3 hours (Figure 4B).

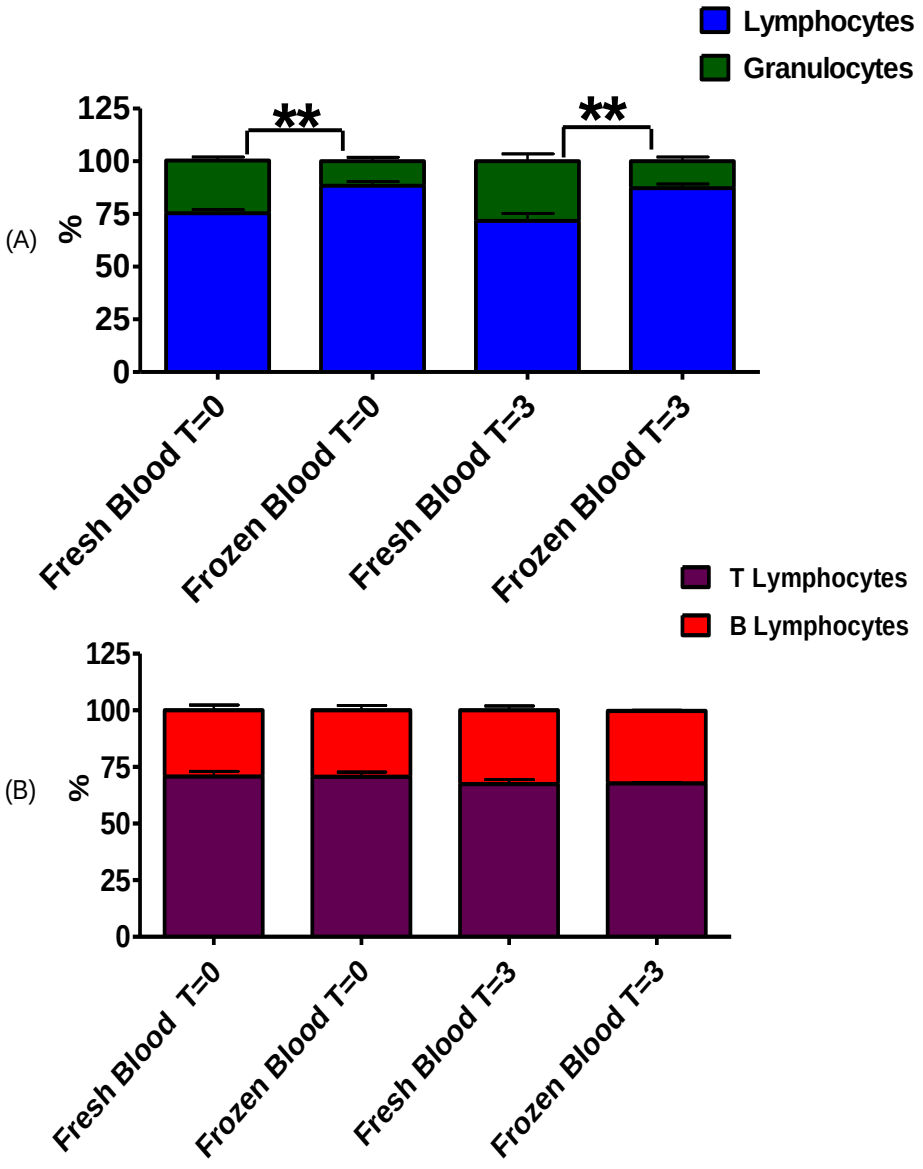


Figure 4. The Effect of Sample Processing on the Relative Proportions of Leukocyte and Lymphocyte Subpopulations before and after 3 Hours in Culture. The proportion of lymphocytes and granulocytes in fresh and frozen blood are shown in Figure 4A. The proportions of T cells and B lymphocytes are shown in Figure 4B. All values are expressed as percentages and represent the mean ± SD of one representative experiment from three independent experiments from 3 different sheep. * Represents a significant difference between the fresh and frozen samples subpopulations $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Effect of Storage in Different Anticoagulants on the Viability of Leukocyte and Lymphocyte Subpopulations in Frozen Blood.

The expression of the cell surface markers, CD5, CD11a and IgM, and the level of propidium iodide exclusion were measured by flow cytometry after 3 hours in culture to assess the % of cell viability of each subpopulation of leukocytes in frozen blood collected in tubes with 2 different anticoagulants, sodium heparin (NaH) and potassium EDTA (KEDTA). Fresh blood samples were not included in this study due to time constraints in dealing with a large number of samples.

The distribution between peripheral blood granulocytes and lymphocytes was identified using the differential expression of CD11a, whereas the distribution between peripheral blood T lymphocytes and B lymphocytes was identified using CD5 expression and IgM expression, respectively. There was no difference between the distribution of the different leukocyte populations for the frozen blood when stored in either of the anticoagulants, sodium heparin or potassium EDTA (data not shown). The viability of each of the leukocyte subpopulations was measured by double staining the leukocytes with the cell surface markers and propidium iodide and gating on granulocytes or lymphocytes or both. The use of KEDTA storage tubes instead of NaH tubes caused a significant reduction in the viability of leukocytes by 7.5% ($p<0.01$) to 83%, lymphocytes by 6.5% ($p<0.01$) to 84%, T lymphocytes by 5% ($p<0.05$) to 80% and B lymphocytes by 8.5% ($p<0.01$) to 81% (Figure 5). There was no significant difference in the viability of granulocytes for the frozen blood stored in the different anticoagulants.

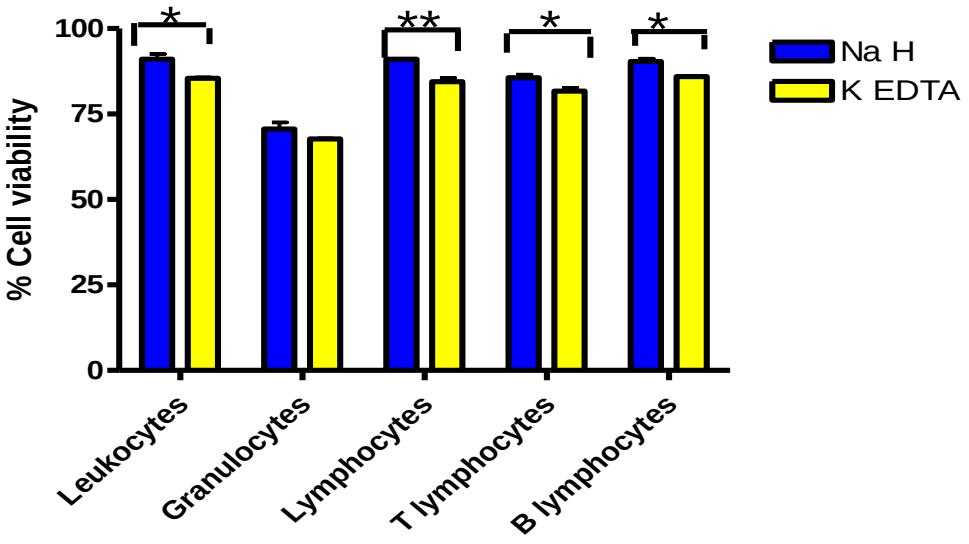


Figure 5. Effect that Storage in Different Anticoagulants has on the Viability of Leukocyte and Lymphocyte Subpopulations in Frozen Blood. Data represent the viability of sheep blood leukocytes, lymphocytes and granulocytes as well as the lymphocyte subpopulations T and B lymphocytes in frozen blood. Each cell surface marker was double stained with propidium iodide (PI) and all values are expressed as a percentage of cell viability of each subpopulation. Values represent the mean ± SD from three independent experiments from 3 different sheep. * Represents a significant difference between sodium heparin (Na H) and potassium EDTA (K EDTA) stored frozen blood samples subpopulations $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Effect of Storage in Different Anticoagulants on the Response of Leukocyte and Lymphocyte Subpopulations in Frozen Blood to Ethyl Methylsulphonate in Cytotoxicity Assays over 3 Hours in Culture.

Because exposure to EMS for more than 3 hours caused substantial cytotoxicity in frozen blood, the 3 hour time point was chosen (data not shown). When frozen blood was collected in sodium heparin (NaH) collection tubes, EMS had no cytotoxic effect on any cell type when the 1 mM dose was used. At a dose of 2.5 mM, there was a significant (6 %) reduction ($p<0.05$) in B lymphocytes but no effect on the other populations. At a dose of 5 mM, there was a significant (10 %) reduction ($p<0.01$) to 80 % in B lymphocytes and a significant (8 %) reduction ($p<0.01$) in granulocytes to 64 %. but no significant effect on the total lymphocytes or the T lymphocytes subpopulation (Table 3).

When frozen blood was collected in potassium EDTA (KEDTA) blood collection tubes, EMS had only a slight cytotoxic effect when the 1 mM dose was used as it did not cause any reduction in the viability of T lymphocytes or granulocytes but it did cause a significant (7 %) reduction ($p<0.05$) to 74 % in B lymphocytes. At a dose of 2.5 mM, there was a significant (10 %) reduction ($p<0.01$) to 71 % in B lymphocytes, a significant (15 %) reduction ($p<0.01$) to 65 % in T lymphocytes and a significant (14 %) reduction ($p<0.01$) in granulocytes to 52 %. At a dose of 5 mM, there was a significant (13 %) reduction ($p<0.01$) to 68 % in B lymphocytes, a significant (20 %) reduction ($p<0.001$) to 60 % in T lymphocytes, a significant (5 %) reduction ($p<0.05$) to 79 % in total lymphocytes and a significant (8 %) reduction ($p<0.01$) in granulocytes to 64 % (Table 3).

