Methods to Detect Apoptosis in Equine Peripheral Blood Neutrophils from Normal Healthy Adult Horses

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ABSTRACT

Apoptosis is a form of ‘‘planned cell death’’ and is an essential component of normal tissue differentiation and functional regulation. Neutrophil apoptosis facilitates down regulation of the inflammatory response while minimizing ‘‘by stander’’ injury to normal tissue, and disruption of this process by various diseases may have a significant negative impact on patient recovery. Consequently, neutrophil apoptosis has been the focus of research in many species. However, methods for measuring apoptosis have not been evaluated in the horse. The goal of this study was to adapt previously reported methods for inducing and measuring both neutrophil apoptosis and necrosis in non-equine species for use in equine peripheral blood neutrophils.

To achieve this goal the experiment was divided into three parts: 1. Induce apoptosis and necrosis in equine peripheral blood neutrophils using previously used known inducers and examine the relationship between exposure time and percentage of affected cells; 2. Measure percentage of apoptosis and necrosis using three methods of detection: a) Annexin-V Fitc PI assay, b) Homogenous caspase 3/7 assay and c) Light microscopy and; 3. Compare the results between the three methods of apoptosis detection to determine if results are comparable

The hypothesis was that previously reported methods for inducing and measuring both neutrophil apoptosis and necrosis in non-equine species can be adapted for use in equine peripheral blood neutrophils.

Venous blood samples were collected aseptically from the jugular vein of eight horses. Isolation of neutrophils was performed using density gradient centrifugation on percoll. In part 1 of the experiment aliquots of the neutrophil suspension were cultured in the presence of four known inducers of apoptosis; actinomycin D, staurosporin, cycloheximide and sodium hypochlorite, at four different concentrations (table 2). A fifth population was to induce necrosis using a freeze-thaw cycle and bleach. A control sample was examined (no inducer) to determine spontaneous rate of apoptosis. The aliquots were cultured and the percentage of apoptosis determined at two sequential time points for each horse. Apoptosis was measured at either 30 minutes and 3 hours or 6 and 12 hours by three simultaneous methods: (1) annexin-V FITC PI assay (AVF), (2) homogenous caspase assay (HC) and (3) light microscopy (MS). The AVF and HC methods detect events associated with early apoptosis whilst MS detects nuclear changes which are late events of apoptosis. Using AVF and MS apoptotic cells are able to be differentiated from necrotic cells.

In part two of the experiment the agreement and reproducibility between AVF and MS was further examined. In this part of the experiment neutrophils were isolated from the peripheral blood of 10 normal healthy adult horses. Each isolated sample was cultured with 80μM Actinomycin D for 12 hours and a control sample (no inducer) also prepared. Three triplicate samples were next set up from both the induced and control sample and apoptosis was determined using both AVF and MS.

In part 3 of the experiment, data was analyzed using the mixed model ANOVA following log transformation of the data. Main effects of treatment, concentration and time were analyzed. Statistical significance was considered if P was < 0.05.
The relationship between the three techniques; light microscopy, flow cytometry and the fluorescent plate reader, was investigated using Spearman rank correlation coefficients (Fisher's Z transformation). The Bland-Altman approach for method analysis was used to further characterize the correlation between results obtained via light microscopy and flow cytometry. Statistical significance was considered if $P < 0.05$.

All inducers increased the percentage of apoptotic cells at either one or more time point and results were most comparable between AVF and MS. Increasing exposure time increased percentage of apoptotic neutrophils for all inducers using AVF and MS ($p<0.0001$). For both AVF and MS, cycloheximide and staurosporin induced apoptosis significantly above control levels at 3, 6 and 12 hours; actinomycin D at 6 and 12 hours and bleach at 3 and 6 hours as well was 12 hours for AVF only. With HC induction of apoptosis was detected earlier with bleach at 30 minutes and 3 hours and staurosporin at 30 minutes, 3 and 6 hours. Apoptosis was detected only at 6 hours for cycloheximide.

Increasing concentration of inducer significantly increased the percentage apoptotic cells for staurosporin and cycloheximide between the lowest and highest concentration using AVF ($p<0.001$). For both AVF and MS, increasing concentration of bleach decreased the percentage of apoptotic cells ($p<0.05$). Increasing the concentration of staurosporin resulted in an increase in apoptosis at 30 minutes and 3 hours.

Both bleach and the freeze-thaw cycle induced necrosis at all time periods excluding 30 minutes for the freeze-thaw cycle ($p<0.0001$).

Spearman rank correlation coefficients revealed a very high correlation for percentage apoptosis and necrosis between AVF and MS ($r^2 = 0.91$, 95% CI 0.89 – 0.93). A high correlation was also present for AVF and HC ($r^2 = 0.75$, 95% CI 0.69 – 0.79) and MS and HC ($r^2 = 0.76$, 95% CI 0.71 – 0.81). The lower limit of the confidence intervals suggests there is some concern about the similarity between AVF, HC and MS, HC.

The Bland and Altman statistical approach indicates that both AVF and MS are highly reproducible methods with minimal variation between the triplicate samples (AVF: 8.9%, 95% CI 6.25 – 11.6%, MS 7.9%, 95% CI 6 – 9.8%). The mean difference between the two methods is 6.7% (95% CI 3.89 – 9.42%). The 95% limits of agreement indicate that results from MS can be 8.7% below to 22% above results from AVF (95% CI -13.41 – 26.7%).

These findings indicate that caspase activation may occur prior to phosphatidylserine externalization and visible nuclear changes, which is in accordance with previously published data. We discovered that actinomycin D induces significant and reproducible equine peripheral blood neutrophil apoptosis in a time dependant fashion. Similarly, necrosis results from a freeze-thaw cycle or high concentration of

bleach and is suitable as a positive control for necrosis. Apoptosis was effectively detected using AVF assay and results indicate good correlation between AVF and MS with an acceptably low mean difference. MS could serve as an inexpensive, simple and quick on site method to rapidly verify results attained from AVF. Induction of apoptosis using the HC was not consistent and can not be recommended based on the results of this study. Future investigation aimed at evaluating assays multiplexed to the AVF which detect other aspects of the apoptotic pathway would lead to increased confidence of results and further evidence of the mode of cell death prior to undertaking clinical studies.

GRANT INFORMATION

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LIST OF ABBREVIATIONS

PMN = polymorphonuclear leukocyte

ATP = adenosine triphosphate

TNF-α = tumor necrosis factor alpha

IL-1ß = interleukin-1-beta

IL-6 = interleukin-6

FADD = fas associated death domain

TNFR1 = tumor necrosis factor receptor

TRADD = TNFR1 associated death domain

Apaf-1 = apoptosis protease activating factor

DNA = deoxyribonucleic acid

TUNEL = terminal deoxynucleotide transferase mediated dUTP nick end labeling

PI = propidium iodide

AVF = annexin-V Fitc PI assay

HC = homogenous caspase 3/7 assay

MS = light microscopy

FACS = Fluorescence activated cell sorter

Mitochondrial $\Delta\Psi$ = mitochondrial transmembrane potential

LITERATURE REVIEW

A. APOPTOSIS

I. Definition and mechanism

1. DEFINITION AND NATURAL OCCURRENCE

For cellular homeostasis to be maintained, a balance between cellular proliferation and cell death must be neatly balanced. If mitosis preceded without cell death an 80 year old person would have 2 tons of bone marrow and lymph nodes and an intestinal tract 16km long [1]. The term 'apoptosis' was first coined in 1972 by Kerr et al and originates from the Greek words *apo* = from and *ptosis* = falling, symbolizing leaves falling from trees or petals falling from flowers, a natural process of death [2]. Apoptosis is a distinct form of regulated cellular death which occurs in all types of tissues. It has been compared to cellular suicide while necrosis has been a likened to cellular murder. The disappearance of a cell by apoptosis creates 'hardly a ripple' whereas necrosis is capable of producing inflammation [2]. Programmed cell death is encoded in the genome. Each cell possesses the necessary molecular machinery required to undergo apoptosis, and the process can be initiated by specific cell signaling events. The decision to live or die contributes to regulation of the immune response so the apoptotic pathways are kept under tight control.

Apoptosis is a widespread phenomenon occurring throughout the animal kingdom [2]. It is becoming clear that many of the genes involved in apoptosis control in mammals also function in plants. Apoptosis is involved in plant gamete fertilization, embryogenesis and development [2]. The process has been described as dynamic, well coordinated but complex and is important in both the development and maintenance of living organisms [3]. Apoptotic processes begin as early as fertilization and are involved in a wide range of critical processes involved in homeostasis and development. Examples include during the development of the nervous system, whereby more nerve cells are produced than required [4]. Apoptosis adjusts the number of neuron, glial and neuroprogenitor cells to the number of target cells and it has been estimated that between 30-50% of all developed neurons die during development [5]. Apoptosis is also involved in neural tube formation,

which in the developing embryo forms the brain, spinal chord, spinal nerves and spinal column.

Apoptosis also occurs during morphogenesis of various body structures including muscle, epithelial, intestinal, gonadal cells and the immune system [6]. In non-aquatic vertebrates including humans, apoptosis of the inter-digital webs in the early embryo is important in the formation of fingers and toes [7]. In amphibians regression of the tail and gills of the tadpole, occurs as a result of substantial apoptosis [8]. In animals which undergo metamorphosis, larval tissues that are no longer required are eliminated by apoptosis whilst the adult body parts emerge [9]. Development of the reproductive tract requires regression of the male (wolffian) or female (müllerian) duct systems to produce a male or female embryo and is another example of apoptosis.

Apoptosis is just as important to the organism during postnatal development into adulthood. It is a mechanism whereby cells which have served their purpose or become nonfunctional can be eliminated safely without causing harm to the host [2]. Follicular atresia of the post-ovulatory follicle and involution of the mammary gland post-weaning are two examples of normal tissue regression achieved by apoptosis [2]. Regulation of the immune system also requires apoptosis [10]. Defective lymphocytes, lymphocytes which threaten an autoimmune attack or those which have performed their duties can all eliminated safely via apoptosis [5, 11].

Although the examples provided above have been greatly simplified, they highlight the importance of apoptosis to development and maintenance of every living species. Apoptosis is a complex interplay between positive and negative regulators as will be discussed in later sections.

2. CAENORHABDITIS ELEGANS

The very first experiments investigating apoptosis utilized the nematode *Caenorhabditis elegans* [13] . The simplicity and transparency of this subject made *C.elegans* a popular genetic model system. *C. elegans* appears as a transparent tube, approximately 1mm long and consists of 1090 cells in an adult hermaphrodite soma [14]. In the 1970's Sulston and Hortvitz used Nomarski optics to visualize live cells of *C. elegans* [13]. They discovered that of the 1090 cells formed during the development of

the nematode, 131 cells undergo apoptosis. They found that the 131 cells were very similar between different nematode's, indicating that the cells die as a part of a predetermined cellular death program. Through a series of investigations led by Sulston and Hortvitz as well as other researchers, the genes responsible for regulating programmed cell death were discovered [15]. Studies moved on to the fruit fly *Drosophila* and select mammalian species where apoptotic genes were also identified [10, 16-18]. The importance of *C. elegans* apoptosis research was fully appreciated when the central components of the apoptotic program were shown to be conserved in all multicellular animals. However the complexity of apoptotic pathways increases from the initial studies on *C. elegans* to drosophila and then further to vertebrates [16].

3. STIMULI WHICH INDUCE APOPTOSIS

A range of stimuli can induce a cell to undergo apoptosis including external insults such as irradiation, oxidative stress, chemotherapeutic drugs or following the ligation of a cell with an external death receptor onto the target cell [2]. The two best understood and researched apoptotic pathways include the 'intrinsic' or 'mitochondrial initiated pathway' and the 'extrinsic' or 'cell surface death receptor mediated pathway'.

4. THE INTRINSIC/ MITOCHONDRIAL INITIATED PATHWAY (fig 1)

The intrinsic pathway can be triggered by either extra- or intracellular insults such as cellular or oxidative stress following generation of ROS, DNA damage induced by irradiation and following increases in intracellular calcium ions through signal transduction [2]. The key step in the mitochondrial pathway involves the release of cytochrome c from the mitochondria to the cytoplasm. Cytochrome c is normally involved in cellular respiration [14]. More specifically cytochrome c functions as an electron carrier in mitochondrial oxidative phosphorylation, the process that couples oxidation of NADH and $FADH_2$ to the production of high energy phosphate bonds in ATP. However cytochrome c is also a potent pro-apoptotic protein. Exactly how cytochrome c manages to cross the mitochondrial membrane is not entirely understood, however it is clear that the Bcl-2 family of proteins is intimately involved in the regulation of this process.

The Bcl-2 family of proteins consists of 24 members which are key regulators of apoptosis [19, 20]. This family has been divided into three groups based on structural similarities and functional criteria. Members of group I (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1) possess anti-apoptotic activity, whereas members of groups II (Bax, Bak) and III (Bid, Bik) promote cell death. The group I members are mostly attached to intracellular membranes whereas many of the group II and III proteins can shuttle between the cytosol and organelles [21, 22]. The Bcl-2 members can interact with each other to form heterodimer combinations which can be thought of as resulting in mutual neutralization of the bound pro- and anti-apoptotic proteins [23]. Activation of pro-apoptotic members can occur through mechanisms such as proteolysis and dephosphorylation [24]. The interactions regulate the release of pro-apoptotic factors, particularly cytochrome c from the mitochondrial intermembrane compartment into the cytosol [24, 25]. When cells are exposed to "stress signals" the resulting balance between pro- and anti-apoptotic members eventually determines the susceptibility to apoptosis.

There are three basic models as to how Bcl-2 family members regulate cytochrome c exit [23]. Based on the structural similarity of Bcl-x_L to the pore forming subunit of diphtheria toxin [26], it has been suggested that Bcl-2 proteins might act by inserting, following a conformational change, into the outer mitochondrial membrane to form channels or large pores [27]. The second theory suggests that pro-apoptotic members recruit other mitochondrial membrane proteins to form a large pore channel. Experimental studies have shown that several Bcl-2 family members can bind and regulate the channel activity of a permeability transition pore (PTP)[27]. The PTP is composed of an inner membrane protein – adenine nucleotide translocator (ANT) and an outer membrane protein – voltage gated ion channel (VDAC or porin). These proteins act together as a pore where the inner and outer membranes are in contact in pathological conditions. An increase in the permeability of the mitochondrial membrane to molecules weighing less than 1500 Daltons, results from opening the PTP [28]. The third model proposes that Bcl-2 members control the homeostasis of the mitochondria [23]. In this model apoptotic signals alter mitochondrial physiology (e.g. ion exchange, oxidative phosphorylation) resulting in a sudden increase in inner mitochondrial membrane permeability which results in osmotic mitochondrial swelling and influx of water into the

matrix. Since the inner membrane is highly convoluted and has a much greater surface area than the outer membrane, swelling of the mitochondrial matrix results in eventual rupture of the outer mitochondrial membrane, releasing proapoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. Other pro-apoptotic factors released from the mitochondria include Apoptosis inducing factor (AIF), Endonuclease (EndoG), Smac/Diablo and Htr/omi [29]. Besides cytochrome c, both AIF and EndoG facilitate DNA fragmentation. The function of AIF is not well known but together with Smac/Diablo and Htr/omi these factors block the inhibitor of apoptosis (IAP)[29], serving as a point of regulation.

In addition to release of proapoptotic factors the mitochondrial $\Delta\Psi$ change causes a loss of biochemical homeostasis involving halting of ATP synthesis, oxidation of redox molecules (NADH, NADPH, glutathione) and increase in generation of reactive oxygen species (ROS) [31]. The ROS in turn cause oxidation of cellular lipids, proteins and nucleic acids and enhance the disruption of the mitochondrial $\Delta\Psi$ in a positive feedback loop.

Once released, cytochrome c forms a complex with apoptotic peptidase activating factor (Apaf-1), a process requiring ATP [29]. The cytochrome c-Apaf-1 complex recruits, binds and allosterically stimulates caspase-9 (upstream caspase), forming an apoptosome, a cytosolic death signaling protein complex [32, 33]. The apoptosome further activates caspases 3,-6, and -7 (downstream caspases). IAPs function as intrinsic regulators of the caspase cascade serving to inhibit function of both downstream and effector caspases and thus serve as a further point of regulation. The levels of IAPs can determine the outcome to the apoptotic signal. If high levels of IAPs are present, caspase activity can be deactivated. Conversely if high enough levels of caspases exist, IAPs are degraded and caspase activity can proceed.

5. THE EXTRINSIC/ CELL SURFACE DEATH RECEPTOR MEDIATED PATHWAY (fig 2)

The extrinsic pathway involves signal molecules known as ligands which are released by other cells and bind to the trimeric transmembrane death receptors on the target cell to induce apoptosis [34]. The death receptors belong to the tumor necrosis

factor receptor gene superfamily (TNFR) and include TNFR-1, Fas/ CD95 and the Trail receptors (DR-4, DR-5) [34, 35]. For example the immune systems natural killer cell possesses the Fas ligand – FasL on its surface [36]. The binding of the FasL to Fas receptors on the target cell will trigger subsequent signaling on the cytoplasmic part of the transmembrane death receptor which contains a conserved sequence termed the death domain (DD) [36, 37]. Adapter molecules such as FADD or TRADD possess their own DD's by which they are recruited to the DD's of the activated death receptor. This series of events forms a death inducing signal complex (DISC). The adaptor molecules also contain death effector domains (DED's) which recruit procaspase 8 to the receptor complex [38]. When bound to the DISC, several procaspase-8 molecules are in close proximity with each other become activated by autoproteolysis. The initiator caspase-8 processes downstream effector caspases 3, -6, and -7 which subsequently cleave specific protein substrates and initiate degradation of the cell [38]. A second example is the ligand TNF, an intercellular signaling molecule produced mainly by activated macrophages [35, 39]. The binding of TNF to TNF-R1 has been shown to propagate the pathway that leads to caspase activation.

Cells which are capable of inducing caspase dependant apoptosis pathways are classified as type 1 cells [40]. In type 2 cells however, the signal from the activated transmembrane receptor does not result in a caspase cascade powerful enough to execute cell death. In this case in order for cell death to occur the signal needs to be amplified by the mitochondria. The link between caspase signaling and mitochondria involves the Bcl-2 family protein Bid [21]. In this process Bid is cleaved by caspase-8 to a truncated form to become tBid and translocates to the mitochondria. This protein acts in conjunction with Bax and Bad to induce the release of cytochrome c and the remainder of the steps follow the intrinsic apoptotic pathway. Under most conditions however it appears that cross-talk is minimal and that the two pathways operate largely independent of each other [21, 41].

The intrinsic and extrinsic pathway converge at the level of caspase-3 activation and activity is antagonized by the IAP proteins which are in turn antagonized by the Smac/ Diablo protein from the mitochondria [23]. Downstream of caspase 3, the

apoptotic program branches into a number of subprogram's involved in the ordered dismantle and removal of the cell.

6. CASPASES – THE CENTRAL EXECUTIONERS OF APOPTOSIS

Both intrinsic and extrinsic pathways have in common the 'central executioners of the apoptotic pathway' the caspases, a term derived from cysteine dependant aspartate specific proteases - which are responsible for most of the morphological hallmarks typical of apoptosis [23].

Caspases are a group of cysteine proteases highly conserved throughout evolution and are found hydra, nematodes and insects as well as complex multicellular animals including humans [42-44]. Over a dozen caspases have been identified in humans with two thirds functioning in apoptosis [23]. All known caspases are synthesized as inactive zymogens (procaspases) and contain an active-site cysteine and cleave substrates after aspartic acid residues. Pro-apoptotic caspases are divided into initiator procaspases 2,8,9,10 and the executioner procaspases 3,6,7. All caspases show a high degree of specificity which results in cleavage of a particular group of proteins in a coordinated manner [45].

Three general mechanisms of caspase activation have been described the first termed autocatalytic activation describes activation of a procaspase through exposure to a activated caspase [46]. This 'caspase cascade' is used extensively by cells for the activation of the three caspases -3, -6 and -7 which are described as the 'workhorses' of the caspase family. The second mechanism is the induced proximity model which poses that accumulation of caspases (for example caspase 8 in the extrinsic pathway) is sufficient to allow the proenzyme proteins to mutually cleave and activate each other [47]. The third model and the most complex mechanism described, used by caspase 9, is an association between a caspase and a dedicated protein cofactor or regulatory subunit (e.g. Apaf-1)[48].

Activation of caspases results in cleavage of a specific set of target proteins at either one or more positions in the primary sequence, always after an aspartate residue [23]. In the majority of cases caspase cleavage results in inactivation of the target protein, however cleavage can also result in activation by directly cleaving a negative regulatory

domain of the protein or by inactivating a regulatory subunit. A number of important caspase substrates have been elucidated in recent years. One of the major discoveries involved the elucidation of the mechanism of activation of the nuclease responsible for the famous nucleosomal ladder [49]. The nuclease cuts the genomic DNA between nucleosomes to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs. In a number of sequential experiments Wang and Nagata revealed that the DNA ladder nuclease (currently known as caspase-activated DNase or CAD) pre-exists in living cells as an inactive complex (ICAD) [36, 50-52]. Activation of CAD occurs following caspase 3 mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit.

Other examples of caspase mediated cleavage include cleavage of the nuclear laminins resulting in the characteristic nuclear shrinking and budding [53, 54]. Cleavage of cytoskeletal proteins and actin filaments such as fodrin and gelsolin leads to overall loss of cell shape [55]. Finally caspase cleavage of PAK2, a member of the p21-activated kinase family appears to be responsible for active blebbing observed in apoptotic cells [56].

II. CELL NECROSIS

The second main form of cell death is necrosis. Analogous to 'cell murder' necrotic cells are characterized by cellular swelling, a rapid compromise of intracellular energy and mitochondrion function and subsequent plasma membrane and nuclear lyses resulting in release of cellular constituents, including proteolytic enzymes, into the tissue spaces [57]. Contrary to apoptosis, this death mechanism triggers an inflammatory response with potentially serious effects. Necrosis of single cells may be minimal impact however necrosis of a large population of cells can not only initiate a severe inflammatory cascade but result in scarring following repair, permanently altering tissue architecture. Both apoptosis and necrosis can occur simultaneously in tissues and cell cultures exposed to the same stimulus. It has been shown that it is often the intensity of the insult which determines the mechanism of cell death (e.g., apoptosis or necrosis) [2]. Although uncommon, apoptotic cells not recognized by phagocytes can undergo

secondary necrosis otherwise termed ‘apoptotic necrosis’ with cells clearly displaying the characteristic features of necrosis [58, 59].

Until recently necrosis and apoptosis have been thought of as two conceptually distinct forms of cell death [60]. More recently this view has been challenged and according to Majne and Joris (1995) necrosis is not a form of cell death but it is the end stage of any cell death, a ‘post mortal event’ versus apoptosis and oncosis which are ‘pre-mortal events’. Thus apoptotic cells which have formed membrane bound vesicles are classified as necrotic under this regime even though these cells produce small amounts of chemoattractant molecules and do not result in an inflammatory response. The authors promote the term ‘oncosis’ for cells passaging through the process of cell death which is a form of cell death characterized by marked cellular swelling.

Another observation that is becoming more apparent is that apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths that are possible [57]. Also termed the apoptosis-necrosis continuum some scientists have suggested that in-between the extremes of the two main forms of cell death being apoptosis and necrosis a large number of hybrids of cell death can be produced, with varying contributions from each form of cell death [57, 61]. Thus it has been stated that it is important to consider that the presence of apoptosis or necrosis may not be strictly ‘black or white’.

III. Dysregulation of Apoptosis and Disease

Apoptosis research has greatly expanded within the last 20 years since it has become well recognized that dysregulation of apoptotic processes is implicated in an extensive variety of diseases. Accelerated levels of apoptosis cause disorders associated with cell loss, whilst delayed apoptosis results in uncontrolled cell proliferation. Examples of specific disease entities from human medicine and equine medicine are presented below.

1. HUMAN DISEASES ASSOCIATED WITH ACCELERATED APOPTOSIS

Human Immunodeficiency virus type 1 (HIV)

HIV infection causes both early and long term effects on the immune system in humans [62]. Early in the disease process infected persons show functional defects of T cells characterized *in vivo* by a loss of cell mediated delayed type hypersensitivity reactions and *in vitro* by a failure of T cells to proliferate in response to stimulation by antigens and mitogens [62-64]. Years later in the course of disease, the infection leads to declining CD4+ T cells as a result of uncontrolled and accelerated apoptosis. Studies have also shown marked acceleration of neutrophil apoptosis in HIV patients [65]. The accelerated levels of apoptosis increase the risk of serious and potentially lethal opportunistic infections due to an inability to mount an effective immune response.

Neurodegenerative diseases
(Alzheimers diseases, Huntingtons disease, Amyotrophic lateral sclerosis)

Neurodegenerative disorders are characterized by a gradual loss of specific types of neurons from various parts of the central nervous system [66]. This loss is associated with accelerated rates of neuronal apoptosis. The four most well studied and described neurodegenerative diseases include: Alzheimer's, Parkinson's, Huntington's and Amyotrophic lateral sclerosis. Two examples, one outlining pathogenesis of disease and the other example relating to treatment, are subsequently presented.

The formation of amyloid plaques in the brain is one of the cardinal features of Alzheimer's disease [66]. The plaques alter the threshold of apoptosis and act to down regulate anti-apoptotic Bcl-2 expression and upregulate pro-apoptotic Bax expression making the neurons more prone to die [67].

Parkinson's disease is characterized by the degeneration of dopaminergic neurons by both apoptotic and necrotic mechanisms [68]. One of the treatments used extensively is selegiline hydrochloride, initially thought to enhance dopamine signaling by blocking monoamine oxidase B, an enzyme responsible for breaking down dopamine [68]. However research suggests that the drug alters gene expression, down regulating genes encoding pro-apoptotic factors. The drug also prevents reduction of the mitochondrial

membrane potential and subsequent release of pro-apoptotic factors from the mitochondria.

Myelodysplastic Syndromes (MDS)

The MDSs are a group of clonal hematopoietic stem cell disorders [69, 70]. Specifically, dysplasia of erythroid, granulocytic and megakaryocytic lineages constitute the hallmarks of MDS [69]. The disease is characterized by a normal or hypercellular bone marrow and peripheral blood cell cytopenias as a result of excessive apoptosis of hemopoetic progenitors [71-73]. Cytokines such as TNF-α derived from marrow cells are believed to be factors predisposing to apoptosis [72, 74]. The identification of TNF-α as a key cytokine involved with accelerated apoptosis has led to investigation of TNF-α inhibitors as treatment in several clinical trials [71].

Host Graft Rejection

Certain areas of the body such as the cornea and testis are sites of 'immune privilege', protected from inflammation and collateral damage associated with vigorous immune responses [73]. Allografts and xenografts transplanted into immune privileged sites are not rejected and instead thrive. This occurs because lymphoid cells which infiltrate into immune privileged sites are induced to undergo apoptosis when their Fas receptors bind to Fas L [75, 76]. Only activated T cells and somatic cells in immune privileged sites express Fas L. In non immune privileged sites, apoptosis by Fas-FasL binding can not occur. Thus reactive T cells of the host are not killed by the graft (does not express Fas L). Instead the infiltrating T cells can induce apoptosis in the graft, killing the graft cells. In strict terms graft rejection is not a primary pathological disease of accelerated apoptosis but a normal response of the host to foreign tissue [76]. This example demonstrated that induction of apoptosis is a common mechanism by which immune effector cells kill invaders.

2. HUMAN DISEASES ASSOCIATED WITH DELAYED APOPTOSIS

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease whereby the immune system attacks multiple joints [77]. RA is described as a chronic and destructive disorder and is characterized by invasive growth of inflamed and hyperplastic synovial tissue into articular cartilage and bone. Although the mechanisms of the disease are not completely understood changes to apoptosis in a number of cell types such as resident synoviocytes, fibroblasts and infiltrating inflammatory cells are associated with the pathogenesis of synovial hyperplasia and chronic inflammation [78].

Infiltrating B and T cells have been shown to exhibit delayed apoptosis [79, 80]. In T cells delayed apoptosis is likely associate with their anergic state, resistance to Fas induced apoptosis and high expression of the Bcl-2 anti-apoptotic family of proteins. In both B and T cells, protection from apoptosis by substances produced from resident fibroblasts and synoviocytes also serves to stimulate further immune cell recruitment [64, 81]. A delay in fibroblast apoptosis has been shown to occur and it is thought that this phenomenon explains the presence of synovial hyperplasia [82].

In conclusion alterations in B and T cell apoptosis lead to accumulation of these cells in the RA synovium and signs of chronic joint inflammation. A delay in fibroblast apoptosis may result in the characteristic feature of synovial hyperplasia.

Cancer

Cancer is an accumulation of abnormal cells due to an excess of proliferation, decrease in apoptosis, or both [83]. Until recently research focused on the model of abnormal proliferation being responsible for causing cancer, however studies have indicated cancers may also arise from dysfunctions in the apoptotic pathway and elucidation of the role specific factors have in tumorigenesis is under intensive investigation [84]. For example certain types of B cell leukemias and lymphomas have been shown to express high levels of the anti-apoptotic protein bcl-2, thus blocking the apoptotic signals they receive [85]. The high levels result from a translocation of the Bcl-2 gene into an enhancer region for antibody production. Melanoma cells have been shown to avoid apoptosis by inhibiting the expression of the gene encoding Apaf-1, thus

preventing formation of the apoptosome and subsequent activation of the caspase cascade [86]. Lastly, specific types of lung and colon cancer cells have been shown to secrete high levels of a 'decoy' molecule which binds to the transmembrane death receptor FasL situated on the cancer cell, preventing binding of the ligand Fas. Thus cytotoxic T cells cannot bind to the cancer cells, and the apoptotic pathway cannot be initiated preventing cancer cell removal [84].

3. EQUINE DISEASES ASSOCIATED WITH APOPTOSIS

There are an increasing number of studies in the equine medical literature evaluating the presence of apoptosis. Although it is now also becoming recognized that apoptosis appears to be involved in the pathophysiology of a number of diseases, the exact importance of the phenomenon in equine medicine is not yet known. Apoptosis has been detected in reproductive physiology [87-89], exercise physiology [90], chondrocyte growth [91, 92] , virus infection [12, 93, 94], melanocytic tumors [95], gastrointestinal disease [96, 97], neurological disease [98], RAO [99] and laminitis [100]. A summary of a select number of these studies is outlined below.

Exercise induced myocyte apoptosis

Training induced apoptosis of equine skeletal muscle myocytes was shown to occur in thoroughbreds exposed to a treadmill training programmed versus non working horses [90]. The authors hypothesized that apoptosis could provide an adaptive mechanism whereby cells are silently removed and replaced by newer and stronger myocytes.

Osteochondrosis

The effect of insulin and insulin like growth factors on neonatal chondrocytes from the lateral trochlear ridge of the distal femur was studied [92]. When cultured in the presence of these factors in vitro a delay in chondrocyte apoptosis was observed compared to controls. This research supported the authors hypothesis that hyperinsulinemia may contribute to equine osteochondrosis.

Effect of phenylbutazone on the right dorsal colon

The response of the right dorsal colon in eight adult ponies to the effects of the phenylbutazone was studied in vitro [96, 97]. Light and emission electron microscopy demonstrated increased apoptosis of mucosal epithelial cells in treated ponies compared to non treated ponies demonstrating a potential mechanism of NSAID injury.

Surgical colic

Biopsies obtained from the intestine of horses with colic stained with H&E and the TUNEL method demonstrated increased apoptotic nuclei from the circular muscle from horses with simple obstruction (NSO) versus horses with strangulating obstruction (SO) or healthy controls [96, 97]. Also intestine distant from the primary strangulating site had increased numbers of apoptotic nuclei versus intestine from a distant site in NSO horses or from the SO/ NSO site. The authors concluded that the increased apoptosis at the distant site in SO horses could be related to a systemic response to ischemia/ reperfusion injury.

Laminitis

Increased apoptosis of epidermal cells in acute cases of naturally occurring laminitis demonstrated by the TUNEL method was shown to occur in comparison to chronic laminitis cases and normal horses [100]. The authors concluded that apoptosis may be important in the pathophysiology of acute laminitis.

Neurological disease

Increased apoptosis of purkinje cerebellar cells demonstrated by the TUNEL method was shown in 3 clinical cases of Arabians diagnosed with cerebellar abiotrophy [98]. There was a concurrent decrease in the number of normal purkinje cells.

B. THE NEUTROPHIL

1. The lifespan of the normal neutrophil

Neutrophils are the most abundant white blood cell, released at a rate of approximately 10^{11} mature cells per day [101] and constitute the innate immunological

line of defense. Their response to the early stages of infection and/or tissue injury is essential for host survival [102, 103]. Under stable, non-infectious conditions their lifespan is short lived and spent in three environments the duration of which has been determined in human peripheral PMNs. Production and maturation in the bone marrow takes approximately 14 days. Upon release into the circulation the cells lose contact with survival factors present in the bone marrow (e.g. G-CSF, GM-CSF), which destines the PMN for a predetermined cell fate. Thus neutrophil apoptosis already appears to be triggered following release into the circulation. The circulation half life, determined using radio-labelled granulocytes, is between 6-9h, after which the cells transmigrate into the extravascular spaces where they are phagocytosed in the spleen and liver [104-106]. This 'constitutive' or spontaneous rate of apoptosis occurs approximately 24-48 hours following migration from the circulation. The entire process of apoptosis occurs rapidly, approximately 20 times faster than the process of mitosis and apoptotic cells are swiftly phagocytosed by neighboring cells. Sightings of cells undergoing apoptosis *in vivo* are therefore rare.

2. *The neutrophil in acute inflammation*

The progression from injury to an acute inflammatory response is characterized by an increased production and release of PMNs from the bone marrow. Subsequent migration to the injured site occurs under the influence of cytokines (e.g. GM-CSF), chemotactic factors (e.g. C5a) and chemokines (e.g. IL-8). Here PMNs exert their defensive role by phagocytosing and killing ingested pathogens by both oxygen-dependant and independent mechanisms which triggers the oxidative burst [107]. Initiation of the PMN mediated acute inflammatory response is vital for resolution of tissue injury and/or infection. However the inflammatory response is non selective in its targets and PMNs are also involved in the pathogenesis of collateral tissue injury and trauma accompanying inflammatory diseases [108]. Therefore timely removal of activated PMNs from affected sites is essential to the resolution of inflammation. Apoptosis provides a probable explanation towards the resolution of inflammation due to PMN activation [109, 110].

Neutrophil apoptosis is exquisitely sensitive to signals received from the environment. These signals are received on the cell surface and either induce or suppress combinations of specific genes and proteins associated with the cell death pathway [103]. In vitro PMN apoptosis can be modulated by survival and death factors, however the relative contribution of these factors to the in vivo model is not yet completely clear [111]. Studies have shown that in the resolution of the acute inflammatory response apoptosis occurs once PMNs have fulfilled their function of phagocytosis and is triggered by reduction of prosurvival factors such as bacterial components (LPS) and host cytokines (GM-CSF, G,CSF, IL-1β) [112].

Although the mature neutrophil is a terminally differentiated cell it remains capable of generating, receiving and responding to signals by altering gene expression both in resting and activated states [113-116]. Previous studies have used oligonucleotide micro arrays to identify gene profiles. These studies have determined that inactivated PMNs isolated from peripheral blood contain transcripts of at least 3000 genes [115]. Shortly following neutrophil activation increased expression of genes encoding numerous inflammatory mediators, including: MIP (macrophage inflammatory protein)-3α (CCL20), TNFα and vascular endothelial growth factor occurs. The function of these cytokines is as signaling molecules, recruiting and activating other neutrophils and immune cells [117]. The same study revealed that within 90 minutes of PMN phagocytosis at least 30 genes encoding pro-apoptotic factors such as the nuclear orphan receptors TR3, NOR1 and NURR1 were expressed. Between 3-6 hours following PMN phagocytosis greater than 800 genes were either up or down regulated. Of these genes, 105 encoded key factors involved in the initiation and execution of apoptosis including TNFα, TNFSF10 (TRAIL, TNF-related apoptosis-inducing ligand), TNFα receptor-associated factor 1 and caspase 1. The onset of apoptosis in activated PMNs correlated well with expression of these apoptosis related genes [114, 115]. Genes encoding 133 key proinflammatory mediators such as IL-6, IL-8Rβ, CXCL2, oncostatin M or signal transduction molecules including phosphoinositide 3-kinase and calcium important in immune cell recruitment were found to be down regulated during the initial process of apoptosis. Furthermore testing a diverse group of bacterial pathogens revealed a common set of genes which are either switched on or off following phagocytosis by PMNs. In

summary following phagocytosis there is a change in gene transcription; genes encoding pro-apoptotic factors are upregulated and genes encoding anti-apoptotic proteins are down regulated. At least in the case of bacterial infection it appears that a global set of genes is involved, thus scientists have adopted the term 'apoptosis differentiation program' to describe the event [114, 115].

Hence the apoptotic machinery becomes initiated and the cells follow the predictable and well choreographed pathway of apoptosis. Apoptotic PMNs are subsequently recognized by antigen presenting cells through reduction in the expression of certain cell surface receptors on the surface of the apoptotic PMNs such as: Fcγ RII (CD32), complement receptor type 1 (CD35), the receptor for TNF-α (CD120b), CD43 and receptors specific to neutrophils such as CD16 and CD16b [118-120]. Ingestion and clearance by APC's occurs without activation of proteases or inflammatory mediators as cells such as macrophages do not undergo stimulation or the respiratory burst. Furthermore because the cell membrane remains intact throughout the apoptotic process there is no release of pro-inflammatory stimuli [121].

The function of PMN apoptosis is to shut down the secretory function of the cell and ensure safe and efficient removal of both activated PMNs and pathogens. This in turn serves to prevent host tissue damage and results in resolution of the inflammatory response. Determining the mechanisms involved which switch the situation from injury and inflammation to apoptosis and resolution of inflammation may yield clues to optimizing the mechanisms of healing and repair.

It is possible that acute inflammation does not resolve, for example in the case that 1) the injury persists 2) neutrophil apoptosis is not initiated 3) Macrophage clearance of apoptotic PMN's does not occur 4) cells undergo lysis and breakdown resulting in liberation of proteases and other enzymes responsible for collateral tissue damage or 5) a combination of the above factors occurs [121].

3. Delayed neutrophil apoptosis and disease

Delayed neutrophil apoptosis is now considered a general feature of neutrophilic inflammation [111]. Persistent delayed neutrophil apoptosis has also been found to be associated with a number of acute inflammatory diseases including: acute respiratory

distress syndrome [122], SIRS [123], acute pancreatitits [124] and inflammatory bowel disease [122].

Delayed PMN apoptosis prolongs the functional longevity of viable PMNs [125], as they continue to produce antibacterial products such as myeloperoxidase [119]. Initially this may be beneficial to the host by preserving the numbers of viable neutrophils at the site of infection [126]. 'Survival-' or 'anti-apoptotic' factors found to prolong PMN lifespan include: cell surface adhesion molecules (e.g. β2-integrin), bacterial cell wall products (lipopolysaccharide or endotoxin, muramyl dipeptide) and host derived pro-inflammatory mediators (IL-1β, IL-6, IL-8, IFN-γ, G-CSF and GM-CSF) [118, 119, 125, 127-130]. The precise intracellular mechanisms by which these factors delay PMN death is far from understood, however progresses are being made. For example there is evidence which shows a link between the effect of LPS and caspase 3 down regulation mediated by the cellular inhibitor of apoptosis protein-2 (cIAP-2) [118]. Furthermore a study evaluating gene expression in a wide range of inflammatory diseases such as cystic fibrosis, pneumonia and cancer associated with neutrophilia among others showed decreased levels of Bax mRNA and protein versus normal control cases [111]. Bax levels were found to be down regulated by an increase in the survival factors GM-CSF, IL-3, G-CSF and IFN-γ. Following survival factor withdrawal Bax was re-expressed in neutrophils.

The question then arises: how long can the process of PMN death be delayed? For at some stage there must come a point where cellular homeostasis can no longer be maintained. Previous studies have reported the occurrence of both apoptosis but also secondary necrosis in a disproportionately high percentage of PMNs following LPS activation. Secondary necrosis implies the apoptotic pathway was initiated but terminated and instead the cells followed a necrotic pathway. This change in cell death occurs following in response to two main situations, when either: 1) the apoptotic load overwhelms the capacity for phagocyte mediated clearance or 2) cellular ATP/ glucose levels are depleted following the energy consuming process of the oxidative burst [131-133]. In vitro this also occurs as phagocytes are usually absent from isolated cells. It has been hypothesized that the presence of secondary necrosis may be initially beneficial to the host by supporting on going inflammation in the face of active injury or persistent

infection [126]. However secondary necrosis may also prolong the inflammatory response beyond an ideal time frame leading to weakening of the immune response, tissue destruction and organ dysfunction and increasing susceptibility to secondary infection.

C. Laboratory Techniques

I. The cell-culture system as a method of studying apoptosis

Two main methods of studying apoptosis are by using cultured cells or a tissue source. There are a number of advantages to using cultured cells in experimental studies of apoptosis. Cultured cells provide a simple system in which the molecular mechanisms and pathways of apoptosis can be investigated. Often intact animals, due to their complexity can not provide such mechanistic answers. Research can focus on specific aspects of apoptosis in an isolated group of cells, as well as determine how they behave in response to stimuli. Cells are also much easier to use as genetic tools versus an entire organism.

Since apoptosis occurs within a defined period of time following release into the circulation, components of specific diseases (e.g. ischemia-reperfusion injury) which have a systemic component can be investigated as to whether they induce or inhibit the apoptotic process. The use of peripheral blood also has the added advantages of being a very simple, low cost sample to obtain with collection resulting in no morbidity or mortality to the host.

There are however a number of limitations. The information provides a snap-shot in time, not a clear delineation of the actual time line of apoptosis in the course of disease. To bridge the gap between laboratory observations about apoptosis and the clinical arena *in vivo* imaging approaches to detect apoptosis will be required. Furthermore the skeptical question "does the cell represent what happens in the intact animal?" should be asked. Logically, how an entire animal functions cannot be explained by changes in a single cell. This can be related to the complex and intricate world of inflammation where there are many factors which likely contribute to the outcome in the host. *In vitro* studies disregard the importance of phagocyte clearance and local factors present at the inflammatory site such as varying tissue pH and the complex interplay

between cytokines and various types of cells all of which are likely to have some effect on the outcome of an inflammatory response. Some would argue, however, that there is one simple pre-requisite to a successful outcome, and that is the safe removal of inflammatory cells. Apoptosis therefore represents one of the key stages in clearance of an inflammatory reaction.

The scientific literature on apoptosis has grown exponentially and has demonstrated that apoptosis is vital in the stages of growth, development and the adult life of an organism. Furthermore the association of apoptosis with a wide spectrum of malignant, inflammatory and degenerative diseases may open up a whole new vista on novel therapeutic approaches.