				Concentrations of EMS			
Blood Tube	**Gate**	**CD marker**	**Cell population**	**0mM**	**1mM**	**2.5mM**	**5mM**
NaH	Granulocytes	$CD11a^{high}$	Granulocytes	72.99 ±2.12	72.41 ±0.91 NS	71.15 ±1.86 NS	64.01 ±1.76 **
NaH	Lymphocytes	$CD11a^{low}$	Lymphocytes	90.98 ±0.43	89.12 ±0.58 NS	85.15 ±1.46 NS	83.40 ±1.56 NS
NaH	Lymphocytes	CD5	T-Lymphocytes	85.86 ±1.41	84.13 ±0.82 NS	80.10 ±3.31 NS	79.42 ±2.46 NS
NaH	Lymphocytes	IgM	B-Lymphocytes	90.61 ±1.39	85.04 ±4.28 NS	83.44 ±3.42 *	80.83 ±3.17 **
K EDTA	Granulocytes	$CD11a^{high}$	Granulocytes	67.69 ±0.27	58.20 ±2.56 NS	52.85 ±4.93 **	52.69 ±3.16 ***
K EDTA	Lymphocytes	$CD11a^{low}$	Lymphocytes	84.43 ±1.04 .	81.35 ±2.02 NS	81.14 ±1.43 NS	80.30 ±1.02 *
K EDTA	Lymphocytes	CD5	T-Lymphocytes	80.70 ±1.70	72.58 ±2.75 NS	65.36 ±4.83 **	60.02 ±2.38 ***
K EDTA	Lymphocytes	IgM	B-Lymphocytes	81.93 ±0.36	74.76 ±0.20 *	71.58 ±2.82 **	68.68 ±3.52 ***

Table 3. **Effect that Storage in Different Anticoagulants has on the Response of Leukocyte and Lymphocyte Subpopulations in Frozen Blood to Ethyl Methylsulphonate in Cytotoxicity Assays over 3 Hours in Culture.** The viability of lymphocytes and granulocytes as well as the lymphocyte subpopulations, T lymphocytes and B lymphocytes after exposure of frozen blood to various EMS concentrations after the blood was collected in either sodium heparin (NaH) or potassium EDTA (KEDTA) are shown in the Table. Each cell surface marker was double stained with propidium iodide (PI) and all values are expressed as a percentage of cell viability of each subpopulation. Values in each column represent the mean ± SD from three independent experiments from 3 different sheep. * Represents a significant difference between control and the EMS treated groups $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Effect of Storage in Different Anticoagulants on the Response of Leukocyte Subpopulations in Frozen Blood to Cadmium Chloride in Cytotoxicity Assays over 3 Hours in Culture.

When frozen blood was collected in sodium heparin (NaH) blood collection tubes, exposure to 100 µM $CdCl_2$ for 3 hours had no cytotoxic effect. At a dose of 333 µM, there was a significant (3 %) reduction ($p<0.05$) to 87 % in B lymphocytes. At a dose of 500 µM, there was a significant (5 %) reduction ($p<0.01$) to 85 % in B lymphocytes and a significant (10 %) reduction ($p<0.01$) to 60 % in granulocytes (Table 4).

When frozen blood was collected in potassium EDTA (KEDTA) blood collection tubes, $CdCl_2$ had no cytotoxic effect when the 100 µM dose was used. At a dose of 333 µM, there was a significant (12 %) reduction ($p<0.05$) in granulocytes to 55 %. but it had no significant effect on any of the lymphocytes subpopulations. At a dose of 500µM, there was a significant (7 %) reduction ($p<0.05$) to 73 % in B lymphocytes, a significant (6 %) reduction ($p<0.01$) to 75 % in T lymphocytes, a significant (5 %) reduction ($p<0.05$) to 79 % in total lymphocytes and a significant (15 %) reduction ($p<0.01$) in granulocytes to 52 % (Table 4).

				Concentrations of $CdCl_2$			
Blood Tube	**Gate**	**CD marker**	**Cell population**	**0 µM**	**100 µM**	**333 µM**	**500 µM**
Na H	Granulocytes	$CD11a^{high}$	Granulocytes	70.66 ± 3.46	65.77 ± 1.93 NS	62.82 ± 3.65 NS	60.06 ± 0.65 **
Na H	Lymphocytes	$CD11a^{low}$	Lymphocytes	87.81 ± 1.08	85.60 ± 0.78 NS	85.32 ± 0.72 NS	84.00 ± 1.84 NS
Na H	Lymphocytes	CD5	T-Lymphocytes	85.86 ± 1.41	85.20 ± 0.29 NS	81.08 ± 2.51 NS	80.16 ± 1.64 NS
Na H	Lymphocytes	IgM	B-Lymphocytes	90.61 ± 1.39	90.61 ± 2.07 NS	87.17 ± 0.47 *	85.14 ± 1.98 **
K EDTA	Granulocytes	$CD11a^{high}$	Granulocytes	67.69 ± 0.27	58.20 ± 2.56 NS	55.72 ± 0.50 *	52.85 ± 4.93 **
K EDTA	Lymphocytes	$CD11a^{low}$	Lymphocytes	84.43 ± 1.04	81.35 ± 2.02 NS	81.14 ± 1.43 NS	79.30 ± 1.02 *
K EDTA	Lymphocytes	CD5	T-Lymphocytes	80.70 ± 1.70	78.18 ± 2.03 NS	77.29 ± 2.15 NS	75.55 ± 0.78 **
K EDTA	Lymphocytes	IgM	B-Lymphocytes	81.93 ± 0.36	79.49 ± 1.07 NS	77.42 ± 3.10 NS	73.44 ± 0.75 **

Table 4. **Effect that Storage in Different Anticoagulants has on the Response of Leukocyte and Lymphocyte Subpopulations in Frozen Blood to Cadmium Chloride in Cytotoxicity Assays over 3 Hours in Culture.** The viability of lymphocytes and granulocytes as well as the lymphocyte subpopulations T cells and B cells after exposure of frozen blood to various concentrations of $CdCl_2$ after the blood was collected in either sodium heparin (NaH) or potassium EDTA (K EDTA) are shown in the table. Each cell surface marker was double stained with propidium iodide (PI) and all values are expressed as a percentage of cell viability of each subpopulation. Values in each column represent the mean ± SD from three independent experiments from 3 different sheep. *represents a significant difference between control and the $CdCl_2$ treated groups $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Flow Cytometry Profiles of Cytokines from Fresh Whole Blood

The cytokines, IL-4, IL-6 and IFN-γ, were measured to assess the immune function of sheep lymphocytes. In pilot studies, there was very low cytokine production detected when the lymphocytes were stimulated for 3 hours (data not shown). A stimulation time of 4 hours was used because it did not cause a decrease in the viability of the lymphocytes (data not shown). Fresh whole sheep blood was cultured in the presence of Brefeldin A (BFA) (untreated) or stimulated with phorbol 12-myrisate 13-acetate (PMA)-ionomycin (I) in the presence of BFA. Lymphocytes (identified through gating) from fresh sheep whole bloods were stained with either the isotype control mouse IgG1: FITC (Figure 6A) or mouse anti-bovine Interferon gamma:FITC (Figure 6B and 6C).

Due to the low production of IL-4 from lymphocytes (identified through gating) from fresh whole sheep blood, the lymphocytes were double stained with mouse anti-Bovine CD5:FITC (IgG1) and mouse anti-bovine IL-4:RPE clone CC303 (IgG2a) antibodies to improve the detection of this cytokine by gating for $CD5^+$ cells (Figure 7B).

Lymphocytes (identified through gating) from fresh sheep whole bloods were stained with either the isotype control mouse IgG1 (IgG1) (Figure 8A) and mouse anti-ovine Interleukin-6 (IgG1) (Figure 8B and 8C) and both were visualised using the polyclonal rabbit F $(ab')_2$ anti-mouse IgG:FITC secondary antibody.

11.5 % of stimulated lymphocytes were IFN-γ positive (Figure 6C) compared to 0.4 % for unstimulated lymphocytes (Figure 6B).

4.5 % of stimulated $CD5^+$lymphocytes (T lymphocytes) were IL-4 positive compared to 0.9 % for unstimulated T lymphocytes (Figures 7A (C) & 7A (B)).

2.0 % of stimulated lymphocytes were IL-6 positive compared to 0.5% for unstimulated lymphocytes (Figures 8B and 8C). All samples were stained using the corresponding isotype control antibody for each cytokine antibody. There was a typical staining pattern for all negative control isotype antibodies (Figures 6A, 7A (A) & 8A).

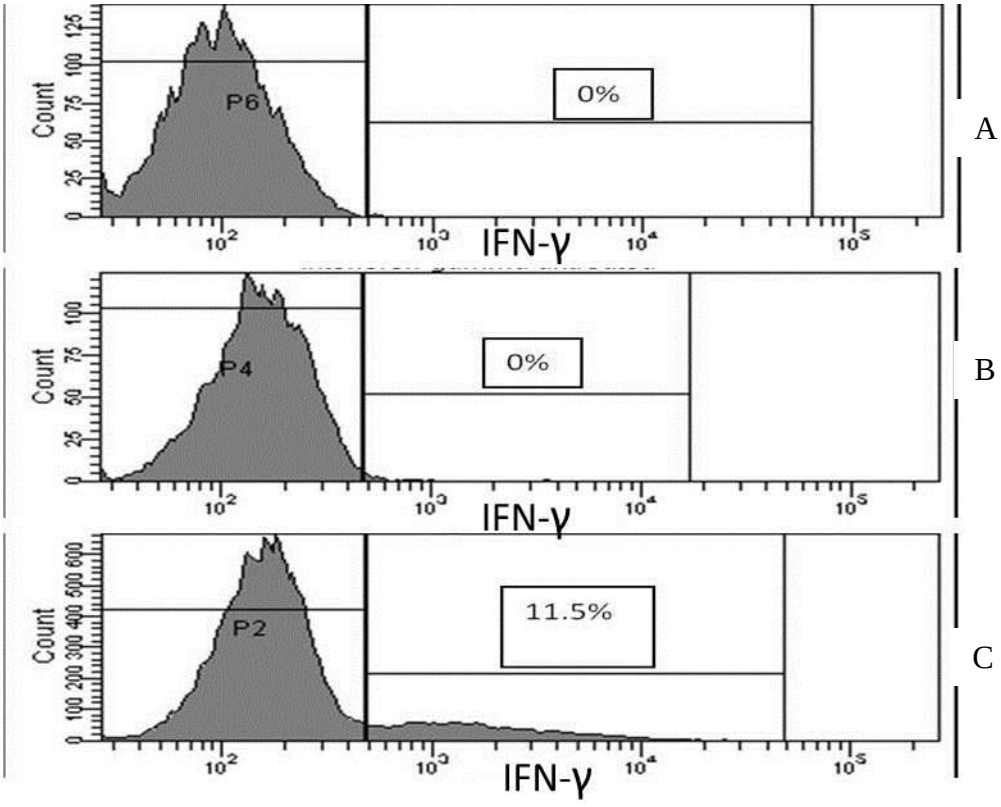
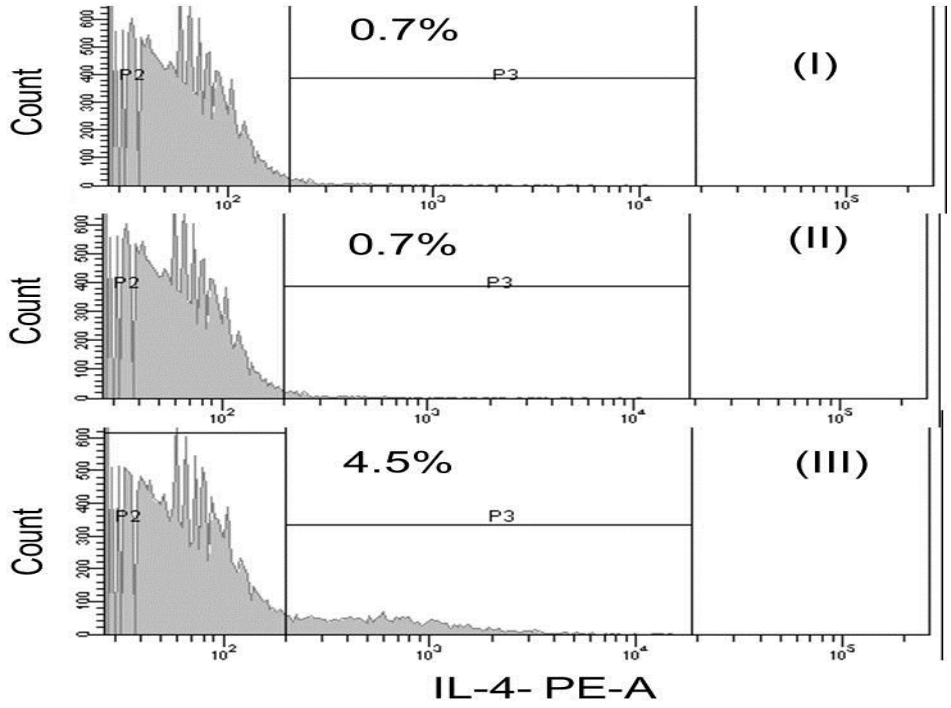
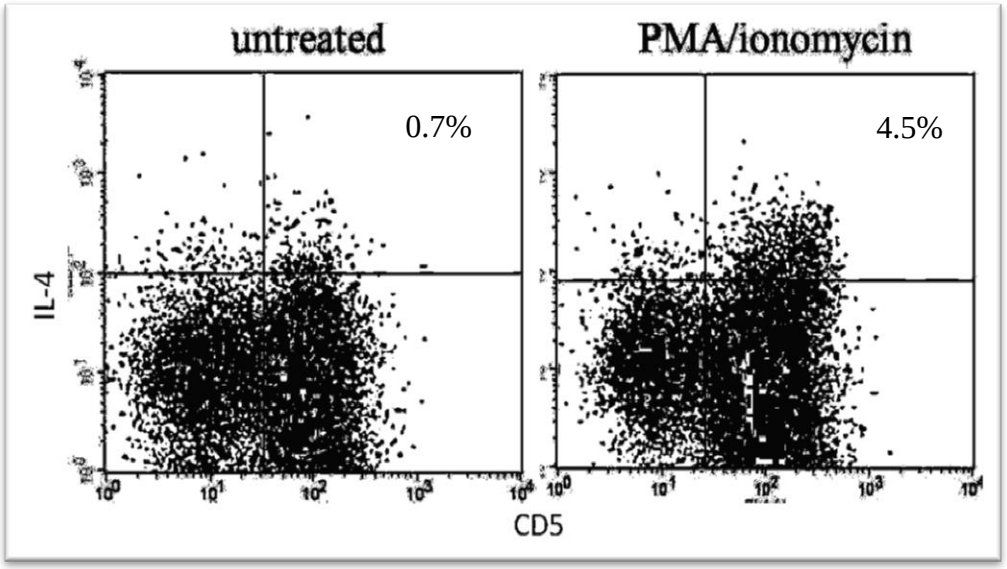


Figure 6. Flow Cytometry Analysis of Intracellular IFN-γ in Lymphocytes from Fresh Whole Blood Following PMA-I Activation. Fresh whole sheep blood cultured for 4 hours in medium in the presence of Brefeldin A (B) or stimulated with PMA-ionomycin in the presence of Brefeldin A (C), was stained with anti-bovine IFN-γ:FITC (IgG1) antibody or stimulated with PMA-ionomycin in the presence of Brefeldin A was stained with mouse IgG1:FITC isotype control (A) antibody.



A



B

Figure 7. Flow Cytometry Analysis of Intracellular IL-4 in CD5+ Lymphocytes from Fresh Whole Blood Following PMA-Ionomycin Activation.

(Figure 7A) Fluorescence (IL-4) vs. frequency histogram shows the distribution of gated peripheral blood $CD5^{+}$ lymphocytes from fresh whole sheep blood cultured for 4 hours in medium in the presence of Brefeldin A (II) or stimulated with PMA-ionomycin in the presence of Brefeldin A (III), was double stained with anti-bovine interleukin-4:RPE (IgG2a) and anti-bovine CD5:FITC (IgG1) antibodies or stimulated with PMA-ionomycin in the presence of Brefeldin A was double stained with mouse IgG2a:RPE isotype control and anti-bovine CD5:FITC (IgG1) antibodies (I).

(Figure 7B) A dot plot of fresh whole sheep blood cultured for 4 hours in medium in the presence of Brefeldin A or stimulated with PMA-ionomycin in the presence of Brefeldin A, was double stained with anti-bovine interleukin-4:RPE (IgG2a) and anti-bovine CD5:FITC (IgG1) antibodies.

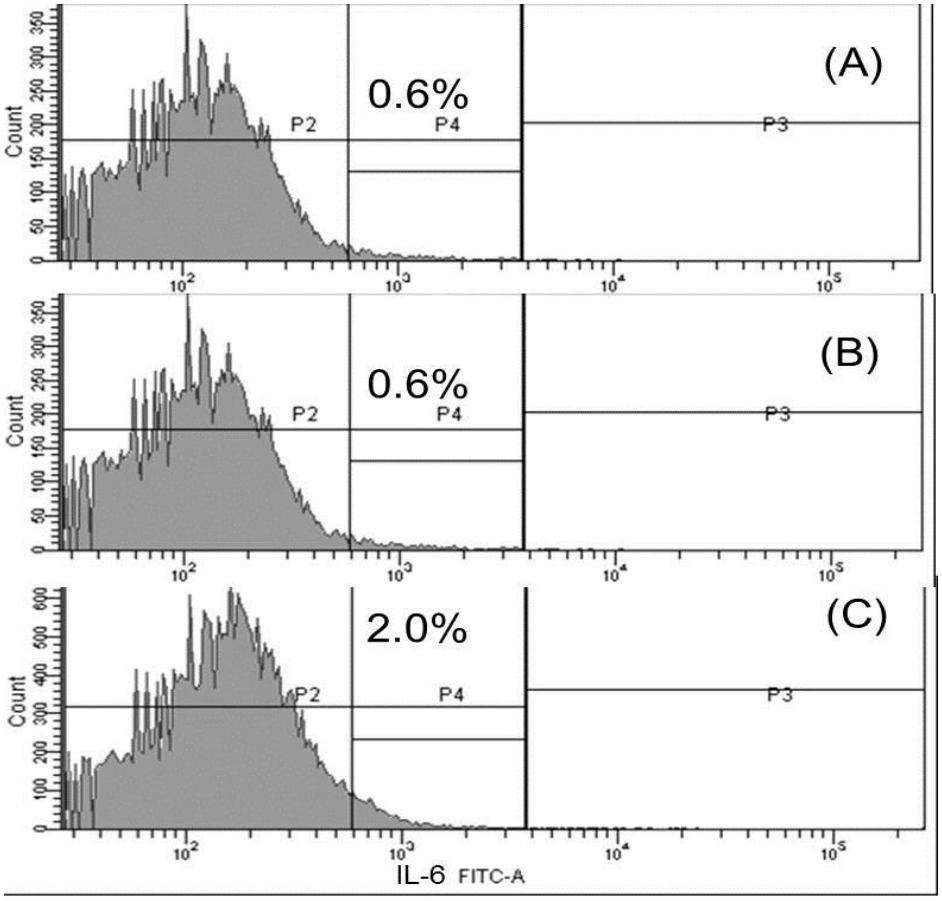
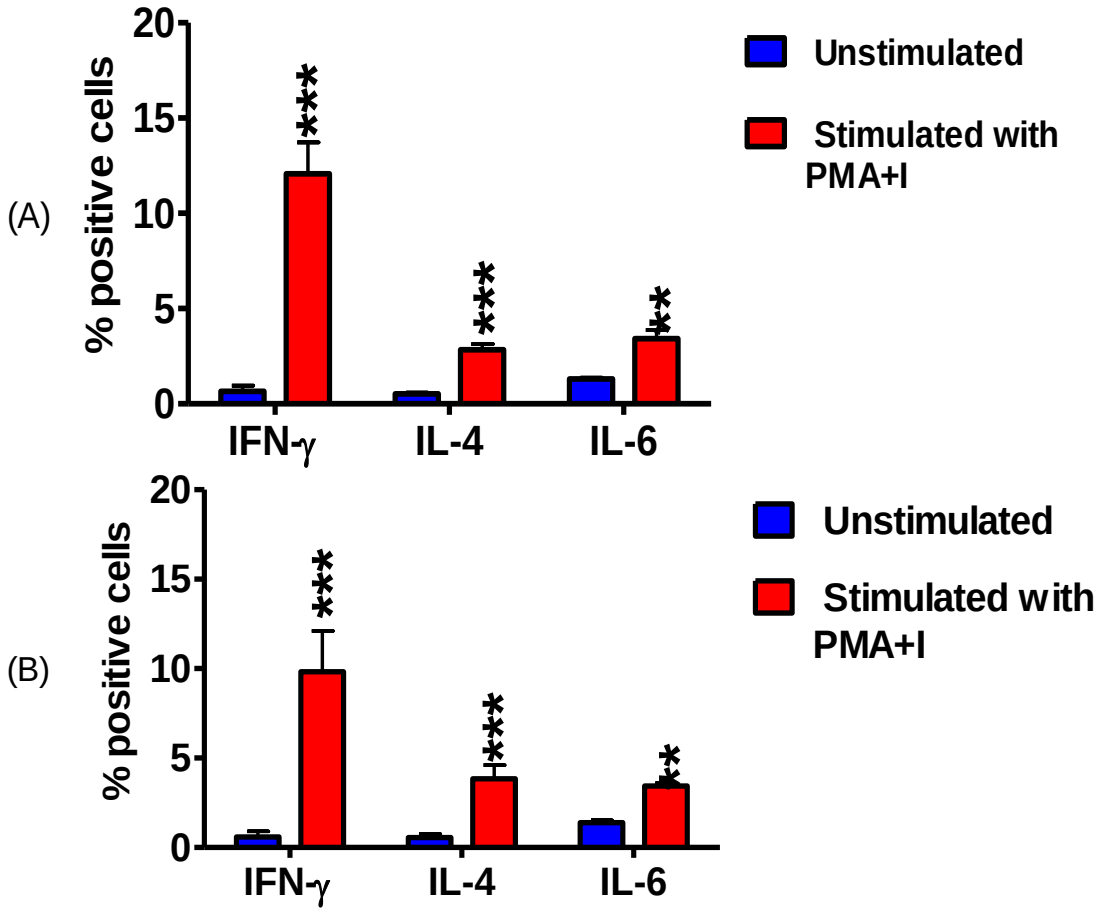


Figure 8. Flow Cytometry Analysis of Intracellular IL-6 in Lymphocytes from Fresh Whole Blood Following PMA-Ionomycin Activation. Fresh whole sheep blood cultured for 4 hours in medium in the presence of Brefeldin A or stimulated with PMA-ionomycin in the presence of Brefeldin A was stained with the mouse anti-ovine interleukin-6 (IgG1) antibody and polyclonal rabbit $F(ab')_2$ anti-mouse IgG:FITC antibody or stimulated with PMA-ionomycin in the presence of Brefeldin A was stained isotype control mouse IgG1 (IgG1) and polyclonal rabbit $F(ab')_2$ anti-mouse IgG:FITC (A).