II. Available apoptosis assays

Apoptosis can be characterized at the molecular, biochemical and cellular level. The methodologies for analyzing apoptosis characteristics are incredibly diverse and there are many assays and techniques from which the researcher can choose (table 1). Characteristic morphologic changes with the characteristic chromatin and cytoplasmic condensation and typical DNA ladder are the gold standard for detection of apoptosis[134]. Flow cytometry can be used to detect light scatter properties and increased DNA sensitivity to denaturation [134]. Other methods to detect apoptosis include antibodies to the pro-apoptotic proteins, the TUNEL assay, DNA laddering, or the Annexin V assay.

The tunnel assay is widely used to detect apoptotic cells. The TUNEL assay is an in-situ terminal transferase-mediated, nick-end labeling assay, that detects DNA strand breaks resulting from cleavage by endonucleases in advanced apoptosis[135]. Examination of histological tissue sections, fluorescence microscopy and flow cytometry are the three methods by which the TUNEL assay can be utilized. Despite the assays widespread use, disadvantages associated with the TUNEL assay include labeling of late necrotic cells resulting in false positive values and the extensive washing, handling and need for fixation of fragile apoptotic cells, furthermore increasing the potential of false positive values.

In general it is recommended to use more than one method to measure apoptosis in order to confirm data as each method used to differentiate between apoptosis and necrosis is considered unable to 'identify unambiguously the type of cell death'[136].

III. Importance of biological controls in apoptosis assays

One of the most fundamental questions asked in flow cytometry experiments is what proportion of cells are positive for a certain marker. Answering this question requires careful instrument set up and implicit is the proper use of controls, prior to undertaking clinical trials [137-139]. The use of biological controls at the same time as test samples from the clinic serves a number of important roles related to quality control of the experiment and instrument.

Experimental quality control

Establishing a set of positive biological controls ensures that the cells under investigation express the feature being measured. Specifically in the case of apoptosis this is important as not all cells undergo certain aspects of the apoptosis pathway or detectable levels for the assay may not be attained [138]. Thus in essence controls show that the assay has worked.

Biological controls should aim to be clinically reproducible. Data obtained from controls then can be expected to fall within a certain range in order for the test data to be considered interpretable. This becomes increasingly important in the case where samples from clinical cases are collected over a prolonged time span, perhaps months or years, and one patient at a time. The controls can ensure the assays are running properly and appropriately on test specimens.

Importantly, biological controls can serve to validate negative results in clinical test samples by ensuring that the experiment was performed in a way that responses could be detected and that the cell sample in question was capable of being activated. The controls protect against misinterpretation of the data in the case that mistakes were made during experimental procedures such as during preparation of cells or where certain regents were not added.

Instrument quality control

Biological controls can be used for instrument set up quality control [137-139]. In the case of flow cytometry controls are used to set-up compensation. Each fluorochrome has a range of fluorescence emission and there will be an overlap between the emission spectra when using more than one fluorochrome is used (i.e. in multi-color studies). This spectral overlap needs to be accounted for; the process referred to as 'compensation' . Compensation can also be explained as the mathematical subtraction of the fluorescence due to one fluorochrome from the fluorescence of another fluorochome. In the case of the annexin-V FITC assay if compensation is not performed then the cells calculated as staining for both FITC and PI would be erroneously high. Compensation ensures only one detector actually detects each fluorochrome. Experimentally it is determined by staining the sample with only one fluorochrome and individually passing these samples for the flow cytometer for all of the fluorochomes. However the samples passed through the flow cytometer need to also be positive for the fluorochrome tested hence the use of samples induced to express/display a certain marker are extremely useful in this regard. Incorrect compensation settings lead to misinterpretation of the data and false positive populations resulting in incorrect analysis and conclusion.

In conclusion for the reasons stated above the use of biological controls is imperative for both experimental and instrumental reasons allowing standardization of the use of different apoptosis assays.

IV. Chemical induction of apoptosis and induction of necrosis

Serum deprivation

The process of neutrophil isolation and culture induces apoptosis as a result of growth factor withdrawal due to serum deprivation [136]. This is termed the spontaneous rate of apoptosis and is used as a control.

Actinomycin D

Actinomycin D is a microbial alkaloid isolated from the bacteria Streptomyces sp. Traditionally used as a chemotherapeutic compound to treat malignant neoplasms such as Wilms tumors and the sarcomas, this use later became unpopular due to the apparent

highly toxic and damaging effects to genetic material [140]. Apoptosis is one mechanism by which regression of the tumor occurs however the actual mechanism(s) of actinomycin D induced tumor regression is not well understood [141, 142]. Documented mechanisms of action include the ability of this antineoplastic antibiotic to bind to DNA via deoxyguanosine residues and interfere with the action of RNA polymerase engaged in cellular replication and transcription. There is also evidence that Actinomycin D promotes induction of apoptosis by TRAIL and Fas (CD95) [143].

Staurosporin

Staurosporin is classified as a microbial alkaloid isolated from Streptomyces species of bacteria. This compound is frequently used as an apoptogenic agent in a diverse range of cells although the precise mechanism of action is still not completely understood [144]. Staurosporin is a potent inhibitor of phospholipid and calcium dependant protein kinases. Protein kinases are enzymes that modify other proteins by adding phosphate groups (i.e. the process of phosphorylation). Staurosporin also inhibits topoisomerase II activity directly by blocking the transfer of phosphodiester bonds from DNA to active site tyrosine. Topoisomerase II is an enzyme involved with DNA replication in eukaryotic cells. This enzyme transiently cuts one or both strands of DNA allowing the DNA coils to relax and extend, thus permitting proper function of enzymes involved in DNA transcription and replication. Evidence shows staurosporin induces apoptosis via a caspase dependant or a caspase independent pathway [145]. Evidence also suggests that this agent may directly activate a Bcl-2 regulated apoptotic pathway [146].

Cycloheximide

Cycloheximide is a classified as an antibiotic and is active against many yeasts and fungi. This compound is a potent inhibitor of protein synthesis in eukaryotic cells by inhibiting translation. Cycloheximide inhibits both chain initiation and chain elongation by acting on the 60s subunit of the eukaryote ribosome, interacting directly with the enzyme translocase [147]. The consequence is arrest of cell growth with induction of cell death. Apoptosis induction has been shown to occur in a variety of cell types [147, 148] however cycloheximide can also delay or inhibit apoptosis also [149].

Sodium hypochlorite (Bleach)

Direct treatment of cells with oxidants such as bleach was originally thought to only result in necrosis however many recent studies have now shown that lower concentrations can trigger apoptosis in a number of different cell lines [150-156]. Bleach affects intracellular signal transduction mechanisms and is capable of modulating downstream signaling events such as calcium mobilization, protein phosphorylation and gene expression.

The main mechanism whereby low concentrations of bleach result in apoptosis is thought to be due to ROS formation induction and oxidative DNA damage with subsequent dysfunction of the mitochondrial membrane potential [157]. Other mechanisms shown to occur involve lysosomal membrane destabilization with release of cytochrome C from the mitochondria in lymphocytes [158]. Lastly caspase activation has also been shown to occur following low doses of hydrogen peroxide in U 937 human promonocytic cells [159]. High concentrations of hydrogen bleach may overwhelm the cells ability to scavenge ROS with rapid decrease in ATP levels and cellular necrosis. Intracellular accumulation or ROS has also been shown to lead to activation of mitogen activated protein kinase (MAPK) leading to mitochondrial dysfunction [160].

Interestingly the effect of dose of bleach also depends on the cell under investigation [155, 157, 161]. It has been shown that ROS formation and DNA damage after irradiation in human articular chondrocytes and the human osteosarcoma cell line is scarce versus human peripheral T cells and malignant lymphoma cells. The latter of which are highly susceptible to apoptosis following a small dose of sodium hypochlorite. The difference in response is thought to involve different degrees of ROS formation and the difference among cells in the ability to scavenge free radicals.

Freezing/ thawing cycle

Cellular necrosis requires severe cellular damage which has been shown possible following a freezing and thawing cycle which has the effect of provoking plasma membrane dysfunctions [136].

V. Evaluation of morphology via light microscopy

The morphological characteristics of both apoptotic and necrotic neutrophils under light microscopy have been studied in conjunction with other methods of measuring apoptosis and are well documented in the literature [136, 162-169]. The standard method of quantitation has been to aliquot equal volumes of sample onto cytospin slides and following centrifugation and staining, count between 200-300 cells per high power field categorizing cells as either apoptotic or non-apoptotic. A variety of stains have been used for prepared mounted specimens some of which have included Wrights [162] or the May Grunwald Giemsa stain [164, 170].

Characteristics of neutrophil apoptosis observed include cell shrinkage and rounding of the cell as a result of digestion of the proteins which form the cytoskeleton by activated caspases [171]. Chromatin condensation or 'karyopyknosis' is characterized by intense nuclear staining which is condensed. Karyopyknosis has been termed as the cytological hallmark of apoptosis [172]. Nuclear fragmentation of 'karyorrhexis' describes fragmentation of DNA; following this stage the nucleus breaks into several discrete chromatin bodies or nucleosomal units. Karryorrhexis is observed as what appear to be intensely stained spherical nuclear fragments within an intact cellular membrane. Other characteristic features which may be seen include plasma membrane blebbing, membrane bound apoptotic bodies and cytoplasmic vacuoles.

Conversely necrotic neutrophils do not possess such sophisticated signs. Cytologically necrosis is observed with one or more of the following: nuclear swelling, ruptured nuclear and plasma membrane, cytoplasmic vacuolization and faintly stained cells with nuclear ghosts [169].

VI. Flow cytometry and the Annexin V-FITC PI assay

Flow cytometry is a technique which is widely used to make rapid measurements of either particles or cells as they flow in a stream one by one though a sensor [173]. Measurements are made on each individual cell rather than just average values for a population. This technique also has the advantage of being able to measure multiple cell parameters based on light scatter and fluorescent properties [174]. Flow cytometry has numerous applications some of which include the ability to measure cellular parameters

such as enzyme activity, pH, calcium flux and membrane potential through the use of fluorescent probes [173]. Determination of the density and distribution of cell surface receptors and the ability to sort cells for functional studies or chromosomes for preparation of gene libraries are two further examples for the use of flow cytometry[174].

Prior to evaluation with the flow cytometer, cells must be appropriately prepared. This is accomplished by generating a suspension of single cells which are stained with dyes (fluorochromes) which can be detected by the sensor of the flow cytometer. The fluorochromes are typically tagged to a specific antibody which allows detection of specific cell receptors or proteins of interest. After the cells are prepared, they are placed into the sample port and the flow cytometer uses a method of hydrodynamic focusing (i.e. laminar flow) to form the sample into a stream of cells in single file through the flow chamber [174]. The cells flow through the flow chamber rapidly, at an average of 10,000 cells per second [175]. The stream of cells is focused into a path of one or more lasers by the laminar flow. The laser beam hits a cell as they pass through the flow chamber in single file and bounces off due to the physical characteristics of the cell. Light bounced at a right angle is termed side scatter and provides information as to whether the cell contains granules (i.e. cell density). Light bounced off at angles less than 90 degrees is termed forward scatter and provides information on the size of the cell. A light detector processes the light signals and sends the information to a computer. Each type of cell in the immune system has a unique combination of forward and side scatter measurements allowing enumeration of each type of cell [174].

As cells pass through a laser, fluorochrome labels become excited and emit light of certain wavelength depending on the type of fluorochrome used. Filters termed photomultiplier tubes (PMT) direct the light emitted by the fluorochromes to the color detectors which collect the different colors of light emitted by the fluorochromes [173]. If multiple fluorochromes are used, any cell can have none, some or all of the fluorochromes present. Data from the light and color detectors is sent to a computer and plotted on a histogram or other formats of graphical display for interpretation and analysis.

Annexin-V FITC PI assay

Translocation of phosphatidylserine from the inner leaflet of the plasma cell membrane to the outer membrane has been determined to be an early event occurring in most cell systems prior to loss of cell membrane integrity [61, 169, 170, 176-178]. Phosphatidylserine is normally sequestered within the cell on the cytoplasmic face of the plasma membrane [178, 179]. Cells undergoing apoptosis expose phosphatidylserine on the outer plasma membrane which is thus exposed to the external environment. The Annexin V-Fitc PI assay is used to detect cells with this membrane change.

The Annexin V-Fitc PI assay coupled with flow cytometric analysis is capable of determining the percentage of cells within a population that are actively undergoing apoptosis. Annexin V is a 35-36 kDa calcium dependant anticoagulant protein [180]. This protein binds to membranes and lipid bilayers containing phosphatidylserine in the presence of free calcium. Annexin V shows minimal binding to other phospholipids such as phosphatidylcholine and sphingomyeline both of which are constitutively expressed on the outer leaflet [181].

Annexin V is conjugated with fluorochromes such as fluorescein isothiocyonate (FITC) which emits green fluorescence. Thus Annexin V-FITC binds to phosphatidylserine expressed on the outer plasma membrane of early apoptotic cells. However phosphatidylserine is also expressed by necrotic cells and therefore Annexin V can also bind to necrotic cells. Therefore an additional marker must be used to differentiate necrotic from apoptotic cells [170]. Propidium Iodide (PI) penetrates into necrotic cells due to the permeable cell membrane and stains the nucleus. Because early apoptotic cells have an intact membrane they are impermeable to PI and therefore do not stain. Cells which stain positive for Annexin V-FITC and negative for PI are undergoing early apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing apoptosis.

There are some limitations associated with this technique [182]. First is the inability to differentiate between late apoptosis and necrosis since cells in either category stain positive for both Annexin V-FITC and PI. Secondly some investigators have found that externalization of phosphatidylserine and thus Annexin V binding only occurs on a

minority of cells or subpopulation of cells during apoptosis [183-185]. So far the reason for this is not clear and it is unknown whether these cells are in some way different from cells which do not expose PS. Furthermore the reliability of annexin-V binding as diagnostic evidence of apoptosis has been challenged recently. It has been reported that oncotic cells like apoptotic cells bind Annexin V without loss of membrane integrity as determined by lack of PI uptake [186].

VII. Fluorescent plate reader and the homogenase caspase 3/7 assay

The Apo-One Homogenous Caspase-3/7 Assay measures the activities of caspase -3 and -7. Both caspase -3 and -7 are classified as downstream effector caspases and may be activated following initiation of either the intrinsic or extrinsic apoptosis pathway. For a detailed summary of each of these processes please refer to the section on apoptosis pathways. In brief, following a stimulus and activation of the intrinsic pathway, the dynamic relationship between the pro and anti apoptotic members of the Bcl-2 family determines whether cytochrome c, a potent pro apoptotic protein is released from the mitochondria [187, 188]. If cytochrome c is released, an apoptosome is formed with Apaf-1 and caspase 9. The apoptosome which is also known as the 'cytosolic death signaling complex' activates downstream effector caspases -3,-6 and -7. The extrinsic pathway involves the binding of ligands released from other cells to transmembrane receptors on the target cell. A series of events occurs one of which involves triggering of intracellular molecules to the cytosolic portion of the death receptor. Procaspase 8 is recruited to this intracellular complex which subsequently activates the downstream effector caspases -3, -6 and -7. Activation of the effector caspases initiates degradation of the cell.

The principle behind the assay is as follows: The Apo-One Homogenous Caspase-3/7 buffer and caspase-3/7 profluorescent substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R100) are mixed and added to a sample of cultured mammalian cells. The buffer is responsible for lysing or permeabilizing the cells. If there is caspase-3/7 activity in the cell sample, cleavage and removal of the DEVD peptides from the substrate occurs. The remaining rhodamine

group becomes intensely fluorescent. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample.

Reasons and objectives for current study

Dysregulation of apoptosis, specifically neutrophil apoptosis has been implicated in the pathogenesis of a large number of human diseases. Research is currently expanding towards investigating methods to control cell death. Interest is also extending to the veterinary field. However, previously reported methods for inducing and measuring neutrophil apoptosis and necrosis in non-equine species have not been critically evaluated in the horse. The objective of this study is to establish a technique for measuring neutrophil apoptosis in normal healthy adult horses that can be applied to clinical cases. The hypothesis is that previously reported methods for inducing and measuring both neutrophil apoptosis and necrosis in non-equine species can be adapted for use in equine peripheral blood neutrophils

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Methods to Detect Apoptosis in Equine Peripheral Blood Neutrophils from Normal Healthy Adult Horses

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E. Attribution

Descriptions of the qualifications of the authors.

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Equine surgery resident at the Marion DuPont Scott Equine Medical center, who was enrolled in graduate studies at Virginia Polytechnic and State University for a Master of Science degree. She performed sample collection, neutrophil isolation and flow cytometry, light microscopy and plate reading techniques. She also analyzed and reported the outcome of this project.

Nathaniel White, DVM, MS,DACVS

Dr. White was Chair of the graduate committee. He helped plan the research project and reviewed the manuscript. He is the Director and Professor at the Marion DuPont Scott Equine Medical Center.

Martin Furr, DVM, ACVIM, PhD

Dr Furr is an Associate Professor at the Equine Medical Center and a board certified specialist in Large Animal Internal Medicine. Dr. Furr has a PhD in cellular immunology. Dr Furr assisted in training associated with the use of the flow cytometer and reviewed the manuscript.

Virginia Buechner-Maxwell, DVM, PhD, DACVIM

Dr Virginia Buechner-Maxwell is an Associate Professor of Large Animal Clinical Sciences with a Masters of Science in Cell and Molecular Biology. Dr Maxwell helped to plan the project and also provided training in technical aspects of the experiment including isolation of neutrophils, culture, use of flow cytometry and both data analysis and interpretation. Dr Maxwell also reviewed the manuscript.

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Assistant professor biomedical science research who performed all the statistical analysis for the project

F. Methods to Detect Apoptosis in Peripheral Blood Neutrophils from Healthy Adult Horses

A. Abstract

Objective: Establish a technique for measuring neutrophil apoptosis in normal healthy adult horses that can be applied to clinical cases. To fulfill this objective the experiment was divided into three parts: 1. Induce apoptosis and necrosis in equine peripheral blood neutrophils using previously used known inducers and examine the relationship between exposure time and percentage of affected cells 2. Measure percentage of apoptosis and necrosis using three methods of detection: a) Annexin-V Fitc PI assay, b) Homogenous caspase 3/7 assay and c) Light microscopy and 3. Compare the results between the three methods of apoptosis detection to determine if results are comparative

Hypothesis: Previously reported methods for inducing and measuring both neutrophil apoptosis and necrosis in non-equine species can be adapted for use in equine peripheral blood neutrophils

Animals: Eighteen healthy adult horses

Procedure: Venous blood samples were collected aseptically from the jugular vein of eight horses. Isolation of neutrophils was performed using density gradient centrifugation on percoll. In part 1 of the experiment aliquots of the neutrophil suspension were cultured in the presence of four known inducers of apoptosis; actinomycin D, staurosporin, cycloheximide and sodium hypochlorite (bleach), at four different concentrations (table 2). A fifth population was to induce necrosis using a freeze-thaw cycle and bleach. A control sample was examined (no inducer) to determine spontaneous rate of apoptosis. The aliquots were cultured and the percentage of apoptosis determined at two sequential time points for each horse. Apoptosis was measured at either 30 minutes and 3 hours or 6 and 12 hours by three simultaneous methods: (1) annexin-V FITC PI assay (AVF), (2) homogenous caspase assay (HC) and (3) light microscopy (MS). The AVF and HC methods detect events associated with early apoptosis whilst MS detects nuclear changes which are late events of apoptosis. Using AVF and MS apoptotic cells are able to be differentiated from necrotic cells.

In part 2 of the experiment the agreement and reproducibility between AVF and MS was further examined. In this part of the experiment neutrophils were isolated from

the peripheral blood of 10 normal healthy adult horses. Each isolated sample was cultured with 80μM Actinomycin D for 12 hours and a control sample (no inducer) also prepared. Three triplicate samples were next set up from both the induced and control sample and apoptosis was determined using both AVF and MS.

Statistics: Data were analyzed using the mixed model ANOVA following log transformation of the data. Main effects of treatment, concentration and time were analyzed. Statistical significance was considered if P was < 0.05.

The relationship between the three techniques; light microscopy, flow cytometry and the fluorescent plate reader, was investigated using Spearman rank correlation coefficients (Fisher's Z transformation). The Bland-Altman approach for method analysis was used to further characterize the correlation between results obtained via light microscopy and flow cytometry. Statistical significance was considered if $P < 0.05$.

Results:

All inducers increased the percentage of apoptotic cells at either one or more time point and results were most comparable between AVF and MS. Increasing exposure time increased percentage of apoptotic neutrophils for all inducers using AVF and MS ($p<0.0001$). For both AVF and MS, cycloheximide and staurosporin induced apoptosis significantly above control levels at 3, 6 and 12 hours; actinomycin D at 6 and 12 hours and bleach at 3 and 6 hours as well was 12 hours for AVF only. With HC induction of apoptosis was detected earlier with bleach at 30 minutes and 3 hours and staurosporin at 30 minutes, 3 and 6 hours. Apoptosis was detected only at 6 hours for cycloheximide.

Increasing concentration of inducer significantly increased percentage apoptotic cells for staurosporin and cycloheximide between the lowest and highest concentration using AVF ($p<0.001$). For both AVF and MS, increasing concentration of bleach decreased the percentage of apoptotic cells ($p<0.05$). Increasing the concentration of staurosporin resulted in an increase in apoptosis at 30 minutes and 3 hours ($p<0.001$).

Both bleach and the freeze-thaw cycle induced necrosis at all time periods excluding 30 minutes for the freeze-thaw cycle ($p<0.0001$).

Spearman rank correlation coefficients revealed a very high correlation for percentage apoptosis and necrosis between AVF and MS ($r^2 = 0.91$, 95% CI 0.89 – 0.93). A high correlation was also present for AVF and HC ($r^2 = 0.75$, 95% CI 0.69 – 0.79) and

MS and HC (r^2 = 0.76, 95% CI 0.71 – 0.81). The lower limit of the confidence intervals suggests there is some concern about the similarity between AVF, HC and MS, HC.

The Bland and Altman statistical approach indicates that both AVF and MS are highly reproducible methods with minimal variation between the triplicate samples (AVF: 8.9%, 95% CI 6.25 – 11.6%, MS 7.9%, 95% CI 6 – 9.8%). The mean difference between the two methods is 6.7% (95% CI 3.89 – 9.42%). The 95% limits of agreement indicate that results from MS can be 8.7% below to 22% above results from AVF (95% CI -13.41 – 26.7%).

Conclusion: These findings indicate that caspase activation may occur prior to phosphatidylserine externalization and visible nuclear changes, which is in accordance with previously published data. We discovered that actinomycin D induces significant and reproducible equine peripheral blood neutrophil apoptosis in a time dependant fashion. Similarly, necrosis results from a freeze-thaw cycle or high concentration of bleach and is suitable as a positive control for necrosis. Apoptosis was effectively detected using AVF and MS and results indicate good correlation between these methods with an acceptably low mean difference. MS could serve as an inexpensive, simple and quick on site method to rapidly verify results attained from AVF. Induction of apoptosis using the HC was not consistent and can not be recommended based on the results of this study. Future investigation aimed at evaluating assays multiplexed to the AVF which detect other aspects of the apoptotic pathway would lead to increased confidence of results and further evidence of the mode of cell death prior to undertaking clinical studies.

B. Introduction

Apoptosis is a process of cell death that is regulated at the genetic level. The realization that that virtually all cells possess the machinery necessary to spontaneously initiate apoptosis has sparked a surge in related research that has persisted for over a decade. Specific areas of investigative research include: 1. The intra- and extra- cellular mechanisms leading to cell death, 2. diseases associated with either induced or delayed apoptosis, and 3. the role of apoptosis in cell growth and regulation, are three major areas of apoptosis research. The rapid growth in apoptosis related research has stimulated the

development of many methods for measuring this process in a wide array of diseases and cell types. There are, to our knowledge, no reports describing *in vitro* methods for inducing apoptosis or necrosis in equine peripheral blood neutrophils. In addition, a comparison of commonly used apoptotic detection methods has not been examined. We predict that previously reported methods for inducing and measuring neutrophil apoptosis and necrosis in non-equine species can be adapted for use in equine peripheral blood neutrophils. To test this prediction (hypothesis) we will 1. induce apoptosis and necrosis in equine peripheral blood neutrophils using previously known inducers and examine the relationship between exposure time and percentage of affected cells. 2. Measure the resulting percentage of apoptotic and necrotic cells using three methods of apoptosis detection (AVF, HC and MS). 3. The results from these methods will be statistically evaluated to determine if methods produce comparative results. These findings will serve as a basis for a multitude of studies aimed at examining the role of apoptosis in neutrophil regulation. These methods will also be readily adaptable for researchers who wish to examine the role of apoptosis in a variety of equine normal and diseased tissues.

C. Materials and Methods

Population: Eighteen normal healthy horses were selected from the teaching herd located at the campus in Blacksburg VA.

Design: Prospective study utilizing blood samples to compare methods of apoptosis determination.

Sample collection: The samples consisted of whole venous blood. From each horse 32.5cc of blood was collected directly into four ACD tubes. Samples were transported to the laboratory and analyzed within 4 hours of collection.

Clinical Evaluation: A complete physical examination was performed and included rectal temperature, heart rate, respiratory rate, mucous membrane refill, and intestinal sounds.

Neutrophil isolation: After blood collection the ACD tubes were centrifuged at 500x g for 15 minutes. The buffy coat was collected and added to 2mls Hanks buffer without calcium or magnesium. The volume of buffy coat was calculated and any adjustments were made so that the final ratio of cells to Hanks solution was 50:50. Iso-osmotic Percoll was made by adding 0.907mls of 10X Hanks solution to 10mls of stock Percoll. This step was important because it reduced the risk of osmotic shock to the cells during the isolation procedure. The iso-osmotic Percoll was added to 1x Hanks solution at different ratios in order to make a 59% and 79% gradient. A double gradient was created by placing 5mls of 59% Percoll® (Sigma-Aldrich) with calcium free modified Hanks solution and a 75% Percoll® solution into the bottom of a tube. The leukocyte rich plasma was then layered over the 59% Percoll® (i.e. over the double gradient) and the tubes were centrifuged at 930- x g for 40 minutes at 22°C. The two resultant bands of leukocytes were removed. The neutrophils were located in the second fraction.

Preparation of neutrophils: Recovered neutrophils were placed in 30cc of DPBS and centrifuged at 600- x g for 15 minutes. The supernatant was decanted after each spin cycle. Following the second cell wash, the cells were then resuspended in 7cc of RPMI culture media with fetal calf serum. The media used for culture included 2-Mercaptoethanol, penicillin (10,000 IU/ml) and streptomycin (10mg/ml), L-Glutamine in NaCl, Na-Pyruvate and gentamicin (10mg/ml). An aliquot (5µL) was taken and the cells be stained with trypan blue and manually counted on a cytometer to determine the number of cells/ ml. The final solution of cells and culture media were adjusted to a concentration of $2x10^6$ cells/ml. Neutrophil purity was (95%) as assessed by microscopy and size and granularity on flow cytometry.

Culture of Neutrophils and inducers: Aliquots of the neutrophil suspension were cultured in the presence of four known inducers of apoptosis at four different concentrations (table 2). Actinomycin D, staurosporin, cycloheximide and bleach induce apoptosis. Actinomycin D and staurosporin stop cell replication, cycloheximide inhibits cell growth and bleach disrupts the mitochondrial transmembrane potential at low concentrations. Bleach is also capable of necrotic induction at high concentrations and a

freeze thaw cycle induces necrosis through provoking plasma membrane disruption. The neutrophil suspension and inducers were cultured in an incubator with room air at 37°C and supplemented with 5% carbon dioxide. The percentage of apoptosis was determined at two time points for each horse, either at (a) 30 minutes and 3 hours or (b) 6 and 12 hours. Thus there were four horses in each group (a and b).

Apoptosis determination: Three independent methods were used to determine the percentage of apoptotic neutrophils: AVF, HC and MS. The AVF method was chosen because differentiation between apoptotic and necrotic/ late apoptotic cell death is possible. The assay is performed using a flow cytometer which provides the possibility of multiplexing the assay and evaluating other steps in the apoptotic pathway. Furthermore it has been widely used in humans and other mammalian animals. The HC method is a newer product which is known for being simple and inexpensive to run. Furthermore it evaluates the key enzymes involved in the execution of apoptosis. Lastly MS was chosen as a method to provide direct visualization of cellular morphology and to verify the other methods of apoptosis detection.

Annexin-V/PI assay

Quantification of apoptotic neutrophils via flow cytometry using a commercial Annexin-V kit[a] (BD Biosciences) was performed according to the manufactures instructions. All reagents were contained in the kit unless otherwise specified. Briefly, the cells were suspended in 1x binding buffer at a concentration of $1x10^6$ cells/ml. From this solution 100 µL aliquots ($5x10^5$) of the cell suspension were collected into a tube. In this tube 5 µl of the Annexin V-FITC binding buffer (0.5 µg/ml) and 5 µl of propidium iodide stock was added. The tubes were mixed, incubated in the dark for 15 minutes and following incubation 400 µl of 1x binding buffer was added to the first tube. Samples were analyzed within one hour using a Coulter Epics XL-MCL[b] (Beckman Coulter Inc) using associated flow software and computer for data collection.

Homogenous caspase 3/7 assay

Quantification of apoptotic neutrophils via the plate reader using a commercial caspase 3/7 assay[b] (Promega) was performed according to the manufactures instructions (Promega). The caspase substrate and Apo-ONE Caspase 3/7 buffer were thawed and mixed (1 substrate: 100 buffer) to make the Apo-ONE caspase 3/7 reagent. In a 384 clear multiwell plate 25µL aliquots of all inducers, control, freeze thaw and DMSO assay samples were pipetted into individual wells at the allocated times. To each well containing a sample, 25µL of caspase reagent was added and the plate was incubated for 2-2.5 hours at room temperature. After incubation the contents of the plate were mixed for 30 seconds using a plate shaker and the fluorescence was measured of each well using an emission wavelength of 485nm and an excitation wavelength of 530nm. Caspase activity is measured as relative linear fluorescence units (RLFU). Samples were analyzed using a Gemini XPS microplate[d] (Molecular Devises Corporation) set at endpoint mode.

Light microscopy

A cytospin slide was prepared from the RPMI suspended neutrophils. A 50 µL aliquot of cells and inducer was added to 150 µL DPBS and 50 µL bovine serum. The entire 200 µL solution was loaded onto Shandon Cytospin Centrifuge®[e] (Thermo Scientific) cytospin unit. All slides were stained using wrights stain. Two hundred cells will be counted and morphologically evaluated by light microscopy. The morphological features used to detect apoptotic neutrophils have previously been described and include: 1) cell shrinkage with 2) nuclear cytoplasmic changes (peripheral condensation of chromatin around the nuclear membrane, nuclear fragmentation, formation of cytoplasmic blebs, membrane bound apoptotic bodies and cytoplasmic vacuoles) [189].

[a] Annexin V FITC Apoptosis Detection kit I®, BD Biosciences, San Jose, CA

[b] Coulter Epics XL-MCL, Beckman Coulter Inc, Miami, FL

[c] Homogenous Caspase 3/7 Assay®, Promega, Madison, WI

[d] Gemini XPS microplate, Molecular Devises Corporation, Sunnyvale, CA

[e] Shandon Cytospin Centrifuge®, Thermo Scientific, Waltham, MA

Determination of assay reproducibility

Neutrophils were isolated from the peripheral blood of ten normal healthy adult horses using the technique described above. Two aliquots from each isolated sample were attained. One aliquot was incubated for 24 hours with no inducer and the other incubated for 12 hours in the presence of 80μM actinomycin D. Following incubation the samples were divided into triplicates (three control tubes and three inducer tubes per horse) and apoptosis was determined via AVF and MS. Reproducibility was evaluated using the standard deviation of the triplicate values against their mean (termed coefficient of variation *CV*).

D. Statistical Analysis

Data were entered into a standard desktop computer and statistical analysis was performed using SAS system[C.] Summary statistics were determined for each treatment group and time, and are reported as mean $\pm$ standard deviation (SD).

To determine if treatments applied induced apoptosis in a proportion of cells greater than the control group, data were analyzed using the mixed model ANOVA following log transformation of the data. Main effects of treatment, concentration and time were analyzed. Statistical significance was considered if P was < 0.05.

The relationship between the three techniques; light microscopy, flow cytometry and the fluorescent plate reader, was investigated using Spearman rank correlation coefficients (Fisher's Z transformation). The Bland-Altman approach for method analysis was used to further characterize the correlation between results obtained via light microscopy and flow cytometry specifically for the highest concentration of Actinomycin D (A4). Statistical significance was considered if $P < 0.05$.

[C] Version 9.1.3, SAS Institute Inc., Cary, NC 27513

E. Results

Apoptosis detection in equine PMN's

Apoptosis was detected by all three independent methods, MS, AVF and HC. By measuring annexin-V FITC binding on cell surfaces and caspase 3/7 activity, we were

able to identify the early stages of apoptosis. Evaluation of nuclear morphology via light microscopy was performed to verify the results of these two techniques.

Induction of apoptosis and dose of inducer

The effect of increasing the concentration of inducer and maintaining time constant was examined for each inducer used as well as each method of detection.

Using FACS analysis increasing the concentration of the inducer actinomycin D, did not significantly increase the percentage of early apoptotic cells detected by AVF. (Figure 3). For staurosporin and cycloheximide, increasing the concentration of inducer did not have an effect for most time periods (Figure 6 and 9 respectively). However, there was a significant difference between the lowest and highest concentration for both inducers at 6 hours. We were able to demonstrate a significant increase of annexin-V AVF positive cells from 14.7% (X1) to 43.1% (X4) and 17.9% (S1) to 57.6% (S4). The spontaneous rate of apoptosis at 6 hours (untreated control) was 4.39% (Figure 2a). For the inducer bleach, increasing concentration significantly decreased the percentage of annexin-V positive cells. At 6 and 12 hours the percentage of annexin-V positive (AVF) cells was significantly higher ($P<0.05$) for concentrations B1 and B2 versus B3 and B4 (figure 12).

The results from light microscopy were paralleled by the results from flow cytometry. For actinomycin D, staurosporin and cycloheximide, increasing inducer concentration did not significantly increase percentage of annexin-V positive stained cells (Figures 4, 7 and 10 respectively). Increasing the concentration of bleach significantly decreased the percentage of annexin-V positive stained cells at 3, 6 and 12 hours.

Using the caspase 3/7 assay a concentration dependant increase in RFLU was observed for the time periods 30 minutes and 3 hours for staurosporin (Figure 8). No other concentration effect was observed for the other inducers.

Induction of apoptosis and duration of incubation

The effect of increasing incubation time and maintaining time constant was examined for each inducer and method of detection (table 3). Using FACS analysis and the inducers actinomycin D, staurosporin, cycloheximide and bleach the effect of increasing incubation time increased the percentage of annexin-V AVF positive cells (Figures 15, 18, 21 and 24 respectively). For most concentrations of actinomycin D, staurosporin, and cycloheximide, the percentage of annexin-V AVF positive cells peaked at 6 hours and was not significantly different to results obtained at 12 hours.

The results of microscopy were similar to FACS analysis in that increasing incubation time increased the percentage of annexin-V positive stained cells for actinomycin D, staurosporin and cycloheximide (Figures 16, 19 and 22 respectively). However unlike flow cytometry the percentage of positive cells continued to increase significantly after 6 hours for actinomycin D. Similarly to FACS analysis increasing incubation time for B1 increased percentage of annexin-V positive AVF cells (Figure 24). There was not a significant effect of time after 30 minutes of incubation with B2. With B3, incubating cells for 12 hours significantly increased the percentage of annexin-V positive cells compared to incubation at 30 minutes, 3 and 6 hours.

Analyzing results obtained from the caspase 3/7 HC assay revealed increasing incubation time did increase caspase 3/7 HC activity for actinomycin D, staurosporin and cycloheximide (Figures 17, 20 and 23). However at many of the time points caspase 3/7 activity was not significantly elevated above control values. There was no effect of increasing incubation time with the inducer bleach.

<u>Actinomycin D</u>

Using FACS analysis, we found a significant increase of annexin-V-AVF positive cells compared to control cells (6hr - 4.39%, 12hr - 5.63%) for concentrations A2 (18.3%), A3 (23.1%) and A4 (22.7%) at 6 hours and for all four concentrations at 12 hours (A1 – 65.3%, A2 – 72.6%, A3 – 70.2%, A4 – 72.9%) (figure 3).

Results from MS were in accordance to FACS analysis. We were able to demonstrate actinomycin D induced apoptosis for all concentrations at both 6 hours (A1 – 15.4%, A2 – 21.5%, A3 – 25.0%, A4 – 20.3%) and 12 hours (A1 – 69.9%, A2 – 82.5%,

A3 – 75.4%, A4 – 64.1%) compared to control values (6hr – 4.25%, 12hr – 6.67%) (figure 4). Apoptotic neutrophils were easily distinguished from surrounding normal neutrophils for each concentration of inducer and for each time period. Figure 33, A-D shows normal, typical control neutrophils at each time period for the concentration A4. Figure 35, A-D demonstrates a representative section of the slide showing altered neutrophils stimulated with actinomycin D. In the actinomycin D treatment group we show shrunken cells, condensed spherical nuclei and an intact plasma membrane.

Caspase 3/7 activity was not above control values for any concentration or time period (figure 5).

Staurosporin

Using FACS analysis we determined a significant increase of annexin-V AVF positive cells for all four concentrations at 3hrs(S1 – 7.51%, S2 – 10.4%, S3 – 8.23%, S4 – 6.55%), 6hrs(S1 – 17.9%, S2 – 24.0%, S3 – 24.0%, S4 – 57.6%) and 12hrs(S1 – 42.1%, S2 – 43.2%, S3 – 46.9%, S4 – 63.9%) compared to control cells (3hr – 1.09%, 6hr – 4.39%, 12hr – 5.63%) (figure 6).

Similarly light microscopy demonstrated induced apoptosis at the above time periods, excluding S1 at 3hrs (figure 7). The percentage of apoptotic cells at 3hrs(S2 – 12%, S3 – 11%, S4 – 10%), 6hrs(S1 – 29.5%, S2 – 44%, S3 – 48.4%, S4 – 66.5%) and 12hrs(S1 – 52.8%, S2 – 66.7%, S3 – 74.2%, S4 – 85.5%) was significantly above control values (3hr – 2%, 6hr – 4.25%, 12hr – 6.67%). Apoptotic neutrophils were easily distinguished from surrounding normal neutrophils for each concentration of inducer and for each time period. Figure 33 , A-D shows normal, typical control neutrophils at each time period. Figure 36, A-D demonstrates a representative section of the slide showing altered neutrophils stimulated with staurosporin. Apoptotic cells usually presented with greater than one nucleoli and apparent cytoplasmic vacuolization. In comparison to the other three inducers; actinomycin D, cycloheximide and bleach the cell membranes were not as distinct when cells were grouped close together. In some sections of the slide the nuclei were so closely clustered that individual cells could not be counted.

Caspase 3/7 HC activity was significantly elevated for at 30 mines (S4 – 1052 RFU), 3hrs (S2 - 148, S3 - 948, S4 – 1665 RFU), and for all four concentrations (S1 –

197, S2 – 201, S3 – 265, S4 – 255 RFU) at 6 hours compared to control values (30mins – 33.9, 3hr – 16.9, 6hr 38.2 RFU) (figure 8).

Cycloheximide

The results of the cycloheximide induced neutrophil group paralleled that of staurosporin. Using FACS analysis we determined a significant increase of annexin-V positive cells for all four concentrations at 3hrs (X1 – 4.57%, X2 – 6.81%, X3 – 6.28%, X4 – 7.18%), 6hrs(X1 – 14.7%, X2 – 28.1%, X3 – 29.5%, X4 – 43.1%) and 12hrs(X1 – 43.5%, X2 – 42.0%, X3 – 37.6%, X4 – 75.4%), compared to control cells (3hr – 1.09, 6hr – 4.39, 12hr – 5.63) (figure 9).

Using light microscopy MS we demonstrated significantly induced apoptosis at 3hrs(X3 – 7%, X4 – 8%), and for all four concentrations at 6hrs(X1 – 19.5%, X2 – 33.4%, X3 – 31.9%, X4 – 52%) and 12hrs(X1 – 46.8%, X2 – 68.5%, X3 – 77.4%, X4 – 88.1%) compared to control cells (3hrs – 2%, 6hrs – 4.25%, 12hrs – 6.67%) (figure 10). Apoptotic neutrophils were easily distinguished from surrounding normal neutrophils for each concentration of inducer and for each time period. Figure 33 , A-D shows normal, typical control neutrophils at each time period. Figure 37, A-D demonstrates a representative section of the slide showing altered neutrophils stimulated with cycloheximide. The morphological features of apoptotic cells was similar to those cells induced by actinomycin D and bleach. However, apoptotic cells also presented with pale staining eosinophilic nucleoli.

Caspase 3/7 activity was detected following induction at 3hrs(X1 – 95.6RFU, X4 – 118) and 6hrs(X1 – 128, X2 – 233, X3 – 312, X4 – 206) (figure 11).

Bleach

The results obtained for bleach differed in comparison to the other three inducers. Using FACS analysis we determined a significant increase of annexin-V positive cells at 3hrs(B1 – 12.9%, B2 – 21.5%, B3 – 4.75%), 6hrs(B1 – 27.7%, B2 - 15.0%) and 12hrs(B1 – 18.5%, B2 – 22.3%) compared to control cells (3hrs – 1.09%, 6hrs – 4.39%, 12hrs – 5.63%) (figure 12). A significantly higher percentage of annexin-V positive cells

was observed for lower concentrations of bleach (B1 and B2) versus higher concentrations of bleach (B3 and B4) at 3, 6 and 12 hours.

Using light microscopy the same trend was observed however only concentrations B1 (11%) and B2 (19%) were significantly above control levels (3hrs – 2%, 6hrs – 4.25%) at 3 hours and B1 (24.6%) at 6 hours (figure 13). Apoptotic neutrophils were easily distinguished from surrounding normal neutrophils for each concentration of inducer and for each time period. Figure 33 , A-D shows normal, typical control neutrophils at each time period for the concentration A4. Figure 38, A-D demonstrates a representative section of the slide showing altered neutrophils stimulated with bleach. At both 30 minutes and 3 hrs apoptotic cells were readily differentiated from normal and necrotic neutrophils. At both 6 and 12 hours however the nucleus of the cell appeared hypertrophied and less well defined and thus distinguishing apoptotic from viable cells was difficult. Caspase 3/7 activity was significantly elevated at 3 hours for B1 (105RFU) versus control cells at 3hrs (17RFU) (figure 14).

NECROSIS

Necrosis in equine neutrophils after stimulation with inducers

We were able to demonstrate neutrophil necrosis with two independent methods. Treatment with bleach and a freeze-thaw cycle induced necrosis in equine neutrophils.