The Effects of Sample Processing on Immune Function as Shown by Cytokine Responses of Lymphocytes

Cytokine Profiles for both Stimulated and Unstimulated Lymphocytes in PBMCs and Fresh Whole Blood

The percentages of cytokine-positive lymphocytes in fresh PBMCs and fresh whole blood after 4 hours incubation time in the 3 sheep studied are shown in Figures 9A and 9B. The synthesis of IFN- γ ($p<0.001$) and IL-6 ($p<0.01$) were significantly enhanced in PMA+Ionomycin (PMA+I) stimulated lymphocytes compared to unstimulated lymphocytes. The synthesis of IL-4 ($p<0.001$) was significantly enhanced in PMA+I stimulated $CD5^+$lymphocytes compared to unstimulated $CD5^+$lymphocytes.



Figures 9A and 9B. Cytokine Profiles for Fresh PBMCs (9A) and Fresh Whole Blood (9B). Percentages of IFN-γ and IL-6 positive sheep lymphocytes or IL-4 positive sheep $CD5^+$lymphocytes which were either resting or PMA+Ionomycin stimulated after 4 hours in culture. Values represent the mean ± SD from 3 different sheep from three independent experiments. Statistics were performed on n=3 mean values ± SD where n is an independent experiment. * Represents a significant difference of treatment groups compared to its corresponding control i.e. untreated * $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Cytokine Profiles for both Stimulated and Unstimulated Lymphocytes in Frozen Blood

The percentages of cytokine-positive lymphocytes in frozen sheep blood after 4 hours incubation time in the 3 sheep studied are shown in Figure 10A. Significantly enhanced IFN-γ synthesis was found for the PMA+I stimulated lymphocytes ($p<0.001$) compared to unstimulated lymphocytes in all 3 sheep studied. When using the mean values of IL-4 expression from the PMA+I stimulated lymphocytes for the 3 sheep, there was a p value just above significance ($p=0.057$). However, there was a significant increase in IL-4 positive lymphocytes following stimulation compared to the unstimulated lymphocytes in each of the 3 sheep measured 401 ($p<0.01$), 440 ($p<0.05$), 642 ($p<0.01$) as shown in Figure 10B. Surprisingly, there was enhanced IL-6 synthesis found in the unstimulated lymphocytes from frozen blood samples compared to unstimulated lymphocytes in the fresh samples in Figure 11. This level of IL-6 synthesis could not be increased to a significant level by PMA+I stimulation in any of the sheep analysed ($p=0.078$).

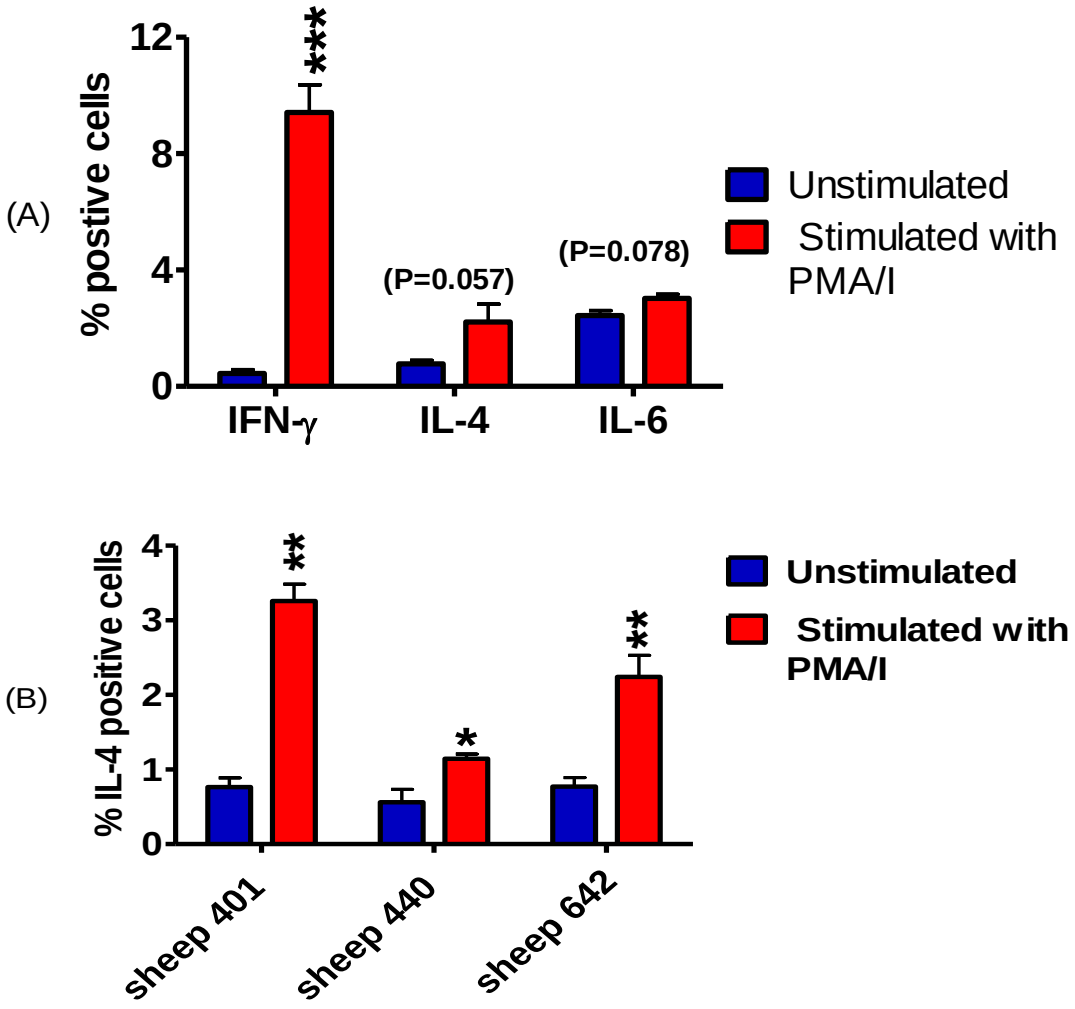


Figure 10A. Cytokine Profiles for Frozen Blood Percentages of IFN- γ and IL-6 postive sheep lymphocytes or IL-4 positive sheep $CD5^+$lymphocytes which were either resting or PMA +Ionomycin stimulated after 4 hours in culture. Values represent the mean ± SD from 3 different sheep from three independent experiments. Statistics were performed on n=3 mean values ± SD where n is an independent experiment * Represents a significant difference of treatment groups compared to its corresponding control i.e. untreated * P< 0.05 ** p< 0.01 *** p< 0.001

Figure 10B. IL-4 Levels for Frozen Blood from Three Different Sheep. Percentages of IL-4 positive resting or PMA +ionomycin stimulated $CD5^+$lymphocytes after 4 hours in culture from three different sheep. Values represent the mean ± SD from 3 independent experiments from each individual sheep. * Represents a significant difference of treatment groups compared to its corresponding control i.e. untreated * P< 0.05 ** p< 0.01 *** p< 0.001

Comparison of the Cytokine Profiles from Blood after 3 Different Sample Processing Methods

There was no significant increase in the lymphocyte or $CD5^+$lymphocyte responses to PMA+I as measured by the increase in IFN-γ or IL-4 production in fresh whole sheep blood, frozen blood and PBMCs after 4 hours in culture as shown in Figures 11A & 11B. The levels in fresh whole blood, frozen blood and PBMCs were compared and there was a significant difference between the level of IL-6 production after PMA+I stimulation in frozen blood compared to those in fresh blood ($p<0.01$) and PBMCs ($p<0.01$) after 4 hours in culture (Figure 11C).

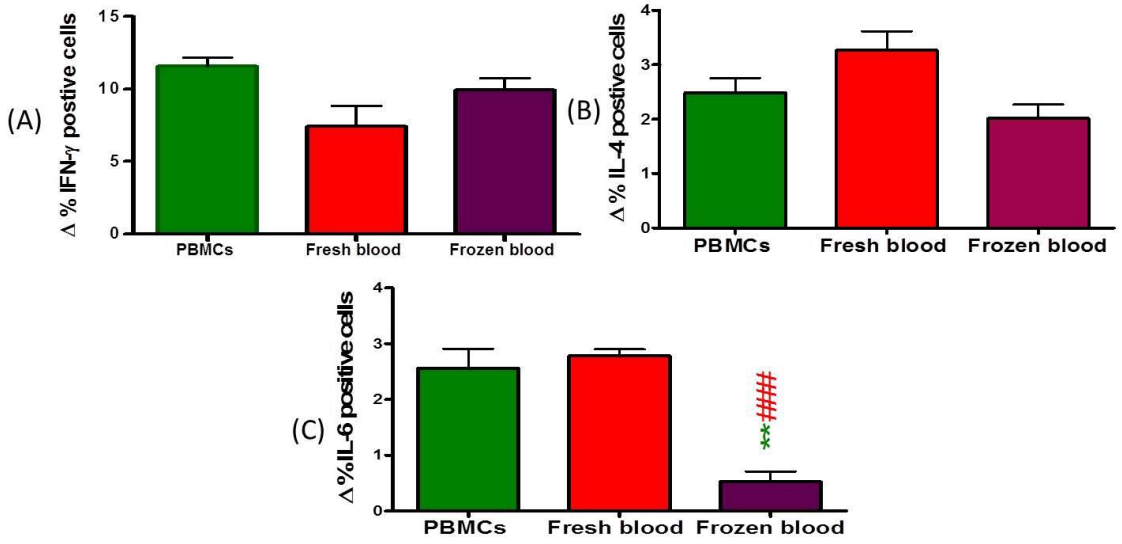


Figure 11. Comparison of Three Different Blood Sample Processing Methods in Terms of the Increase in IFN-γ, IL-6, and IL-4 Positive Cells. Data represent the Δ IFN-γ (Figure 11A) or Δ IL-6 (Figure 11B) Positive Cells (IFN-γ or IL-6 positive PMA+ionomycin stimulated lymphocytes minus the IFN-γ or IL-6 positive unstimulated lymphocytes) after 4-hours in culture. Data represent Δ IL-4 Positive **Cells** (Figure 11C) (IL-4 positive PMA+ionomycin stimulated $CD5^+$lymphocytes minus the IL-4 positive unstimulated $CD5^+$lymphocytes) after 4-hours in culture. Values represent the mean ± SD from 3 different sheep from three independent experiments. Statistics were performed on n=3 mean values ± SD where n is an independent experiment.* Represents a significant difference between PBMCs versus the other sample preparations * $P< 0.05$. # Represents a significant difference between fresh blood versus the other sample preparations $^{\#}$ $P< 0.05$.