Using FACS analysis, we determined a significant increase of PI-positive cells compared to control cells for all concentrations of bleach as well as for all time periods measured (figure 27). Necrosis at 30mins(B1 – 2.29%, B2 – 3.21%,B3 – 11.6%,B4 – 56.2%), 3hrs(B1 – 25.9%, B2 – 32.9%,B3 – 76.4%,B4 – 94.6%), 6hrs(B1 – 52.0%, B2 – 69.3%, B3 – 91.0%, B4 – 92%) and 12hrs(B1 – 61.0%, B2 – 72.5%, B3 – 85.6%, B4 – 88.9%) was significantly elevated above control cells (30mins – 1.32%, 3hrs – 1.22%, 6hrs – 2.23%, 12hrs – 1.8%). The freeze thaw cycle induced necrosis for all time periods excluding 30 minutes (3hrs – 40.4%, 6hrs – 42.6%, 12hrs – 84.6%) (figure 29). Using the microscopy method, we were able to verify the results attained from FACS analysis for both bleach and freeze-thaw (figure 28 and 30). Using light microscopy a significant increase in necrotic neutrophils was observed for all concentrations and time periods for bleach. Necrosis at 30mins(B1 – 6.25%, B2 – 9.50%, B3 – 18.0%, B4 – 59.3%), 3hrs(B1

– 28.5%, B2 – 31.0%, B3 – 79.3%, B4 – 95%), 6hrs(B1 – 52.8%, B2 – 65.4%, B3 – 92.0%, B4 – 92.0%) and 12hrs(B1 – 60.5%, B2 – 71.4%, B3 – 83.9%, B4 – 88.6%) was significantly elevated above control cells (30mins – 1.50%, 3hrs – 1.63%, 6hrs – 1.75%, 12hrs – 0.67%). The freeze thaw cycle induced necrosis for all time periods excluding 30 minutes (3hrs – 43.0%, 6hrs – 38.0%, 12hrs – 97.7%). Necrotic neutrophils were easily distinguished from surrounding normal or apoptotic neutrophils for each concentration and time period of bleach and freeze-thaw induction. Figure x , A-D shows normal, typical control neutrophils at each time period. Figure x, E-H demonstrates a representative section of the slide showing altered neutrophils stimulated with Bleach and Figure x, E-H demonstrates sections induced by a freeze-thaw cycle. In both the bleach and freeze-thaw treatment group, the nucleus of necrotic cells appeared enlarged with accompanying rupture of the plasma membrane. Variations of necrotic cells included faintly stained nuclear ghosts.

Induction of necrosis and dose of inducer

Results from FACS analysis revealed increasing percentage of annexin-V Fitc PI cells (either late apoptotic or necrotic) and results from light microscopy demonstrated increasing percentage of necrotic cells with increasing concentrations of bleach (figure 27 and 28). At all time periods the percentage of cells which stained annexin-V Fitc PI positive using the higher concentration of inducer (B3 and B4) was significantly greater than when lower concentrations of bleach (B1 and B2) were used.

Induction of necrosis and duration of incubation

Increasing incubation time increased the percentage of PI-positive cells for concentrations B1 and B2 (Figure 31). There was a less apparent increase in PI-positive cells with increasing incubation time for B3 and B4. Increasing the length of the freeze-thaw cycle also increased the percentage of PI-positive cells, however there was no significant difference between 3 and 6 hours.

COMPARISON OF METHODS OF MEASUREMENT

Correlation coefficients

Spearman rank correlation coefficient indicated a very strong positive correlation between flow cytometry and light microscopy ($r = 0.91$, $p < 0.0001$). A strong correlation was demonstrated between light microscopy and the caspase 3/7 assay ($r = 0.76$, $p<0.0001$) and a similarly strong correlation between the annexin V-FITC assay and the caspase 3/7 assay ($r=0.75$, $p<0.0001$). Considering the data specifically for the highest concentrations of actinomycin D, from the line of equality plot it can be seen that the two readings from flow cytometry and light microscopy give different results and that most of the observations for light microscopy lie above the line of equality (figure 33).

Bland and Altman statistical approach

Agreement

For both flow cytometry (FC) and light microscopy (LM) the difference in % apoptosis (flow – microscopy) was plotted on the y-axis versus the average % apoptosis [(flow + microscopy) /2] on the x-axis (Figure 34). For this data, the differences LM – FC have a mean difference (bias) 6.66 and a standard deviation 7.82, the latter representing the variation around the mean difference. The 95% CI for the bias is between 3.89 and 9.426%

The 95% limits of agreement for the bias are -8.68 [(6.66 – (1.96*7.82))] and 22 [(6.66 + (1.96*7.82))]. The 95% lower level of agreement is between -13.41 to -3.95 and the 95% upper level of agreement is between 17.3 to 26.7. Hence a measurement by method LM would be between 8.68% (95% CI -13.41 to -3.95) less than a measurement by method FC and 22% (95% CI 17.3 – 26.7) greater. The width of the 95% limits of agreement, -8.68 to 22, is 30.7%.

Reproducibility

The reproducibility in each of the two methods was evaluated by examining the standard deviation of triplicate values against their mean, termed coefficient of variation (CV). The mean CV for flow cytometry was 8.94. The 95% limits of reproducibility for FC are 6.25 – 11.6. The mean CV for was 7.90. The 95% limits of reproducibility for LM

are 6.00 – 9.80. The ranges are narrow for both methods, i.e. the measurements for FC and LM could vary up to 11.6% and 9.8% respectively. The mean difference between FC CV and CV is 1.04 with a 95% limit of agreement between -4.13 and 5.44.

Discussion

Objective 1: Induce apoptosis and necrosis in equine peripheral blood neutrophils using previously known inducers and examine the relationship between exposure time and percentage of affected cells.

Apoptosis and necrosis was consistently induced in equine peripheral blood neutrophils and results detected with AVF and MS. Increasing incubation time increased percentage of affected cells. Both actinomycin D and cycloheximide induced a high percentage of apoptosis and morphological changes were easily visualized by MS. Both actinomycin D and cycloheximide can be recommended as suitable positive controls for apoptosis. The highest percentage of apoptosis was attained at 12 hours following treatment with actinomycin D (A2: AVF 73%, MS 64%) and cycloheximide (X4: AVF 88%, MS 75%). Therefore a 12 hour incubation period is an adequate time period to induce a high number of apoptotic cells. In contrast at 12 hours apoptotic cells were difficult to differentiate from normal and necrotic cells using bleach and staurosporin with MS and are not recommended as inducing agents.

Freezing cells for 12 hours and subsequent thawing resulted in a high percentage of necrotic cells (AVF 85%, MS 97%). A freeze thaw cycle is a suitable positive control for necrosis.

Objective 2 and 3: Measure resulting percentage of apoptosis and necrosis using three methods of detection: AVF, HC and MS. Subsequently compare the results between the three methods of apoptosis detection to determine if results are comparative.

Results obtained from AVF and MS exhibited the greatest similarity when displayed graphically and analyzed using descriptive statistics. Conversely, there was a discrepancy when comparing results attained between HC and MS, and HC and AVF.

Results from HC showed either no significant induction or low numbers of samples which induced caspase 3/7 activity. For example actinomycin D did not induce significant caspase activity for any time period whereas significant apoptosis was detected with both AVF and MS after 30 minutes of incubation. Using cycloheximide, significant caspase activity was only detected for three out of four samples at 6 hours. Whereas, significant apoptosis was detected after 30 minutes of incubation using both AVF and MS.

There are a number of potential reasons for the discrepancy in results between HC and both MS and AVF. Firstly, apoptosis may be occurring via a caspase independent pathway. Caspase independent phosphatidylserine exposure has been observed in human peripheral T cells induced with etoposimide and staurosporin [190, 191], B cells [192] and human retinal cells [193]. As in apoptosis, the mitochondrion can play a key role but also other organelles such as lysosomes and the endoplasmic reticulum have an important function in the release and activation of death factors such as cathepsins, calpains, endonucleases and other proteases. These organelles can act independently or collaborate with each other [193].

The second reason why there was a discrepancy between HC and the other two assays could be related to the small sample size and the wide variation in control values at 12 hours (between 120 – 1591RFU). Most of the induced samples were also within the range of the control samples thus there was no statistical significance and caspase 3/7 activity was not induced. The reason for the wide range in values at 12 hours is not known as sample preparation did not change throughout the experiment and the same person performed all experiments. However, an experimental error can not be ruled out. A third reason for the possible discrepancy could be that caspase activation occurs either earlier than 30 minutes or later than 12 hours, that is at times not studied. In order to

verify the results of HC, a caspase assay could be performed using another technique such as flow cytometry.

Spearman rank correlation statistics suggests a good correlation between HC and both the MS and AVF. However the lower limits of the confidence intervals indicate that there may be some concern with the strength of association between these techniques. Furthermore evaluation of graphical data associated with each concentration of inducer, time period and caspase activity did not reveal a suitable positive control for apoptosis. Based on the results of this experiment we can not make recommendations as to the use of this assay in clinical equine cases. Conversely a very strong correlation was observed for results attained via AVF and MS. This statistic gives us the information that as one variable increases in one method the other also increases. Thus it tells us whether the measurements in both assays go up and down together. However, this statistic does not say anything about the magnitude of difference between the paired measurements which would be of value to know when assessing two methods of measurement in this case in order to determine which event occurs earlier. Furthermore the correlation coefficient is an arbitrary unit and provides little information about the numerical relationship between two data devices. There can be clear and significant mean differences which can be shown graphically between two methods and a correlation coefficient would not portray such a difference.

The literature supports the use of the Bland-Altman statistical approach for method comparison studies. This approach is different because it focuses exclusively on the differences between two methods. The approach uses the average of the new and reference measurement as the 'reference' value against which the new method is compared. This approach is very useful if a gold standard does not exist and thus when the true values of the quantity being measured is unknown.

The approach utilizes graphical interpretation of the data. A scatter diagram (difference versus mean) is constructed with the results scattered around a horizontal line versus the traditional line of equality for correlation coefficients. This is because the eye is better at judging departures from a horizontal versus a diagonal line. If the measurements are comparable the differences should be small, ideally centered around zero.

Calculation of the bias and precision limits estimates the limits of agreement between the two techniques. The limits of agreement give the range of difference in which 95% of the observations are expected to be included. The limits of agreement are then used to predict the range of discrepancies to be expected between the new and reference technique.

The Bland-Altman statistics show that the mean difference between the two techniques; LM and FC is 6.7% with a 95% CI of 3.89 – 9.42%. This LM tends to give a higher reading, by between 3.89 and 9.42%. This difference is practically small. The 95% limits of agreement are -8.7% - 22%. Therefore results from LM may be -8.7% below or 22% above FC results. The precision estimates for the limits of agreement are narrow (-13.41 to -3.95%, 17.3 to 26.7%). These results indicate a possible discrepancy between the two results, up to a difference of 26.7%. Therefore although these methods are not interchangeable the results of MS could still be used to verify the presence of apoptotic cells.

Conclusion

The results of this study indicate that equine peripheral blood neutrophils can be stimulated *in vitro* to undergo apoptosis and necrosis. Apoptotic and necrotic neutrophils express annexin-V Fitc and annexin-V Fitc PI respectively and display a characteristic morphology both in a consistent and reproducible manner. Thus the results described verify the use of the AVF and MS methods for peripheral blood apoptosis and necrosis neutrophil detection. The determined positive inducers of apoptosis and necrosis can be added to healthy control horses, incubated and then run alongside samples of isolated neutrophils from clinical cases to ensure appropriate instrument and experimental set-up. The inducers can also be used in conjunction with other assays evaluating different aspects of the apoptosis pathway to allow standardization of methods. Advantages of AVF is the early detection and quantification of apoptosis. MS provides a rapid, simple, inexpensive bedside test to verify results attained from AVF.

Tables

Approach	Theory/comments	Advantages	Disadvantages
Cell viability			
Plasma membrane integrity	Vital dye exclusion assays (e.g. trypan blue, PI)	Simple, low cost Can be used with flow cytometry for quantitative assessment	Not useful for apoptosis alone because plasma membrane remains intact
Morphological exam	Gold standard Considered most reliable in defining apoptosis		
Light microscopy		Quick, convenient, on-site method, low cost	Subject to individual bias, semi-quantitative if images of representative fields captured
Electron microscopy		Unequivocal	Tedious Not quantitative
Alterations in plasma membrane			
Annexin-V FITC	Measures membrane lipid redistribution Binds to apoptotic cells with PS externalization	Quantitative Can discriminate between necrotic cells Detects apoptotic cells earlier than TUNEL Allows for double or triple labeling	Expensive Variable success with whole animal injections
Cytosolic changes			
Caspases	Activation considered biological hallmark of apoptosis	Commercial kits widely available Imaging approaches being developed for in vivo detection Quantitative	Kits are not specific for a particular caspase Technique lyses/ fixes cells
Bcl-2 family proteins Bad phosphorylation Bid cleavage Translocation to mitochondria	Expression levels or activation status evaluated	Can use antibodies specific to individual members	
Mitochondrial changes	Most important organelle in apoptosis initiation and regulation	Changes occur in apoptosis and necrosis	Several parameters can be measured simultaneously
Release of apoptotic proteins	i.e. Cytochrome C, Smac/Diablo, Htr/Omi, endonuclease G, AIF	Amount necessary to trigger apoptosis unknown	
Alterations in mitochondrial function:	Many assays available: - change in transmembrane potential - permeability transition - free radical generation		

	- calcium content - pH changes		
Changes in nucleus			
Nuclear condensation and fragmentation	Visualized by light/electron microscopy	Simple, quick method	
DNA fragmentation: TUNEL	DNA breaks detected Late apoptotic event	Well accepted assay	False positive staining of necrotic cells with DNA damage Expensive Time consuming Often requires harsh pretreatment
DNA fragmentation: Internucleosomal DNA cleavage (IDNAC)	First biochemical hallmark is cleavage of DNA at internucleosomal sites leading to nucleosomal fragments 180-200bp lengths Late apoptotic event	Further nucleosomal fragmentation can occur during preparation, hence may be difficult to see DNA ladder	Poor sensitivity IDNAC not always associated with apoptosis false positive results due to staining of necrotic cells, qualitative

Table 1: Techniques and assays available for apoptosis determination

Inducer	Tube 1	Tube 2	Tube 3	Tube 4
Actinomycin D (µM)	0.8	8	16	80
Bleach (1%)	0.01%	0.3%	0.6%	1%
Cyclohexamide (µM)	3.6	36	72	360
Staurosporin (µM)	0.2	2	4	20
Control	-	-	-	-
DMSO (vehicle, µM)	360	-	-	-

Table 2 Inducers of apoptosis and concentrations used

Treatment	Flow Cytometry	Light Microscopy	Plate Reader
A1	12	6, 12	-
A2	6, 12	6, 12	6
A3	6, 12	6, 12	-
A4	6, 12	6, 12	-
B1	3, 6, 12	3, 6	3
B2	0.5, 3, 6, 12	3	-
B3	3	-	0.5
B4	-	-	-
X1	3, 6, 12	6, 12	3
X2	3, 6, 12	6, 12	6
X3	3, 6, 12	3, 6, 12	6
X4	3, 6, 12	3, 6, 12	3, 6
S1	3, 6, 12	6, 12	6
S2	3, 6, 12	3, 6, 12	3, 6
S3	3, 6, 12	3, 6, 12	3, 6
S4	3, 6, 12	3, 6, 12	0.5, 6
Freeze-thaw	-	3	-
DMSO	-	-	-

Table 3 – Treatments and incubation periods which significantly increased the percentage of annexin-V positive cells above control levels

Figures

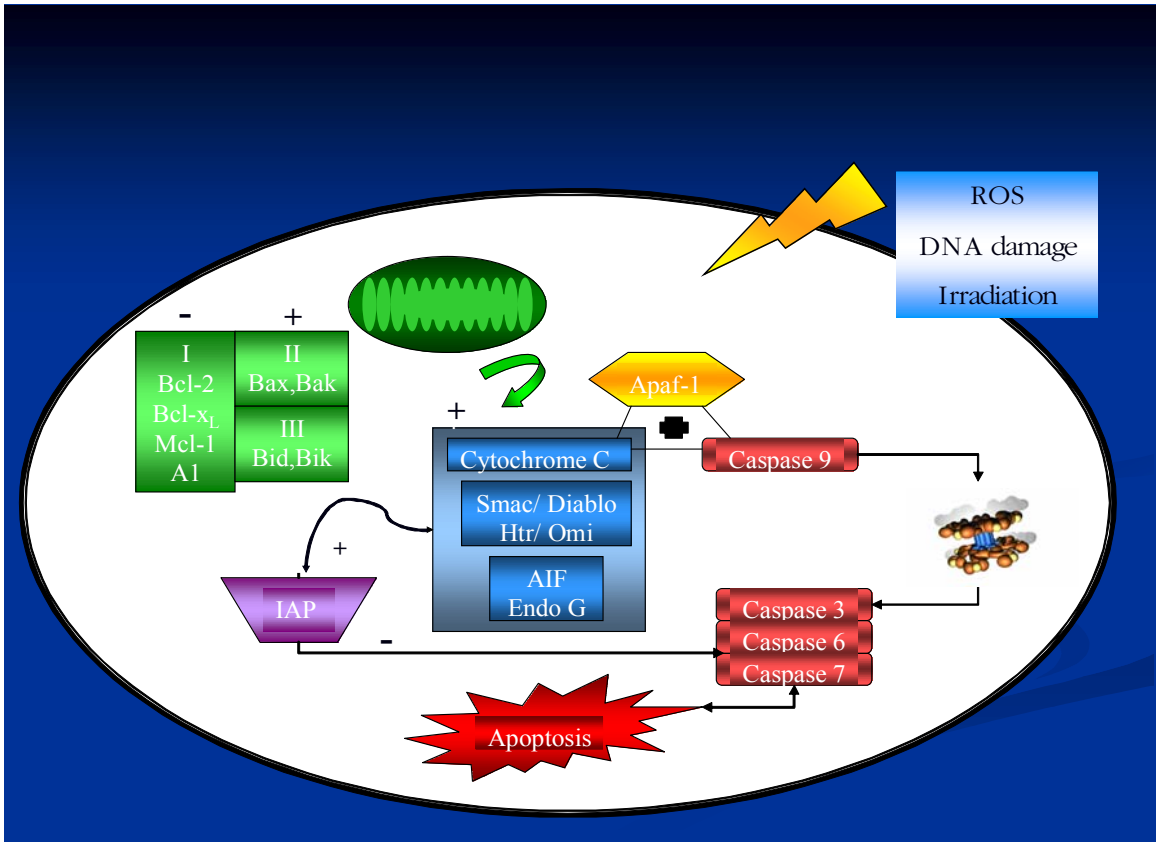


Figure 1a: Intrinsic/ mitochondrial initiated apoptosis pathway

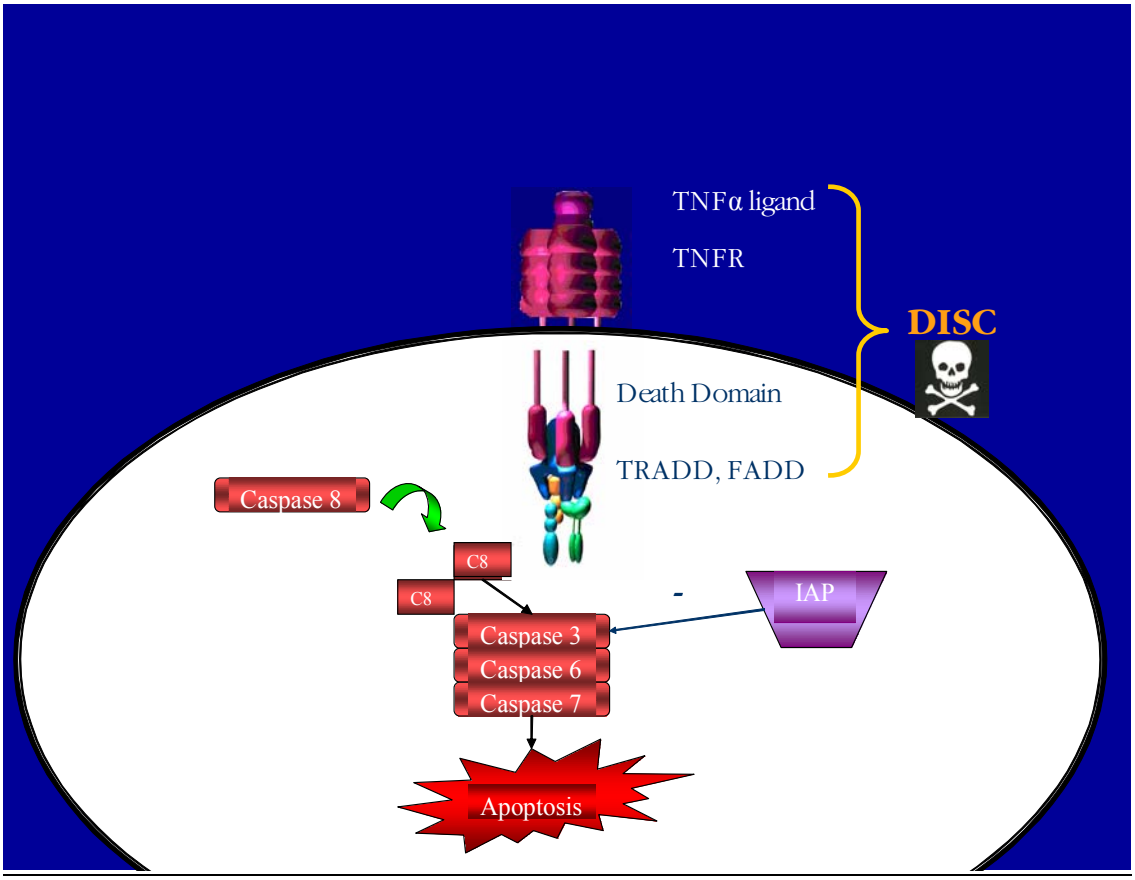


Figure 1b: Extrinsic/ cell surface death receptor mediated pathway

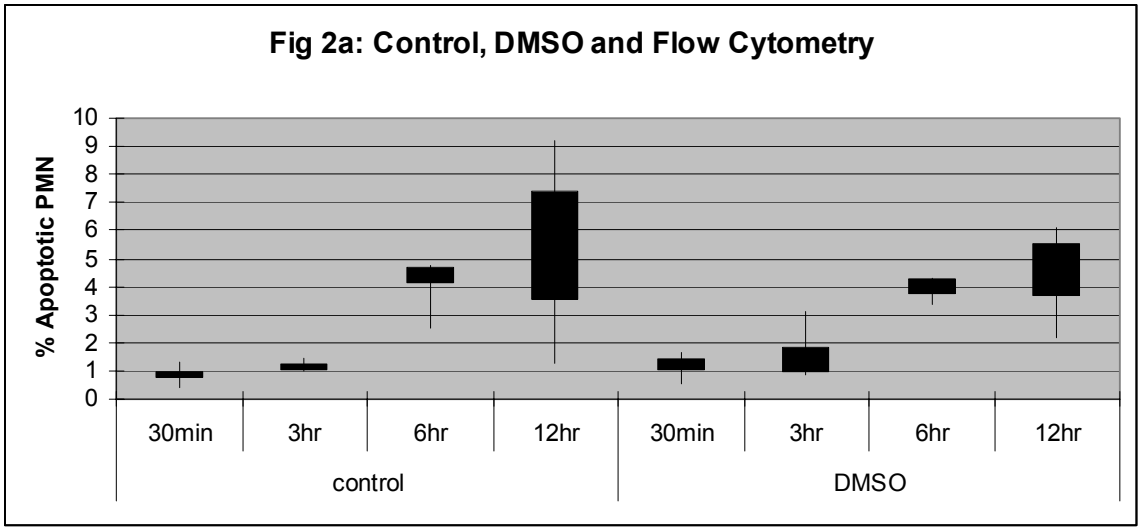


Fig 2a: Control, DMSO and Flow Cytometry
% Apoptotic PMN
10
9
8
7
6
5
4
3
2
1
0
30min
3hr
6hr
12hr
30min
3hr
6hr
12hr
control
DMSO