Assessment of the Levels of IFN-γ and IL-4 Positive Cells in Frozen Blood after One Week and Six Months Storage in Liquid Nitrogen

The levels of IFN-γ and IL-4 positive cells in frozen blood taken from sheep and stored for 1 week and 6 months in liquid nitrogen are shown in Figures 12A and 12B, respectively. Significantly enhanced IFN-γ ($p<0.001$) synthesis was found for the PMA+I stimulated compared to unstimulated lymphocytes after both 1 week and 6 months storage. Similarly, significantly enhanced synthesis of IL-4 ($p<0.01$) was found for the PMA+I stimulated compared to unstimulated $CD5^+$lymphocytes after 6 months storage but there was no significant increase in IL-4 ($p<0.057$) after 1 week in storage. There was no significant difference between the amount of IFN-γ and IL-4 synthesis from stimulated lymphocytes after 1 week and after 6 month storage (Figures 12A and 12B). The measurement of IL-6 was not included in this long term storage experiment because PMA+I stimulation had no effect on this cytokine in frozen blood samples (Figure 10A).

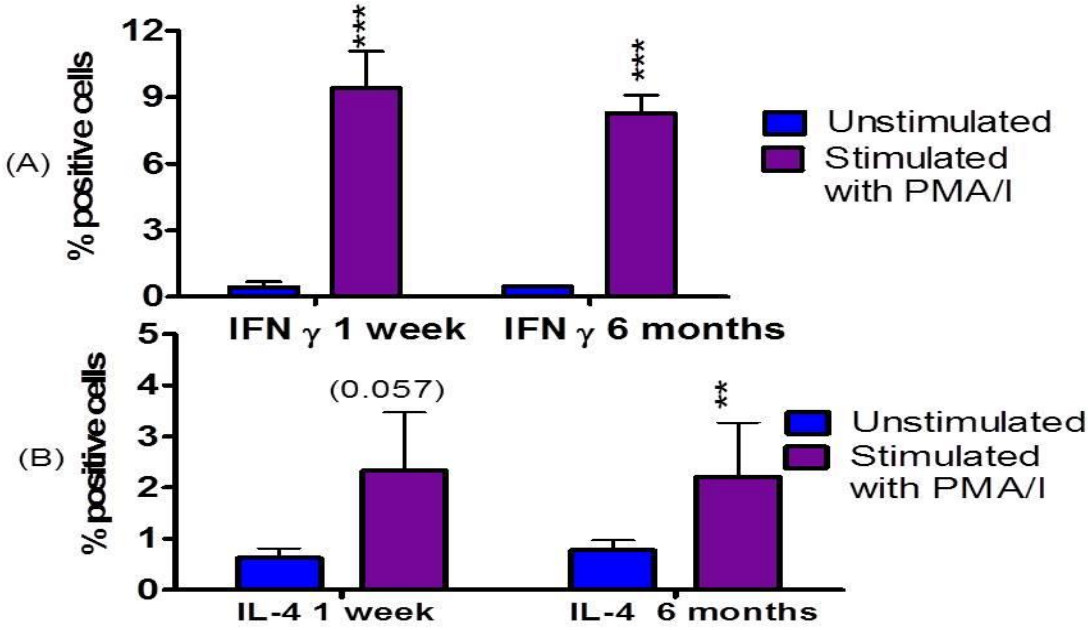


Figure 12. Comparison of the Levels of IFN-γ and IL-4 Positive Cells in Frozen Blood Stored for 1 Week and 6 Months. Percentages of IFN-γ (Figure 12A) and IL-4 (Figure 12B) positive resting or PMA+ionomycin stimulated lymphocytes or $CD5^+$PMA+ionomycin lymphocytes after 4-hours in culture. Values represent the mean ± SD from 3 different sheep from three independent experiments. Statistics were performed on n=3 mean values ± SD where n is an independent experiment. * Represents a significant difference of treatment groups compared to its corresponding control i.e. untreated * $P< 0.05$ ** $p< 0.01$ *** $p< 0.001$

Discussion

The effects of cryopreservation on leukocyte subpopulations and immune function parameters in sheep blood were examined in this study. These methods were chosen as the focus of the current investigation as they can evaluate immunotoxicological effects of chemical exposure. Both functional and non-functional assessments have been included where the non-functional tests normally provide information about changes in the subpopulation distribution of different leukocytes, whereas functional tests, such as cytokine expression in response to stimuli, can reflect the nature of potential paracrine interactions among lymphocytes (Muller-Steinhardt et al., 2009). Taken together, these analyses should provide an insight into the effects on the immune system of animals.

In the first approach to the study of the sheep immune system, cell surface marker analysis was used to assess the proportions of lymphocytes and granulocytes as well as lymphocyte subsets (T and B) in fresh and frozen blood. Fluorescent antibodies were used to identify granulocytes ($CD11^{high}$), lymphocytes ($CD11^{low}$) in samples by flow cytometry. This method of quantification of leukocyte subsets in sheep blood gave similar profiles to those obtained with automated haematology analysis, the standard method. In addition, the distribution of T lymphocytes (CD5) and B lymphocytes (IgM) was analysed in the lymphocyte population, information which cannot be obtained from automated haematology analysis.

$CD5^+$lymphocytes (T cells) are the major lymphocyte subpopulation and occur at values ranging from 45 % to 65 % in the peripheral blood of mammalian species (Mackay et al., 1985, Birkebak et al., 1994, Brodersen et al., 1998, Boppana et al., 2004). In this study, the value for $CD5^+$lymphocytes (60 %) detected in fresh whole sheep blood is very similar to those reported in two previous studies in sheep (Mackay et al., 1985, Boppana et al., 2004).

Similar variations in the number of IgM^+lymphocytes (B cells) in the peripheral blood of the sheep are reported in the literature. In this study, 29 % of IgM^+lymphocytes were observed in fresh whole sheep blood whereas previous published values were at a range of 30 % to 39 % of ovine peripheral blood lymphocytes (Mackay et al., 1986, Birkebak

et al., 1994, Smith et al., 1994). Surface immunoglobulin measurement may be a relatively insensitive method for the determination of lineage specificity in other parts of the body, since only mature B-lymphocytes express immunoglobulins, but for identification of B lymphocytes in the peripheral blood of sheep it is the most accurate method (Mackay et al., 1986).

Controlled-rate cryopreservation significantly reduced the percentage of granulocytes in the blood samples in this study and enriched the percentage of lymphocytes in the frozen blood preparations but did not cause substantial changes in the proportions of T and B lymphocytes. Therefore, frozen blood, when processed according to the method described in this study, does not reflect the fresh whole blood sample due to this decreased granulocyte and red blood cell distribution. Previous studies reported that the surface properties of neutrophilic granulocytes were affected by the cryopreservation procedure and lose their surface markers, such as CD11a, and this affects their function (Schindler et al., 2004). However, in this study the percentage of granulocytes was reduced when measured by the flow cytometer parameters forward scatter and side scatter as well as by CD11a staining.

There have been a number of studies in the literature regarding the effect of storage of blood samples on lymphocyte subset distribution. These have highlighted the fact that the way in which blood samples are stored can have a marked effect on the results obtained (Grunow et al., 1976, Dzik and Neckers, 1983, Shield et al., 1983, Weiblen et al., 1983, Bongers and Bertrams, 1984, Nicholson et al., 1984, Prince and Arens, 1986, Ashmore et al., 1989, Ekong et al., 1992, Ekong et al., 1993). However, controlled rate cryopreservation does not cause changes in lymphocyte subset proportions (Callery et al., 1980, Glassman and Christopher, 1984, Kawai et al., 1988, Letellier et al., 1991, Truax et al., 1993, Sobota et al., 1997) and this is in agreement with the findings reported in this study.

In contrast to the absence of change in the lymphocytes expressing CD5 or IgM in fresh and frozen blood in this study after 3 hours in culture, Lloyd et al., (1995) found that the percentage of sheep lymphocytes expressing CD4, CD5 and CD8 markers were all decreased significantly after freezing. Other studies have found a reduction in $CD4^+$ and $CD5^+$lymphocytes in sheep after a 24 hours culture (Lloyd et al., 1995). Differences in

the methods of cryopreservation as well as the samples used (frozen whole blood vs. frozen leukocytes) may explain the inconsistencies

The characterisation of the CD cell surface antigens on leukocytes, granulocytes, T lymphocytes and B lymphocytes in both fresh and frozen sheep blood samples provides the opportunity to monitor host responses either straight away after collection or after long term storage. A number of environmental contaminants can have detrimental effects on the immune system. Some of these contaminants may influence the progression of autoimmune disease through either immune activation or suppression. The ability of contaminant exposures to affect autoimmunity appears to depend on genetic susceptibility, the duration of exposure, the timing of the exposure and the dose of the contaminant involved (Inadera, 2006). Immunophenotyping of sheep blood leukocytes using limited numbers of CD antigens enables the differentiation states of the immune system cells to be determined. In future, more extended phenotyping involving parallel and/or simultaneous measurement of multiple CD antigens may help identify expression pattern signatures associated with perturbations of host immunity, pathogenesis, disease progression, or specific environmental contaminant exposures.

Sodium heparin (NaH) was the anticoagulant of choice for the studies in Chapters 2-5 and the majority of this chapter. Potassium EDTA (KEDTA), an alternative anticoagulant which is extensively used in clinical and veterinary settings, was included in these investigations to determine the effects of anticoagulant on cell viability and leukocytes profile. When NaH was used as the anti-coagulant, the cell viabilities of greater than 90 % for the leukocytes, total lymphocytes and B lymphocytes and the viability of 85 % for T lymphocytes after 3 hours in culture in frozen blood are consistent with the findings of Anderson et al, (1996), Cheng et al, (2001) and Stevens et al, (2007). When KEDTA was used as the anti-coagulant, T lymphocytes and B lymphocytes had cell viabilities of 80 %, whereas the viabilities of leukocytes and total lymphocytes were 83 % post thawing at 3 hours and significantly lower than when NaH was used. However, while the viability of the granulocytes (70 %) was lower than that of lymphocytes, there was no significant difference between the anticoagulants used. However, in a further study, no differences were detected in cell viability and immune function when EDTA, acid citrate dextrose (ACD) and sodium heparin were used as

anticoagulants in the collection of blood samples for the cryopreservation of PBMCs (Bull et al., 2007) However, Bull et al., (2007) measured the viability of total PBMCs and the viability of different leukocyte subpopulations were analysed in this study.

The effects of ethyl methanesulfonate (EMS) and cadmium chloride ($CdCl_2$) on the different leukocyte subpopulations of frozen blood collected in two different storage tubes were investigated. When frozen blood samples were collected in NaH tubes, the addition of EMS reduced viability in a dose–dependent manner in all leukocyte subsets. The 5 mM dose caused a significant reduction in the viability of granulocytes and B lymphocytes. The frozen blood samples that were collected in KEDTA tubes proved more sensitive to EMS exposure and both the 2.5 mM and 5 mM doses caused significant reductions in the viability of granulocytes, total lymphocytes, T lymphocytes and B lymphocytes with the latter again proving the most sensitive cell type. In NaH tubes, at a concentration of 500 μM, $CdCl_2$ caused a significant reduction in the viability of granulocytes and B lymphocytes in frozen blood samples that were exposed for 3 hours. In contrast, in KEDTA tubes, at concentrations of 333 and 500 μM, $CdCl_2$ caused a significant reduction in the viability of granulocytes, total lymphocytes, T lymphocytes and B lymphocytes after exposure for 3 hours.

The reduction in the viability of T and B lymphocytes but not the viability of the gated lymphocytes was not explained by insufficient gating or sensitivity/specificity of antibodies staining as shown in Figure 1. A possible explanation could be the presence of another population in the lymphocyte gate which are both CD5 and IgM negative. Recent studies identified a cell population which is 10% of ovine peripheral blood lymphocytes and characterised by the cell surface markers $NKp46^{+}CD16^{+}CD14^{-}$ $CD5^{-}$ IgM^{-} and has the morphological and functional characteristics of NK cells (Elhmouzi-Younes et al., 2010, Connelley et al., 2011).

Overall, NaH appears to be a better anticoagulant for immunophenotyping of leukocytes in frozen blood since KEDTA has a greater effect on the cell viability and causes a greater sensitivity to DNA-damaging agents as observed in this study. What was also interesting was that B lymphocytes were the subpopulation that were most sensitive to EMS and $CdCl_2$. B-cell chronic lymphocytic leukaemia may arise following environmental exposures with chemicals or radiation (Shim et al., 2007). Rats exposed

in vivo to $CdCl_2$ had decreased B lymphocytes in peripheral blood with doses of 5 and 10 ppm whereas doses from 25 to 100 ppm increased B lymphocytes (Lafuente et al., 2004). When human whole blood was treated with doses as low as 0.3μM $CdCl_2$ for 12 hours *in vitro,* T lymphocytes produced higher levels of metallothionein, a cadmium binding protein, than did B lymphocytes (Yurkow and Makhijani, 1998). The lower level of this protective protein in B lymphocytes could possibly account for the greater sensitivity of B lymphocytes to $CdCl_2$ compared to T lymphocytes. In addition, doses as low as 0.1μM $CdCl_2$ inhibited IgE synthesis in human B lymphocytes after 96 hours in culture (Marth et al., 2001). All of these effects could be due to the induction of reactive oxygen species and apoptosis in B lymphocytes by $CdCl_2$ leading to cell death (Nemmiche et al., 2012).

In order to obtain sufficient sensitivity in the measurement of the intracellular cytokines, IL-4, IL-6 and IFN-γ, at the single-cell level by flow cytometry, it was necessary to cultivate purified PBMCs or whole blood and culture them in the presence of the strong cellular stimulants, phorbol 12-myrisate 13-acetate (PMA) and ionomycin (I), combined with Brefeldin A blockage of protein transport from the endoplasmic reticulum to the Golgi complex (Foster et al., 2007).

The suitability of using gates with forward scatter and side scatter to measure the cytokine levels in lymphocyte populations was verified using CD11a staining. Both IL-4 and IFN-γ are mainly produced by T lymphocytes, which are the majority (60 %.) of the lymphocytes population. However, since IL-4 was produced by a low percentage of lymphocytes in healthy animals, the cells were also stained with the pan T cell marker (CD5) to increase the detection levels. Since IFN-γ is produced at a much higher level it was not as necessary to stain for CD5. Double staining of both IFN-γ and CD5 was not possible at the time of the study due to the limited number of fluorochromes conjugated to antibodies commercially available for sheep.

As expected, only barely detectable levels of spontaneous lymphocyte cytokine production were observed in unstimulated whole blood and PBMCs for all 3 cytokines. The addition of PMA+I significantly enhanced the synthesis of IL-4, IL-6 and IFN-γ compared to the levels found in lymphocytes treated only with Brefeldin A (BFA). These results are in agreement with observations reported for sheep PBMCs (Weynants et al., 1998, McWaters et al., 2000, Pedersen et al., 2002) but this is the first study to

report successful induction of these cytokines in fresh whole and frozen sheep blood. In this study, the level of cytokine production was comparable for fresh whole blood and PBMCs when PMA+I stimulation acted directly on lymphocytes. In contrast, previous studies which used different stimuli, such as mitogens or pyrogens, reported a difference in level of cytokines between fresh whole blood and PBMCs. One possible reason for this difference could be the culture composition, since whole blood cultures contain large numbers of erythrocytes and granulocytes that are more vulnerable to sample processing than lymphocytes and monocytes and die rapidly, producing debris and other substances that may disturb and inhibit the function of lymphocytes (Gaines et al., 1996, Baran et al., 2001). PMA+I are potent stimulators of IFN-γ in both PBMCs and whole blood cultures (Prussin, 1997, Rostaing et al., 1999). Stimulated cultures from adult humans revealed a biphasic kinetic production pattern for IFN-γ with peaks occurring 6 and 24-30 hours after initiation of the cultures and approximately 20 % of the lymphocytes produced IFN-γ (Andersson et al., 1990), which was higher than the amount produced by either fresh whole blood or PBMCs after 4 hours stimulation in this study. The amount of PMA+I induced IFN-γ^+ lymphocytes in frozen blood and fresh whole blood were comparable at 9.5 % and 10.5 %, respectively and slightly lower than the 12 % observed in PBMCs. These findings are in agreement with Kilani et al., (2005) who reported that the levels of IFN-γ^+ lymphocytes detected after PMA+I activation were very similar for fresh and frozen PBMCs. Likewise, other studies are in agreement with our findings and showed no difference in IFN-γ secretion in frozen PBMCs compared to fresh PBMCs, even when using different freezing methods (Knight et al., 1972, Friberg et al., 1994, Venkataraman, 1995, Sobota et al., 1997, Wang et al., 1998, Smith et al., 2001).

One of the main advantages of having a well characterised intracellular detection method of IFN-γ production in domestic animals is that it can be used as a more rapid and robust indicator of general immunocompetence. IFN-γ is an important component of the host immune response to common intracellular pathogens in sheep including *Chlamydophia abortus* and *Toxoplasma gondii* or viruses i.e. Foot and Mouth Disease Virus (FMDV) and so the ability to detect this marker in the plasma or serum or in an *ex vivo* whole blood assay could enhance the ability to reveal the presence of pathogens (Graham et al., 1995, Innes et al., 1995) and viruses (Shtrichman and Samuel, 2001, Parida et al., 2006, Billiau and Matthys, 2009) as measuring of IFN-γ can be effect

specific marker for infection. IFN-γ has antiviral activity against FMDV (Zhang et al., 2002a, Zhang et al., 2002b, Moraes et al., 2007, Summerfield et al., 2009) and also promotes natural killer and macrophage activation, which are likely to contribute to controlling FMDV replication and spread within the host (Summerfield et al., 2009). The methods used in this chapter could be transferred to cattle where the IFN-γ secretion assay has been used for measuring the immune modulation associated with bovine tuberculosis (Wood and Rothel, 1994), bovine brucellosis (Weynants et al., 1995) and Johnes disease (Billman-Jacobe et al., 1992) . The intracellular production of IFN-γ was measured by Antas et al., (2004) to monitor cattle for the above infections.

IL-4 is the Th2 cytokine and is typically produced in smaller quantities than IFN-γ in human lymphocytes (Jung et al., 1993) and is only produced in a very small percentage of lymphocytes from healthy animals (Weynants et al., 1998, Sopp and Howard, 2001). As discussed earlier, PMA+I induce Th1 cytokine (IFN-γ) profiles, whereas PHA stimulated both the Th1 and the Th2 cytokines (IL-4) profiles, although with different kinetics (Baran et al., 2001). In this study, the 4 hours in culture time point was chosen for all samples because the activation of lymphocytes after PMA+I stimulation was rapid and robust at this time and lymphocyte viability is unchanged but decreased in the case of frozen samples beyond 4 hours.

The levels of PMA+I induced IL-4^+ lymphocytes expressing CD5 in fresh whole blood and PBMCs were comparable at 3.5 % and 2.75 %, respectively. This is in agreement with reported values for which the maximum level of IL-4^+lymphocytes induction was after 6 hours of PMA+I stimulation (Andersson et al., 1990). The level of induction of PMA+I induced IL-4^+ lymphocytes expressing CD5 in frozen blood was lower at 2.3 % but it was not significantly different from fresh whole blood and PBMCs. These findings are in agreement with Kilani et al., (2005) who reported that the levels of IL-4^+ lymphocytes were similar between fresh and frozen human PBMCs. However, the values reported were less than 1 %, considerably less than those in this study. It is noteworthy that Kilani et al., (2005) analysed total lymphocytes rather than the $CD5^+$ cells (T lymphocytes) which were analysed using double staining in the present study and which would be the major producers of IL-4. In addition, Kilani et al., (2005) used a longer stimulation time of 16 hours which is not optimum for IL-4 production. Likewise other studies are in agreement with our findings that there was no change in the levels of

IL-4 produced in fresh and frozen PBMCs following stimulation with mitogens (Kreher et al., 2003) or specific antigens (Grogan et al., 1998). In contrast with our results and the other studies described above, a decrease in IL-4 secretion in frozen PBMCs compared with fresh PBMCs following auto antigen and allergen induced secretion was shown by Bailey et al., (2002) and Kvamstrom et al., (2004). Suboptimal freezing conditions for the activated cells may explain the decrease in IL-4 production in these studies.

The availability of a reproducible method to measure IL-4 production in peripheral blood is beneficial as the assessment of this cytokine in human clinical trials and molecular epidemiology of environmental health has facilitated a better understanding of the mechanisms that underlie associations between environmental exposures and immune-mediated disorders, such as asthma, and atophy (Heaton et al., 2005, Duramad et al., 2006, Robroeks et al., 2006). IL-4 can also be used to measure the immune modulation of livestock infected with Fasciola *hepatica*, a prevalent helminthic parasite. The immune response to *F. hepatica* is skewed toward type 2 helper (Th2) dominance, characterized by interleukin-4 (IL-4) production (Brown et al., 1994) and its infection down regulates Th1 responses in mice (O'Neill et al., 2000).

While IL-6, a pro-inflammatory cytokine, is generally not produced constitutively in lymphocytes or monocytes, it can be upregulated by a number of factors including viral infection, lipopolysaccharide (LPS) and cytokines such as IL-1β, tumour necrosis factor-alpha (TNF-α), IFN-γ and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Van Snick, 1990). However, all these stimulations require longer incubation times than those used in the present study. For example, stimulation with PMA+I for 24 hours increased the production of IL-6^{+}lymphocytes to 40 % in sheep PBMCs (McWaters et al., 2000).