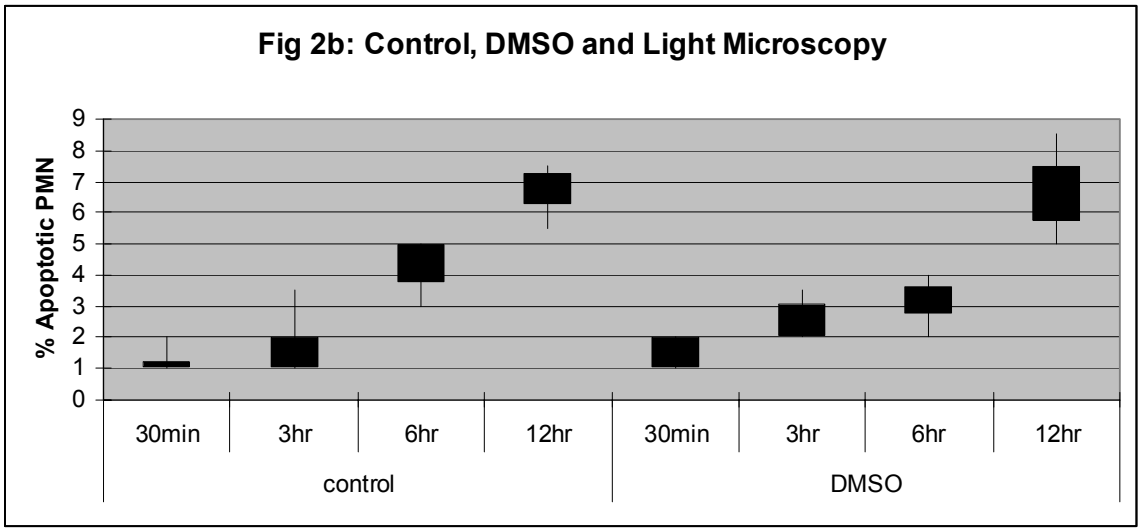


Fig 2b: Control, DMSO and Light Microscopy
% Apoptotic PMN
9
8
7
6
5
4
3
2
1
0
30min
3hr
6hr
12hr
30min
3hr
6hr
12hr
control
DMSO

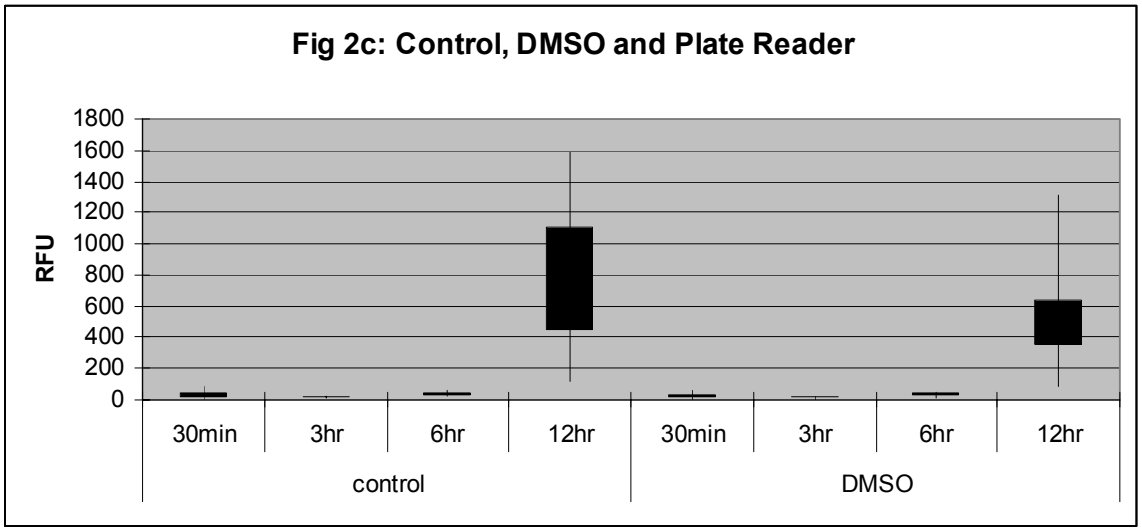
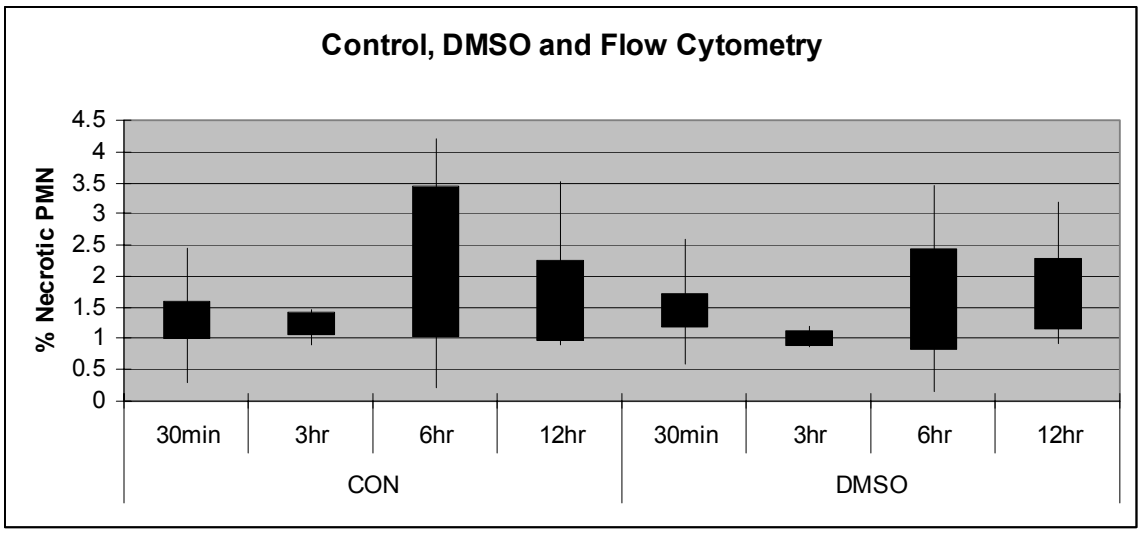


Fig 2c: Control, DMSO and Plate Reader
RFU
1800
1600
1400
1200
1000
800
600
400
200
0
30min
3hr
6hr
12hr
30min
3hr
6hr
12hr
control
DMSO


Control, DMSO and Flow Cytometry
% Necrotic PMN
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
30min
3hr
6hr
12hr
30min
3hr
6hr
12hr
CON
DMSO

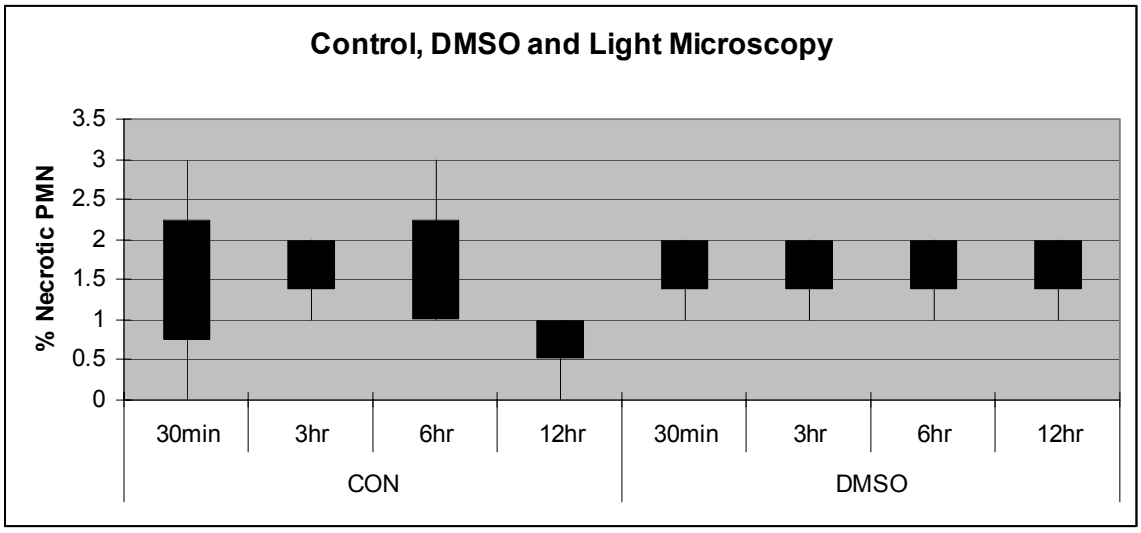

Control, DMSO and Light Microscopy
% Necrotic PMN
3.5
3
2.5
2
1.5
1
0.5
0
30min
3hr
6hr
12hr
30min
3hr
6hr
12hr
CON
DMSO