The levels of PMA+I induced IL-6^{+}lymphocytes in PBMCs and fresh whole blood were 3 % while the level in frozen blood was 2 %. The level of IL-6^{+} lymphocytes in unstimulated lymphocytes was much higher than that in PBMCs and fresh whole blood and could not be increased further with PMA+I treatment. These findings are in agreement with previous studies that reported the level of IL-6 in frozen T lymphocytes was elevated following cryopreservation (Venkataraman, 1994, Wang et al., 1998). This observation could be explained by the fact that IL-6 is produced by a variety of cells

with varying degrees of sensitivity to freezing and thawing. Moreover, the larger quantities of IL-1 and IL-2 secreted by the frozen, mitogen activated cells (Venkataraman and Westerman, 1990, Venkataraman, 1992) might also enhance the IL-6 secreting activities of the frozen cells, because these cytokines are known to induce IL-6 production in the peripheral blood monocytes and T lymphocytes secrete IL-6 in a monocyte dependent manner (Horii et al., 1988, Nakajima et al., 1989, Tosato and Jones, 1990).

When frozen blood was stored for 1 week or for 6 months and then stimulated with PMA+I after thawing, there were comparable levels of IFN-γ and IL-4^{+}lymphocytes after 4 hours in culture. The results of this study suggest that whole sheep blood can be cryopreserved for as long as 6 months without losing its capacity for activation or altering the proportion of Th1 and Th2 cytokines. These findings are in agreement with other observations which suggest that human PBMCs can be cryopreserved without compromising the ability of the cells to respond to activation signals for up to 1 week (Sobota et al., 1997) and to 12 months (Kilani et al., 2005) of cold storage. Previous studies have shown that the freezing and thawing processes, and not the time of storage, exert the main effects on the lymphocyte response (Wang et al., 1998, Kleeberger et al., 1999, Smith et al., 2001).

When frozen blood samples were exposed to EMS and $CdCl_2$, the gentotoxic effects, and the effects on different leukocyte subpopulations were examined. However the production of cytokines after PMA/I stimulation of frozen blood exposed to EMS and $CdCl_2$ was not studied. To do so, would require either *in vivo* or *in vitro* chemical exposure before PMA/I stimulation and neither of these scenarios was feasible. Chemical exposure *in vivo* was outside the scope of this project and *in vitro* exposure would require prolonged culture which would lead to a loss in viability of the frozen blood samples.

Profiling the intracellular cytokine production after stimulation in sheep PBMC's, fresh whole and frozen sheep blood, offers a very important technique for monitoring host responses and general immunocompetence. The IFN-γ and IL-4 assays can be performed on sheep that could possibly carry a disease or that have been subjected to environmental exposures either straight after blood collection or after long term storage. What is beneficial about the intracellular lymphocyte cytokine staining by flow

cytometry is that more cytokines can be included in the future and also multiple cytokines from specific lymphocyte subsets can be analysed concurrently, providing a quantitative and phenotypic assessment of responding lymphocytes. Eventually this technique, in combination with immunophenotyping, could become part of routine analysis in clinical or veterinary laboratories. Also, the recent availability of mobile flow-cytometer units may allow the use of this assay under field investigation conditions instead of having to bring the blood samples back to the laboratory (Mbopi-Kéou, 2012).

Main Conclusions

This study demonstrated that the analysis of lymphocyte subsets can be successfully undertaken on frozen sheep blood as there were no alterations following the freezing and thawing process compared to fresh sheep whole blood. These observations are of prime interest due to the amount of clinical investigations taking place on cryopreserved lymphocytes which have been stored and this allows all the blood samples to be analysed in one batch to reduce variability. One can analyse lymphocyte subsets without the need for prior isolation, as in the case of PBMCs, while maintaining the lymphocyte subpopulation proportions similar to those of fresh whole blood. However, cryopreservation of sheep blood led to a major decrease in the amount of granulocytes present compared to fresh whole blood as shown by a reduction in $CD11a^{high}$ cells as well in the viability of the these granulocytes. The different anticoagulants blood tubes were shown to alter the sensitivity of leukocytes from frozen blood to EMS and $CdCl_2$. B lymphocytes seemed to be the most sensitive cell type to these genotoxic compounds especially when the frozen blood was collected in potassium EDTA tubes. Future studies could assess the T lymphocyte subpopulations of sheep such as $CD4^{+,}$ $CD8^{+}$ and gamma delta T lymphocytes, as well as some subsets of $CD4^{+}$ such as T regulatory cells and T helper 17 cells, in frozen sheep blood compared to fresh blood.

In this chapter, the merits of a number of sample preparations after short term culturing with PMA+I and their cytokine responses were assessed. It was demonstrated that four hour stimulations can be undertaken without considerable alterations to the levels of 2 out of 3 cytokines (IFN-γ, IL-4) produced by lymphocytes after different sample processing methods described (PBMCs, fresh whole blood and frozen blood). This

demonstrates the importance of investigating the effects of freezing for each cytokine and stimuli before using frozen cells in studies of *in vitro* cytokine secretion. The characterization of cytokine production at the cellular level after short-time stimulation by flow cytometry should be beneficial for the study of functional aspects of ovine lymphocytes and cytokine patterns of specific immunological reactions.

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Chapter 7

General Discussion& Conclusions

The overall aim of this study was to explore and develop biomarkers which are suitable for biological effect monitoring of domestic farm animals related to environmental exposure. These biomarkers would ideally be used for the detection of different biological or toxicological effects. They should be biomarkers of effect and would not necessarily be compound specific as the composition and degree of contamination that occurs in many cases of environmental exposure is unknown and may involve a range of compounds with different biological/toxicological properties. Biological effect monitoring should ideally identify some molecular and cellular alterations that occur before serious health incidences are apparent in the domestic farm animals that are grazing close to factories and other manufacturing industries. The benefit of this approach is that the knowledge of individual compounds and their concentrations is not required and this might also provide information on the interaction of chemicals related to the endpoint in question. The sheer scope of environmental contamination and its effects on the flora and fauna highlights the need for a multi-faceted approach to monitoring of animals involving environmental, biological and biological effect monitoring.

Environmental and biological monitoring investigations of possible chemical exposures in Ireland in Askeaton Co. Limerick, Castlecomer Co. Kilkenny and the Silvermines area in Co. Tipperary, were unable to identify the cause of poor animal health. In addition to the possible presence of chemicals, there are other explanations for the changes in animal health which are equally valid, including infections and the poor nutritional status of the soils (EPA, 2002, Jorgensen et al., 2009, Li et al., 2011, Qin et al., 2011). However, the introduction of biological effect monitoring should be considered as an option for the future to investigate the impact of environmental exposure on animals that have infections or health problems.

Sheep were the environmental sentinels chosen in this study for practical and economic reasons and because they are considered to be suitable environmental bioindicators (Parada and Jaszczak, 1993, Rubes et al., 1997, Stahl Jr, 1997). In addition, some exposure specific biomarkers are common to sheep and humans and can be detected by antibodies and other biochemical and cellular tools which are commercially available. Blood samples can be easily obtained from sheep as they are relatively small and, therefore, easy to house and to handle and more economical than cattle. However, many

of the methods described in this study could easily be applicable to the monitoring of other domestic animals. This could lead to more economical methods of biomonitoring as the animals can be monitored repeatedly without requiring their destruction. Although quantitative studies that describe the biological effect monitoring of environmental exposure of sheep are rare, they have been used to monitor the bioavailability and bioaccumulation of chemical contaminants and integrate exposures across their entire environment (van Kampen, 1969, Pistl, 2002, Mohammad, 2007, Duffy, 2009).

Both invasive (blood) and non-invasive (urine, exhaled air, hair, skin, saliva and milk) biological sampling can be used for measuring biomarkers. Blood was the sample of choice for this study after the pros and cons of different biological samples were considered. Urine sampling cannot be controlled easily in animals as they do not excrete in a regular fashion. The grazing of sheep in pastures instead of enclosed housing renders breath sampling obsolete as the detection of the relatively low concentrations of environmental contaminants in the lung would require analytical sensitivity which is not common place at the present. Hair, milk and saliva have also been used for biological effect monitoring in humans, but the biomarkers available are limited (Timchalk et al., 2004, Esteban and Castano, 2009).

After exposure to chemicals, chemical specific or effect specific biomarkers can be monitored at the cellular, protein and gene level in peripheral blood lymphocytes (Liew et al., 2006). Biochemical and haematological parameters are measured in blood and blood serum, respectively, in animals as part of routine clinical diagnosis. However, it can be argued that many of the biochemical parameters in the blood are too non-specific to be used as biomarkers as the alterations of these parameters are not specific to animals which have been exposed to chemical contaminants in the environment but are known to be changed in the case of infection, disease and a number other factors. Therefore, it is useful to measure other biomarkers in the blood including, genotoxicity markers, immune markers and enzyme markers, that are related to specific biochemical responses to chemical exposure. Enzyme/protein markers related to compound specific responses have used extensively in laboratory-based toxicological studies in which the levels of these proteins have been measured in specific organs such as the kidneys and liver. Since this normally requires the destruction of the animals, another alternative

would be the use of peripheral blood lymphocytes. However, the levels of cytochrome P450 1A (CYP1A) and glutathione S-transferases (GST) activity and the expression of metallothioneins (MT) in peripheral blood lymphocytes are low and extremely difficult to measure compared to their measurement in microsomes of the liver and in the kidneys and also there is considerable inter-individual variation in expression and inducibility (van Duursen et al., 2005, Chang et al., 2009, Sharma et al., 2013). The immune and genotoxicity biomarkers would be more effect specific and hence were the markers of choice in this study

The implementation of large scale field-based biomonitoring effect studies has been hindered by the problems of sample collection, transport, and storage, especially when attempting to collect multiple blood samples from a flock of sheep. It proved difficult to validate a strategy for the collection, processing and cryopreservation of PBMCs from multiple sheep blood samples because a major loss in the viability of the lymphocytes was encountered. The observation that viability was greatly reduced by delayed processing is in agreement with the findings of Kristal et al. (2005) who found that when processing time was extended, the viability of the cells was below the required 80% for *in vitro* or *ex vivo* cell culture assays.

When using isolated PBMCs, it is important to maintain a high viability of lymphocytes before measuring different biomarkers and this proved very difficult with the current methods in place. For example, if insufficient sampling is performed to detect slight changes in biomarkers, the programme will be inconclusive and not protect or effectively monitor environmental health (Lam, 2003). In this study, conditions for the optimum preparation of blood samples were established. Based on these the ideal sample preparation for field- based biomonitoring should include 1) ease of collection 2) minimum amount of sample processing time 3) suitability to perform processing in batches 4) ability for long term storage and 5) ability to be able to measure multiple endpoints at the same time. Comparison of the different types of sample preparations allowed robust conclusions as to the best sample preparation to be drawn.