Fig 3: Actinomycin D and Flow Cytometry

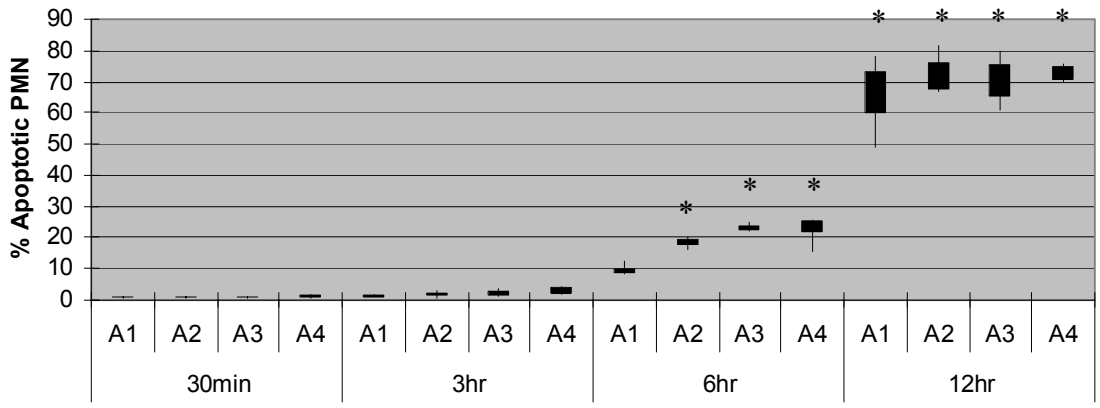


Fig 4: Actinomycin D and Light Microscopy

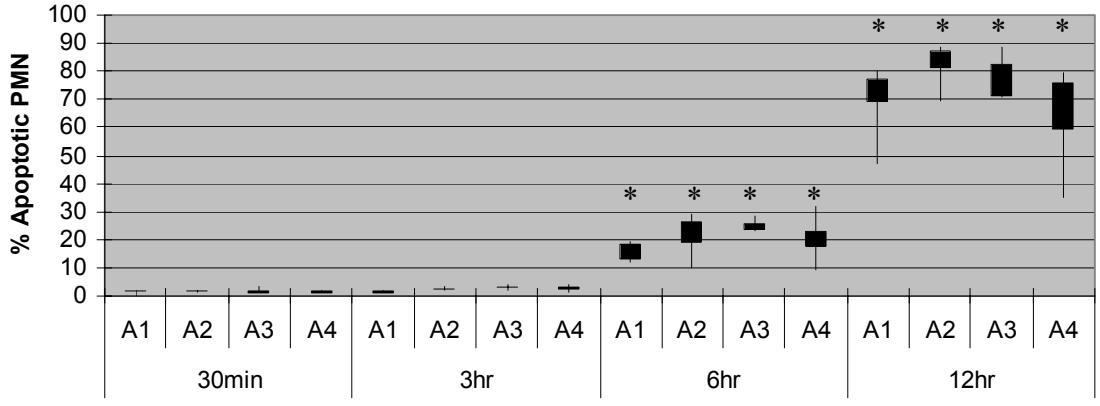


Fig 5: Actinomycin D and Plate Reader

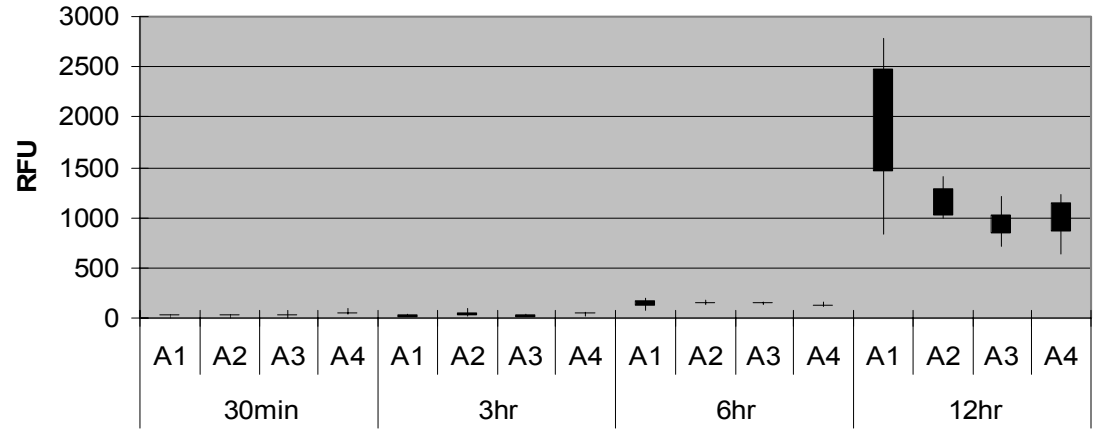


Fig 6: Staurosporin and Flow Cytometry

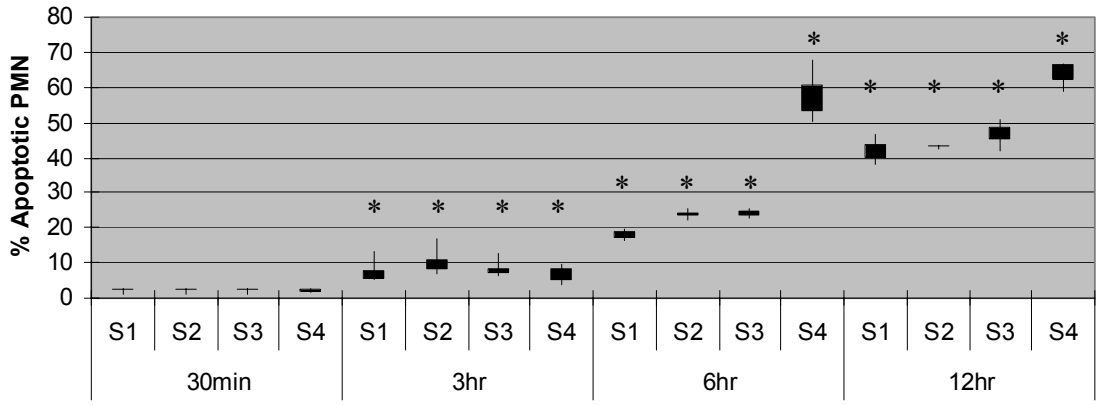


Fig 7: Staurosporin and Light Microscopy

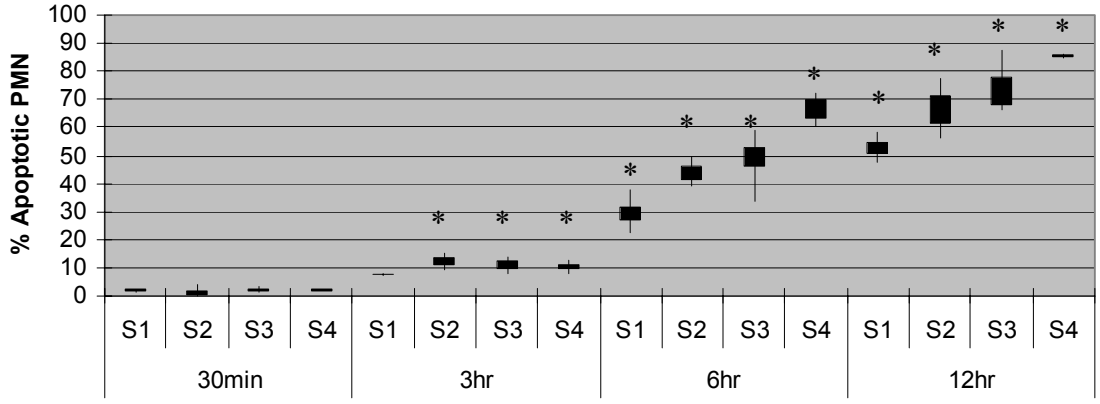


Fig 8: Staurosporin and Plate Reader

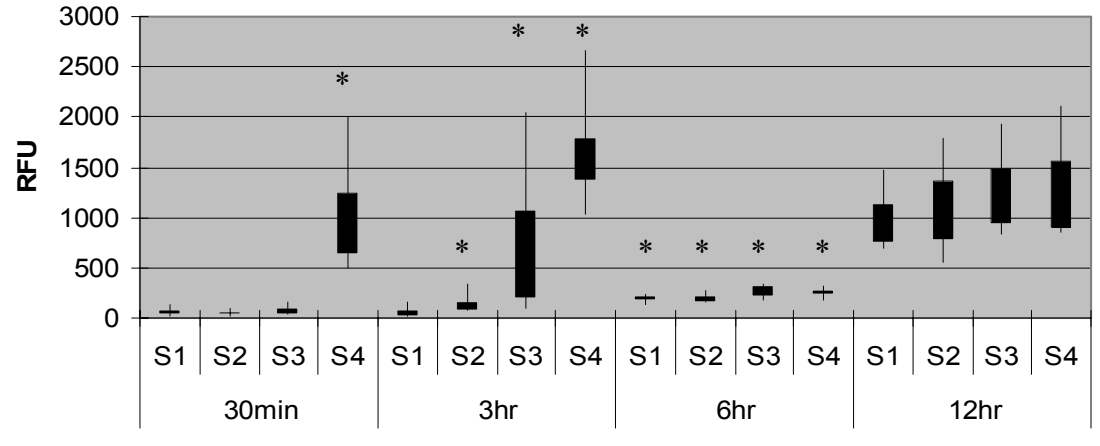


Fig 9: Cyclohexamide and Flow Cytometry

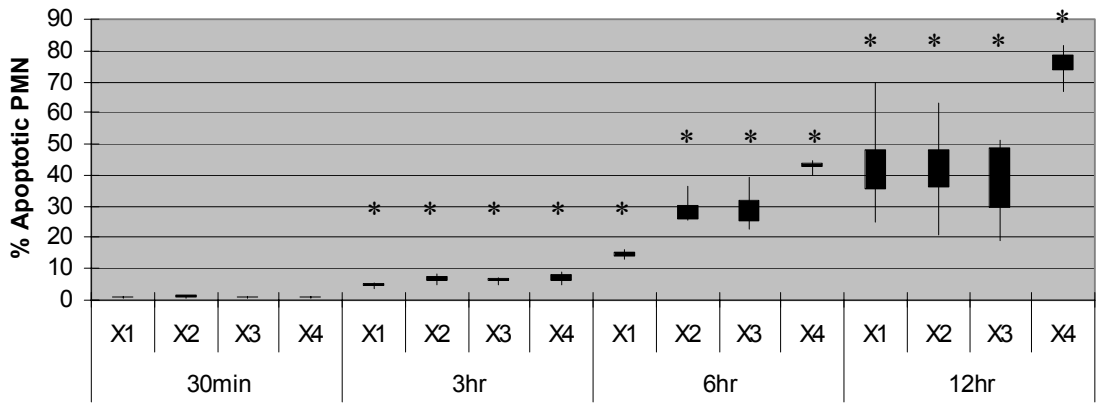


Fig 10: Cyclohexamide and Light Microscopy

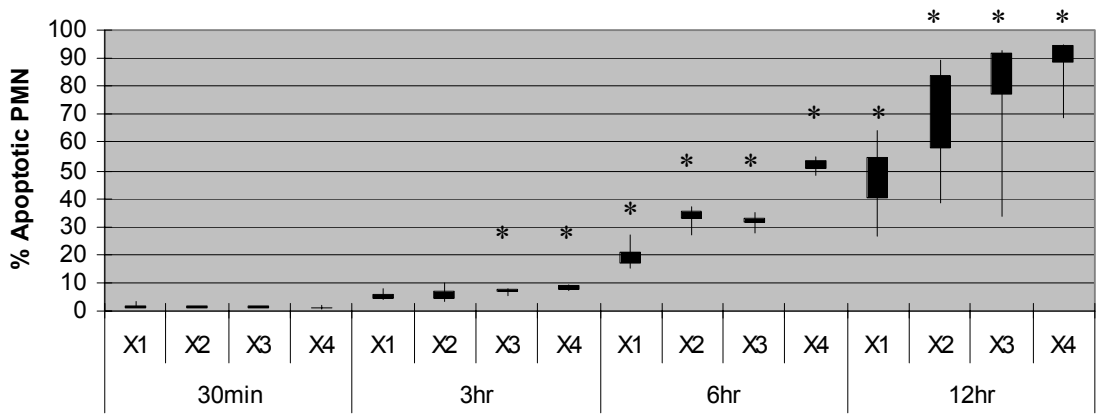


Fig 11: Cyclohexamide and Plate Reader

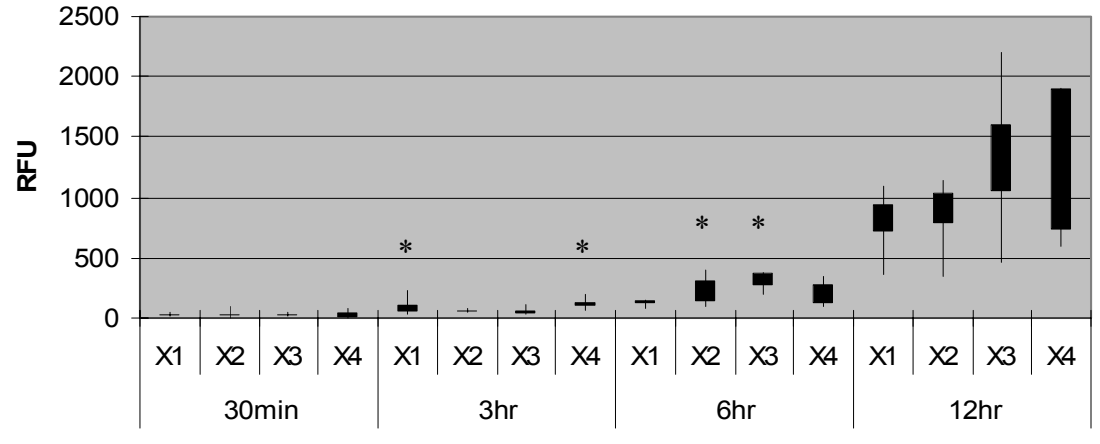


Fig 12: Bleach and Flow Cytometry

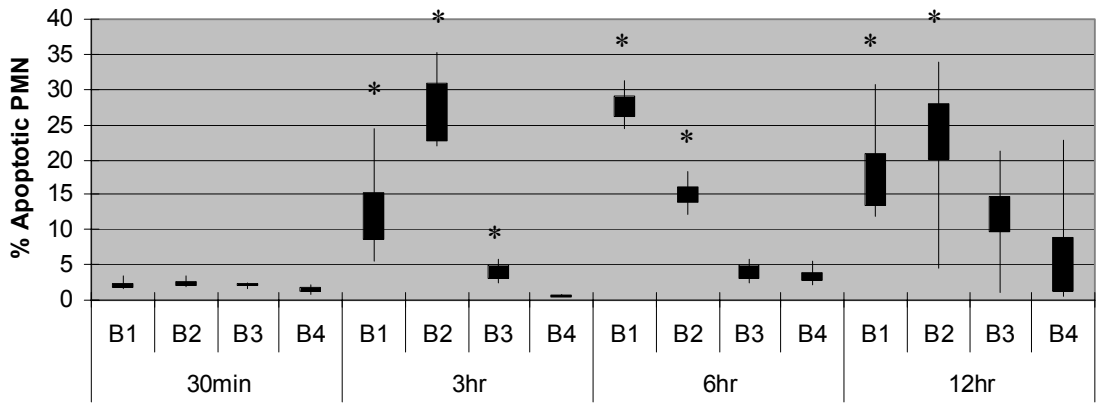


Fig 13: Bleach and Light Microscopy

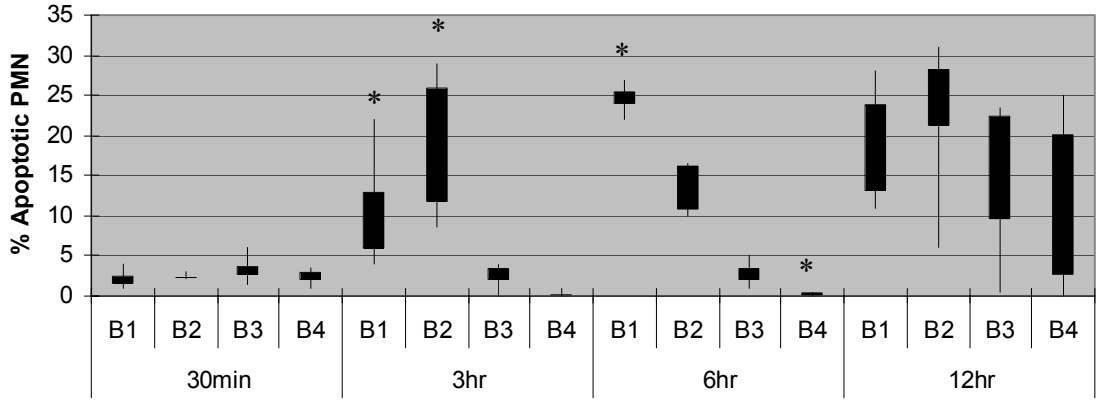


Fig 14: Bleach and Plate Reader

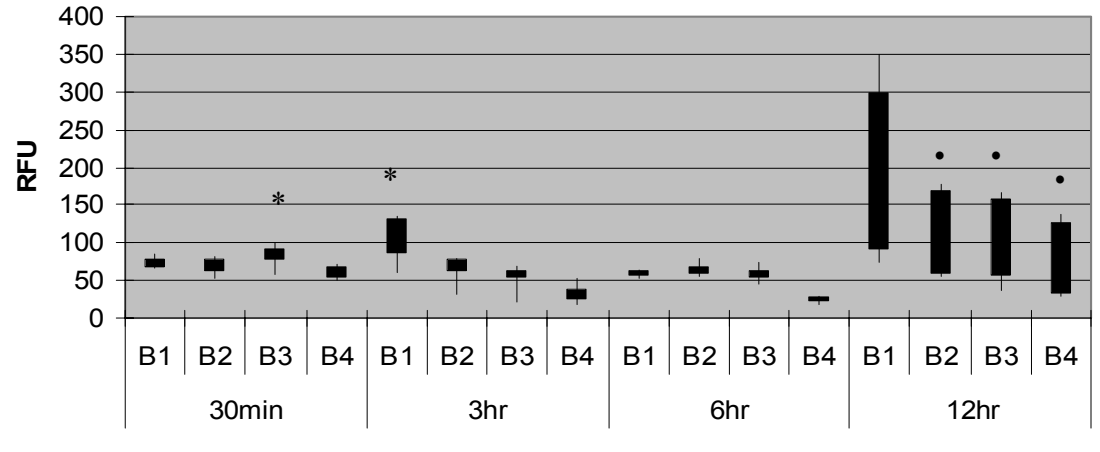


Fig 15: Actinomycin D and Flow Cytometry

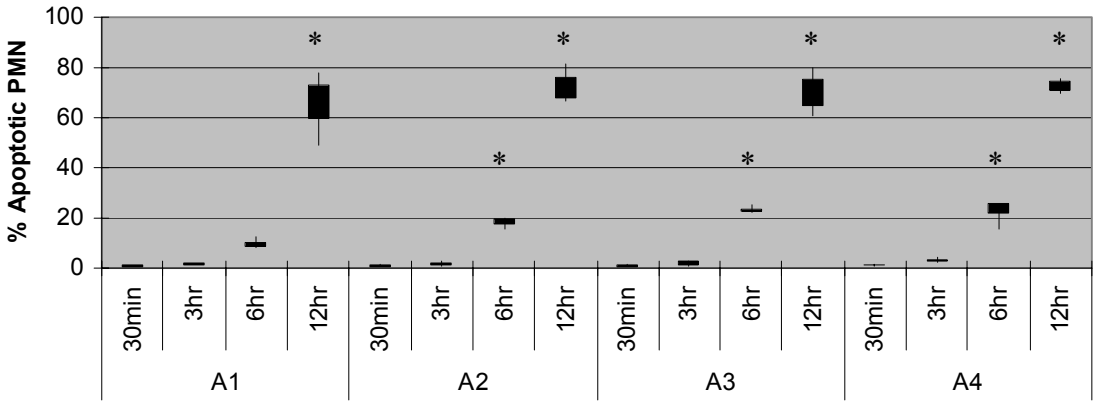


Fig 16: Actinomycin D and Light Microscopy

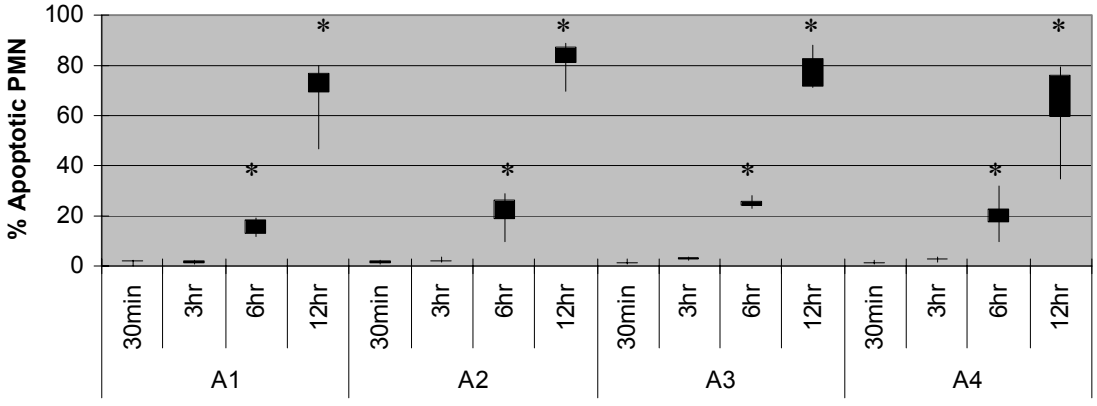


Fig 17: Actinomycin D and Plate Reader

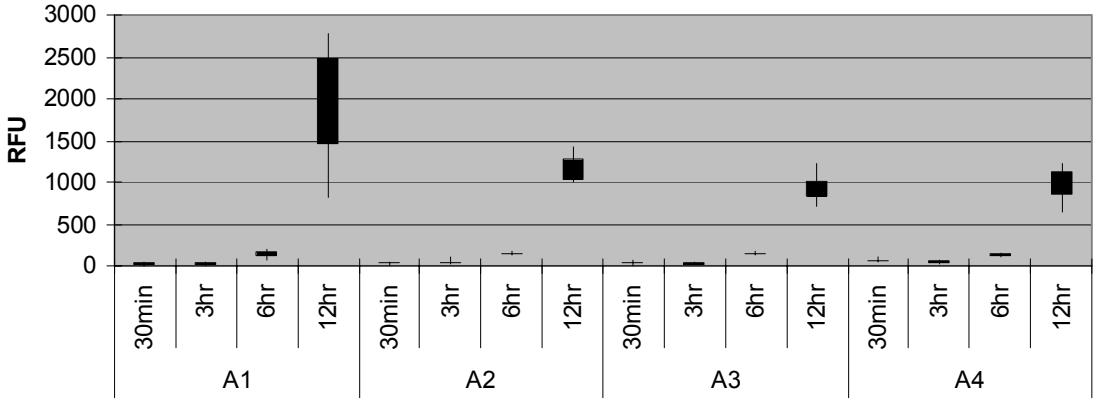


Fig 18: Staurosporin and Flow cytometry

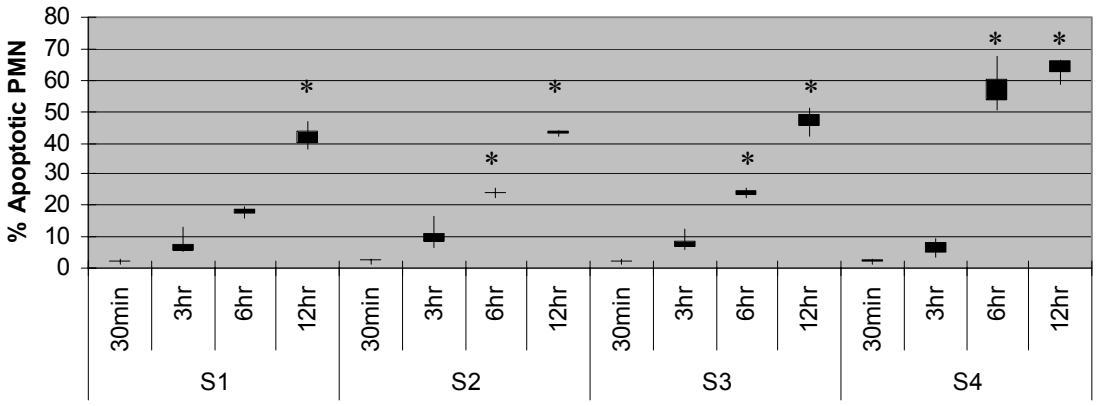