Taken together, the studies in Chapters 3, 4 and 5 provide the first comparative *in vitro* characterisation of peripheral blood lymphocytes in fresh and frozen blood in terms of viability, reactive oxygen species (ROS) production, apoptosis induction and responsiveness to genotoxic compounds. When collecting field-based sheep blood

samples, fresh whole and frozen blood processing protocols are encouraging because these methodologies have additional advantages of simplicity, economy and speed and involve less sample manipulation than the isolation of PBMCs. Frozen blood, fresh blood and PBMCs show similar viability and DNA integrity levels in their lymphocyte populations after 3-4 hours in culture. Longer cultures (24 hours) of frozen blood lead to a decrease in the lymphocyte viability and a decrease in DNA integrity so biomarkers should be measured after less than 4 hours after incubation or can be potentially measured in the serum or plasma. These results are consistent with the findings of the following studies that found comparable levels of viability or DNA integrity between fresh and frozen blood or PBMC samples (Visvardis et al., 1997, Duthie et al., 2002, Hayes et al., 2002, Schindler et al., 2004, Villavicencio, 2006, Stevens et al., 2007).

The decrease in the viability of lymphocytes from frozen blood after 24 hours in culture was examined with a view to establishing factors involved in the death of cells. The findings suggest the involvement of ROS formation (associated with haemolysis during freeze-thawing) and apoptosis. The role of apoptosis in the cell death was demonstrated although this was not the only determining factor in the fate of the lymphocytes. While some reports have suggested that apoptosis represented the principal effector of cell death of frozen blood lymphocytes (Fowke et al., 2000, Maecker et al., 2005), the results of this study suggest that both ROS and apoptosis may mediate cryopreservation-induced cell death in a hierarchal manner, with ROS playing the dominant role in dealing the terminal insult to frozen blood lymphocytes, while apoptosis may occur after the initial production of the ROS and result in the cell death evident at the later time points.

In this study, a number of critical steps were included to maintain frozen blood samples of the highest quality. These included collection in the least toxic anticoagulant blood tube (sodium heparin) and the use of a slow freezing rate and thawing procedure involving a slow dilution with medium containing DNAse and 20% foetal bovine serum (v/v). Other studies indicate that the addition of antioxidants and some anti-apoptotic caspase inhibitors to the freezing media might contribute to the quality of the frozen blood (Sarkar et al., 2003, Fujita et al., 2005). A combination of these additions may be useful for the field-based biomonitoring involving the collection of large batches of samples.

In vitro models were used to investigate the response of model compounds in terms of cell death and single strand DNA damage and also their effect on specific immune cells in whole blood. While the direct acting alkylating agent, EMS, and the indirect DNA damaging agent, $CdCl_2$, represent some of the major categories of genotoxins, another group of genotoxins which could be included in further investigations would be pro-mutagens such as aflatoxin B1and benzo [a] pyrene to be used with the S9 fraction prepared from the liver of rats. These compounds were not used in this study due to time constraints. When measuring the response to genotoxic compounds, the Comet assay was used in this study to measure single stranded DNA breaks that can be caused by exposure to genotoxic compounds directly or indirectly through the formation of intracellular reactive oxygen species generated by redox sensitive metals such as copper, iron and cadmium or to UV and ionising radiations which act directly on the DNA (Polle, 1993, Schutzendubel and Polle, 2002). This technique has not been used to-date in the biomonitoring of environmental chemicals in domestic animals. However, other genotoxic biomarkers have been measured in lymphocytes from peripheral blood, including chromosome aberrations, sister chromatid exchanges, micronuclei, double stranded DNA breaks and DNA adducts to monitor chemical exposure. Although some of these biomarkers have been used in the investigations of genotoxic exposure in farm animals, there are limitations in their potential (Burrows and Borchard, 1982, Rubes et al., 1992, Perucatti et al., 2006). Low level exposure to environmental toxicants can induce an increased frequency of chromosome aberrations but this is difficult to detect statistically, probably because the noise in the system is larger than the number of compound-induced aberrations. Even where there is exposure to potent alkylating agents, only a small elevation in sister chromosomal exchange frequency can be observed (Rubes et al., 1992).

This study focused on the *in vitro* characterisation of different sample preparations after blood collection under controlled laboratory and field-based conditions because without viable lymphocytes in blood samples it would be very difficult to obtain accurate and validated measurements in *ex vivo* assays when determining the effects of animals exposed in the field (*in vivo*). The number of sheep used in the study was chosen as the minimum number of animals that could comfortably provide sufficient replicates while being easy to manage under controlled conditions on a farm close to the laboratory,

The findings in the study suggest that frozen blood may be a useful sample preparation for toxicologists involved in large biomonitoring trials and for veterinarians involved in the monitoring of the health status of animals in the case of infection and other diseases. The samples can be easily prepared and can be stored for up to 6 months in liquid nitrogen while maintaining viability, immune phenotype and function when cultured for up to 4 hours. When the relative numbers of leukocytes and T and B lymphocytes were measured in different blood sample preparations, the % of $CD5^+$ (T lymphocytes) and IgM^+ (B lymphocytes) were in agreement with the percentage of these cells reported in the literature (Mackay et al., 1985, Mackay et al., 1986, Birkebak et al., 1994, Smith et al., 1994, Dharmendra K V Boppana, 2004).

The loss of erythrocytes through haemolysis that occurred during the freeze-thawing procedure used in this study has both advantages and disadvantages in the context of biological effect monitoring. On the one hand, the extra step of red blood cell lysis is not required when antibody staining of lymphocytes is used to measure changes in cytokine or leukocyte subpopulations; however, delta-aminolevulinic acid dehydratase (ALAD) and acetylcholinesterase enzymes are measured in erythrocytes. These enzyme biomarkers have been measured in domestic animal species and are considered specific for heavy metals and pesticides, respectively. However, their activity levels show high variability even in individual animals (Ahamed et al., 2006, Askar, 2011).

There were no alterations in the levels of the T and B lymphocytes when comparing fresh and frozen blood samples. In contrast, Lloyd et al., (1995) found a reduction in $CD4^+$ and $CD5^+$ lymphocytes in frozen sheep leukocytes. Different freezing methods and sample preparations could explain the differences between the two studies. T lymphocytes have major roles in autoimmunity and allergy. Therefore, the measurement of T lymphocytes subpopulations such as $CD4^+$ (helper), $CD8^+$ (cytotoxic /suppressor) T regulatory cells, and gamma delta T lymphocytes of frozen sheep blood could be used outside the remit of environmental monitoring, for example in immune health monitoring of individuals with cancer or autoimmune disease or after exposure to pathogens or viruses.

This study provided an approach to efficiently maintain the number of IFN-γ and IL-4 positive cells after cryopreservation for up to 6 months and subsequent restimulation. These findings are in agreement with Kilani et al., (2005) who reported that the levels of

IL-4^+ lymphocytes and IFN-γ^+ lymphocytes were similar between fresh and frozen human PBMCs. Kilani et al., (2005) did not, however, measure the cytokine response in frozen blood which was the sample of choice in this study. The characterization of cytokine production at the cellular level after short-time stimulation by flow cytometry should be beneficial for the study of functional aspects of ovine lymphocytes and cytokine patterns of specific immunological reaction. Although the cell culture method can be said to be artificial, the relative comparisons of the percentage of cytokine-producing cells have been shown to reflect closely the *in vivo* health status of an animal (Langezaal et al., 2001, Langezaal et al., 2002).

One of the main advantages of having a well characterised intracellular detection method of IFN-γ and IL-4 production in domestic animals is that it can also be used to demonstrate immune modulation following infection with bacteria, viruses or parasites. The methods used in this study could be transferred to cattle in which intracellular production of IFN-γ has been used to diagnose bovine tuberculosis, brucellosis, and Johnes disease (Antas et al., 2004). In addition, intracellular detection of IL-4 was used to diagnose asthma and atophy (Heaton et al., 2005, Duramad et al., 2006, Robroeks et al., 2006) and to diagnose *Fasciola hepatica*, which is a prevalent helminthic parasite of livestock (O'Neill et al., 2000). A possible limitation of this study is that only three cytokines were measured while there are many cytokines and chemokines produced by lymphocytes which are valid choices to measure including IL-2, IL-5, IL-10, IL-13, TNF-α and GM-CSF ,RANTES, IP-10, MIP-1α, MIP-1β, (Sordillo et al., 1997, Tizard, 2000). However, there were no commercially available fluorochrome conjugated – antibodies to any of these markers for sheep at the time of this study.

While cryopresereved PBMCs have been used in in immunotoxicology studies of harbour seals (Ross et al., 1993, Levin et al., 2005), chickens and American coots (Finkelstein et al., 2003), these studies did not use frozen blood and they did not use blood from domestic animals. It is, perhaps, surprising that cryopreservation strategies have not been employed more frequently in field-based biomonitoring studies. There may be a number of reasons for this. Firstly, monitoring of the environment has, until now, focused primarily on detection and quantitation of chemical contaminants in the soil, water and air or in affected animals or humans and has not focused on the biomarker approach. Secondly, field-based monitoring investigations are very

expensive and labour intensive. Furthermore, to date only a limited range of biomarkers for biological effect monitoring (in animals) have been developed and even fewer have been validated. Cryopreservation will have to undergo a peer reviewed standardisation of the technique in order for it to become the mainstream method for storing samples for biological effect monitoring. The standardisation of blood specimen collection, processing, storage (at ambient temperature, cooled or frozen) and shipping blood should be should be conducted under a strict quality assurance program, including standard operating procedures and regular quality control reviews enforced and well documented.

In addition to the sample preparations of choice and the biomarker approaches suggested in this study, additional strategies may be worthy of consideration.These include molecular and cellular biomarkers such as the use of mRNA probes, cDNA microarrays (transcriptomics) or advanced proteomics approaches (Maldi-Tof-Mass Spectrometry) to detect many of the proteins and mRNAs whose synthesis is stimulated or inhibited by pollutants. These approaches are potentially more sensitive than the traditional cytological/biochemical "core" biomarkers suggested in traditional biological effect monitoring programs. However, widespread validation of both the traditional and novel approaches in the domestic animal field is still required (Jamers et al., 2006). High-throughput proteomic methods based on liquid chromatography coupled to 2-dimensional gel electrophoresis (LC/2-DE) have been developed for environmental monitoring of marine pollution in a field experiment (Amelina et al., 2007).

The present study represents a significant addition to the current body of knowledge in the developing area of biological effect monitoring of domestic farm animals in the field. While genotoxic and immune biomarkers have been measured in sheep and cows using fresh PBMCS or fresh whole blood, this is the first time that frozen blood has been used to measure selected biomarkers and to show similar response to fresh blood sample preparations.

This study also provides valuable information for researchers in the area of environmental monitoring and veterinary medicine. The approaches described can be useful for the biological effect monitoring of other domestic animals like cows, goats and buffalo. In addition, some of the techniques described, especially those that measure immune biomarkers, may be useful in the health monitoring of domestic animals

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