Fig 19: Staurosporin and Light Microscopy

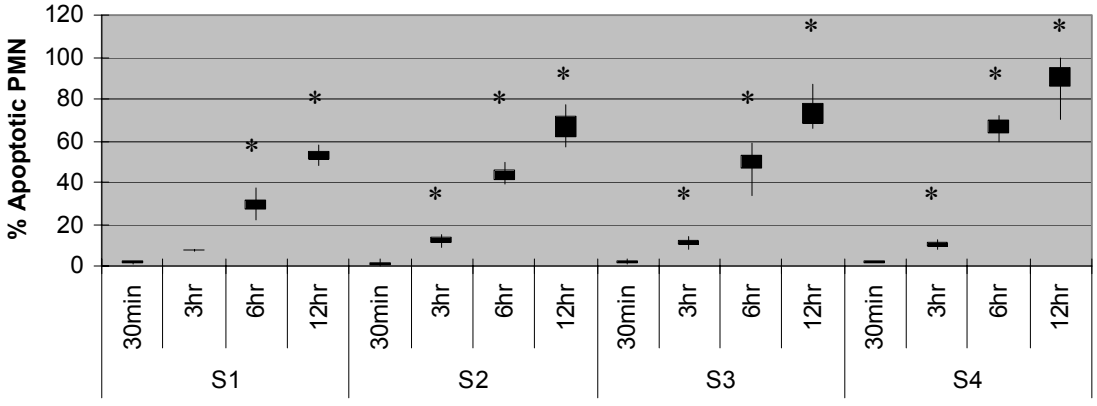


Fig 20: Staurosporin and Plate Reader

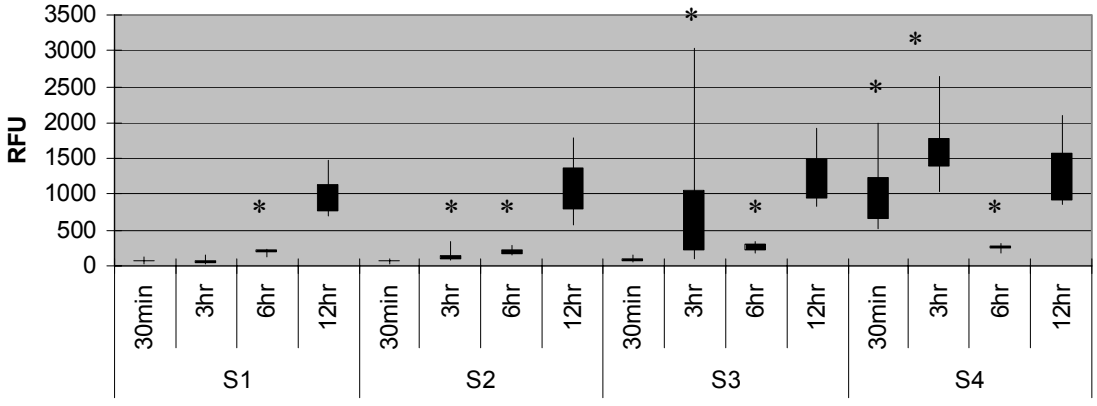


Fig 21: Cyclohexamide and Flow Cytometry

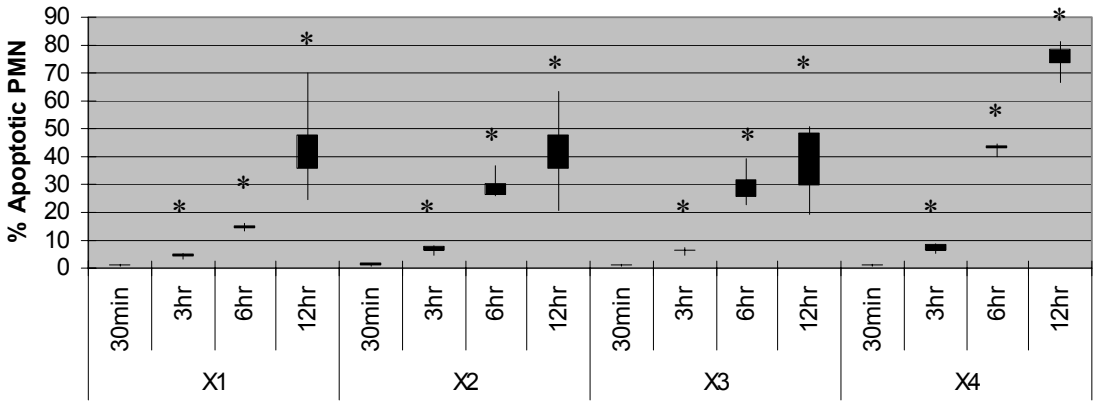


Fig 22: Cyclohexamide and Light Microscopy

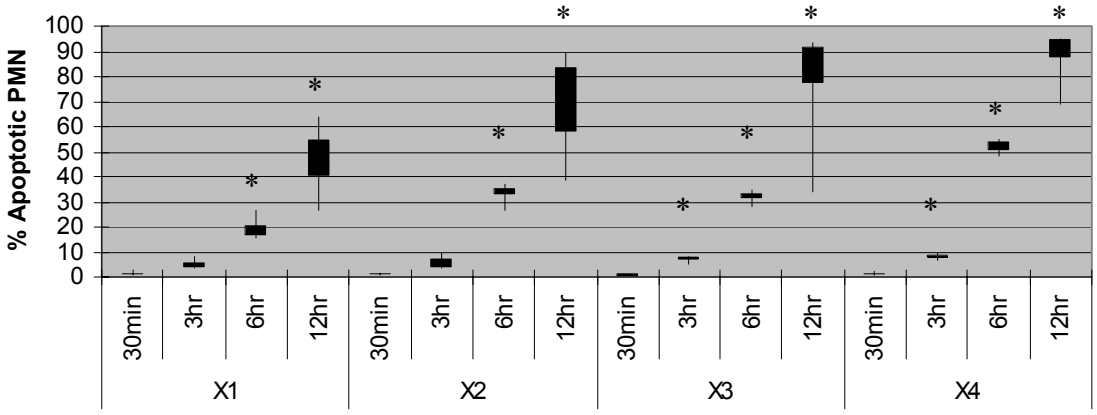


Fig 23: Cyclohexmide and Plate Reader

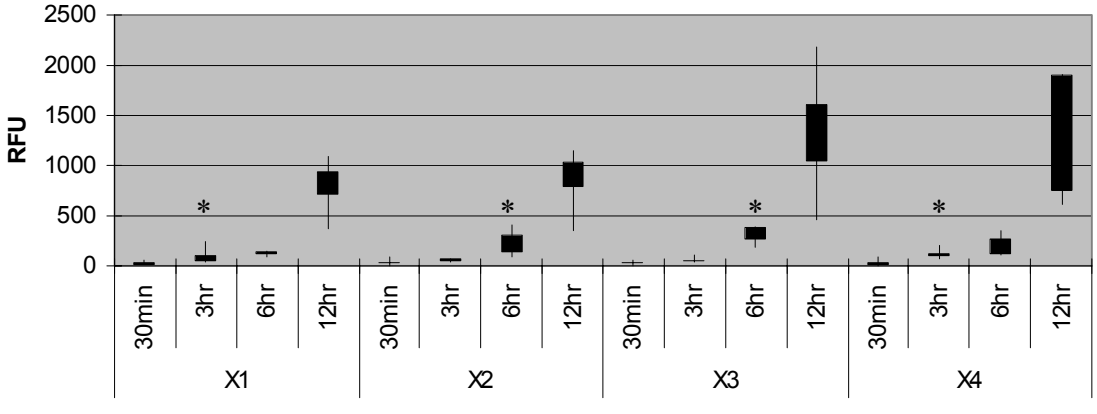


Fig 24: Bleach and Flow cytometry

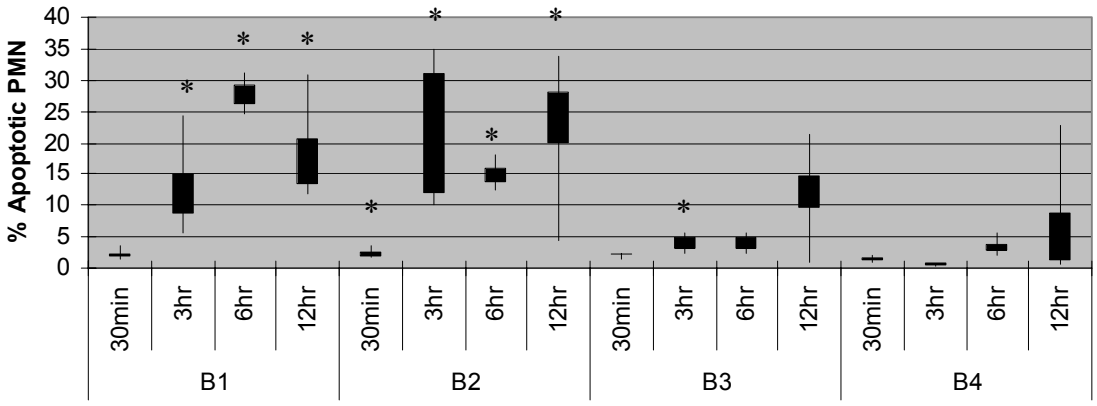


Fig 25: Bleach and Light Microscopy

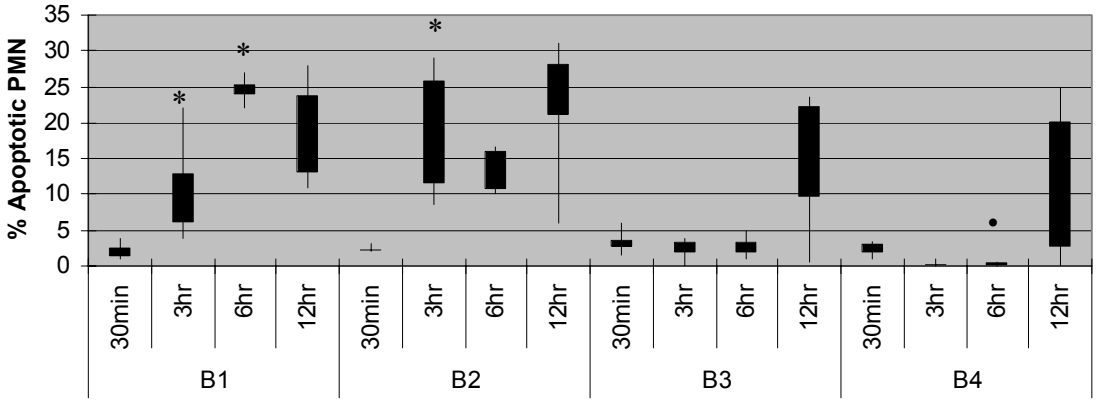


Fig 26: Bleach and Plate Reader

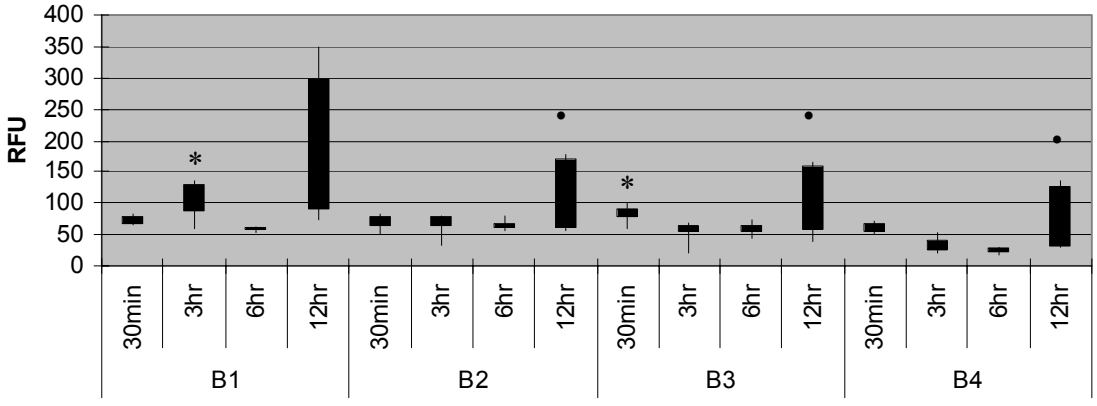


Fig 27: Bleach and Flow Cytometry

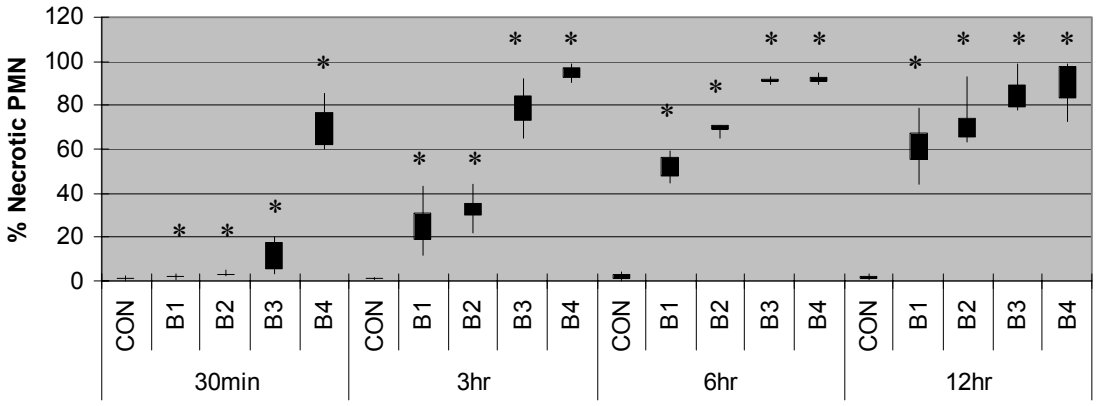


Fig 28: Bleach and Light Microscopy

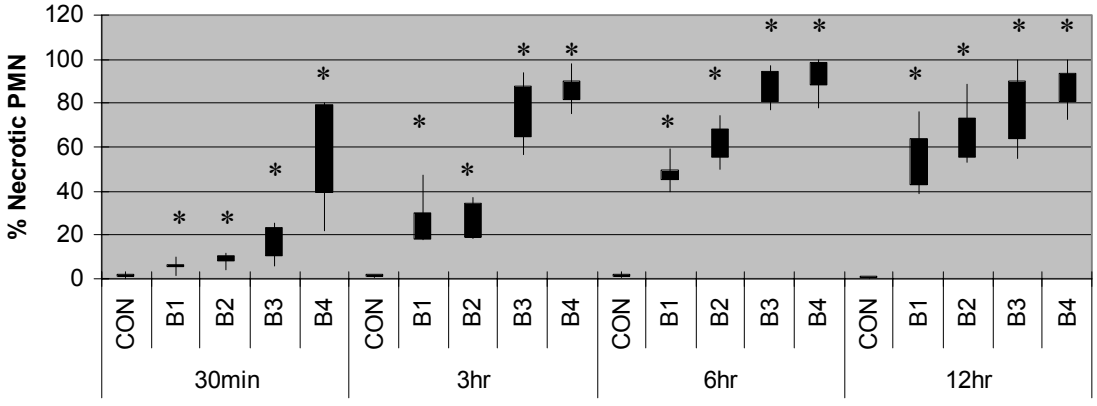


Fig 29: Freeze-thaw and Flow Cytometry

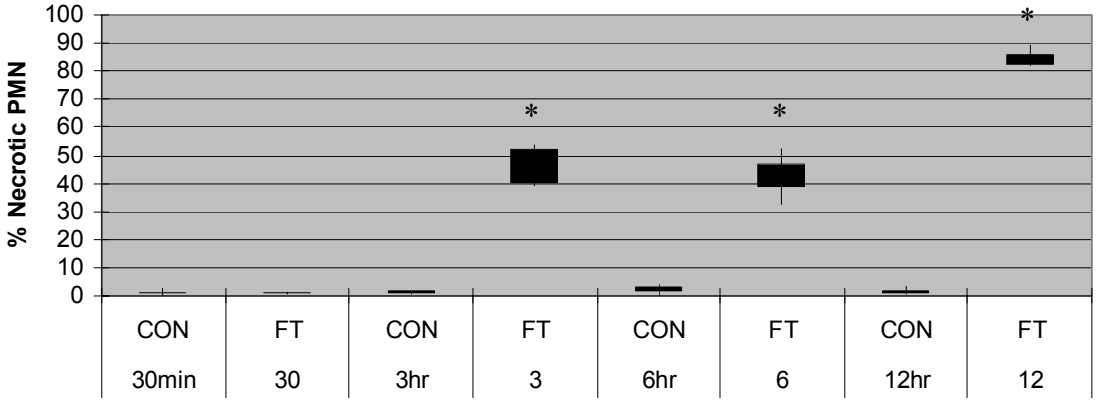


Fig 30: Freeze-thaw and Light Microscopy

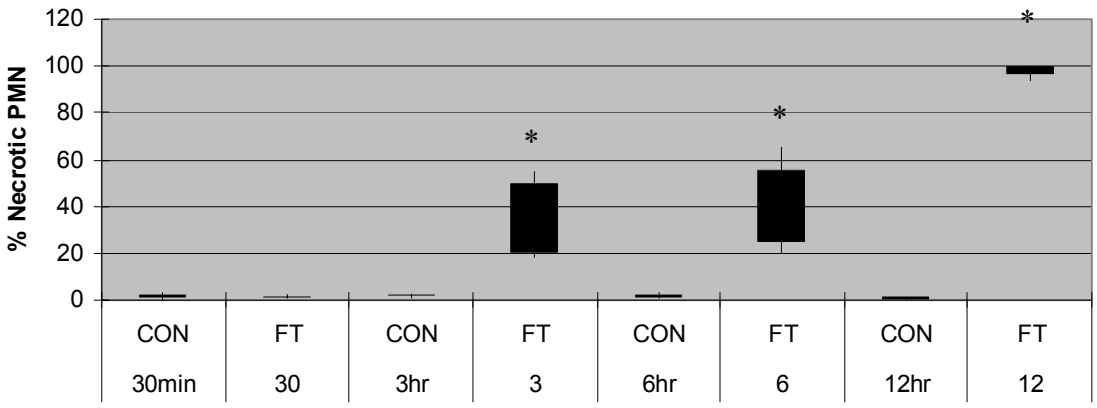


Fig 31: Bleach and Flow cytometry

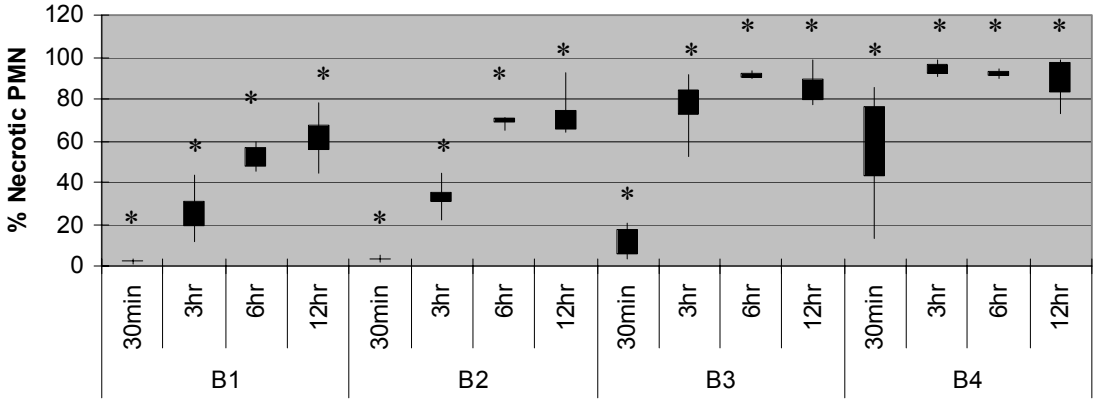
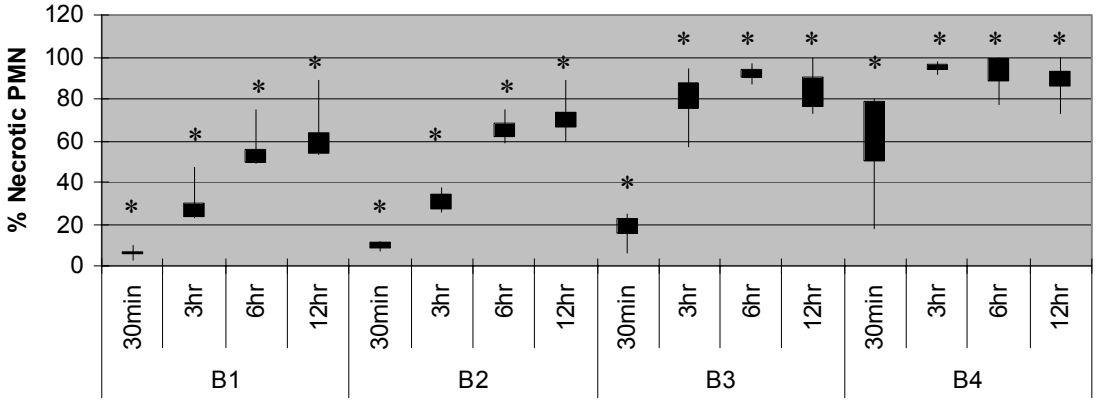


Fig 32: Bleach and Light Microscopy



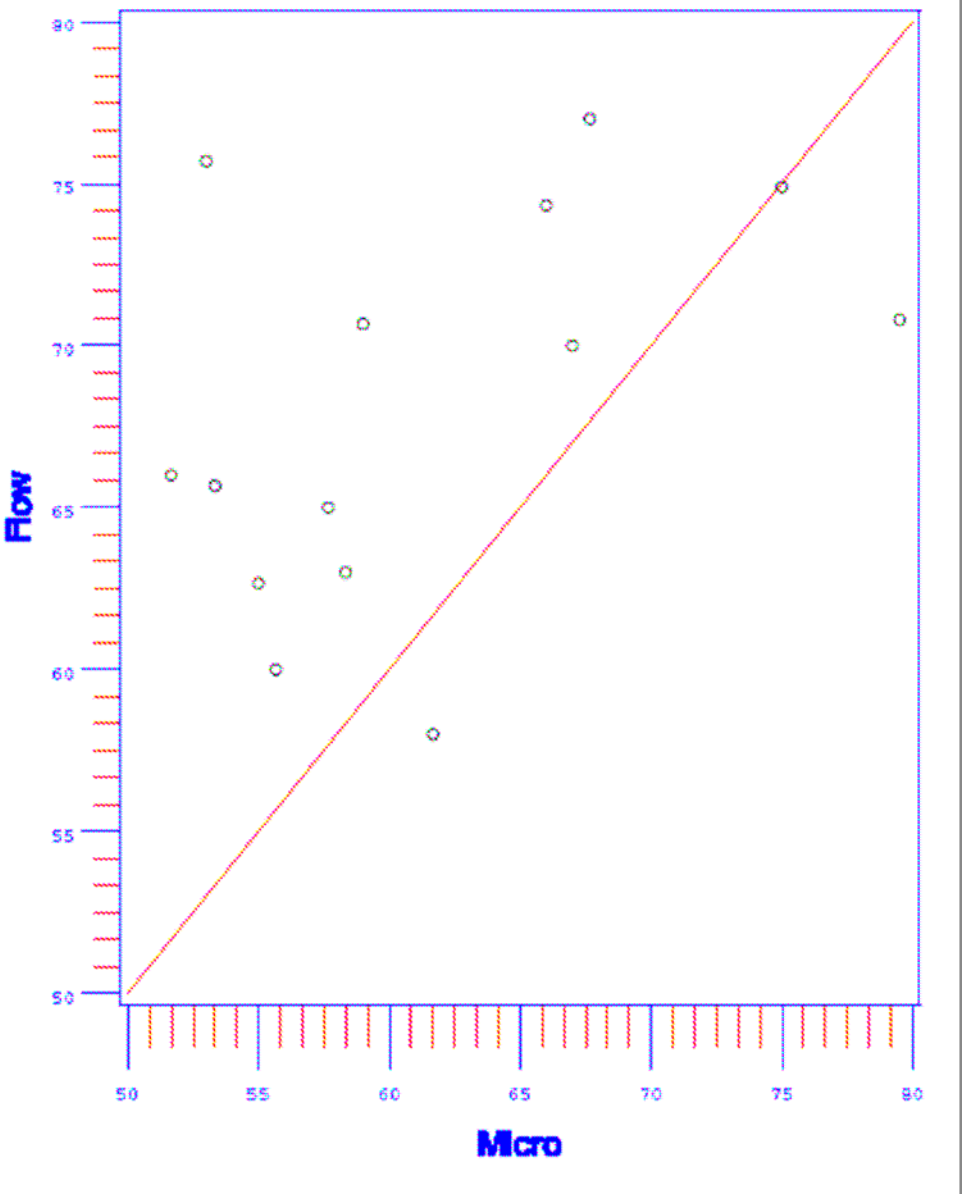


Figure 33 – Percentage apoptosis measured by flow cytometry and light microscopy, with line of equality

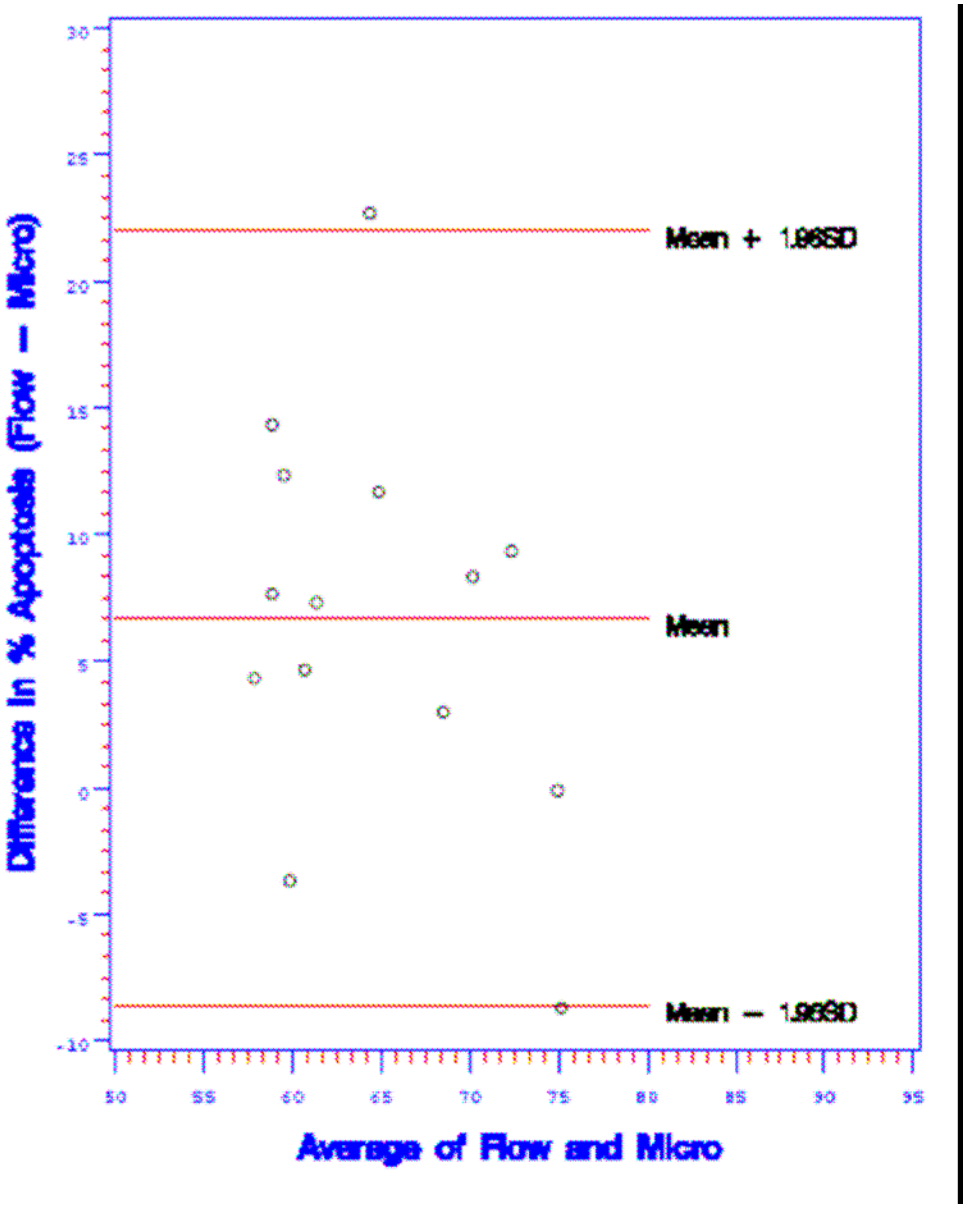
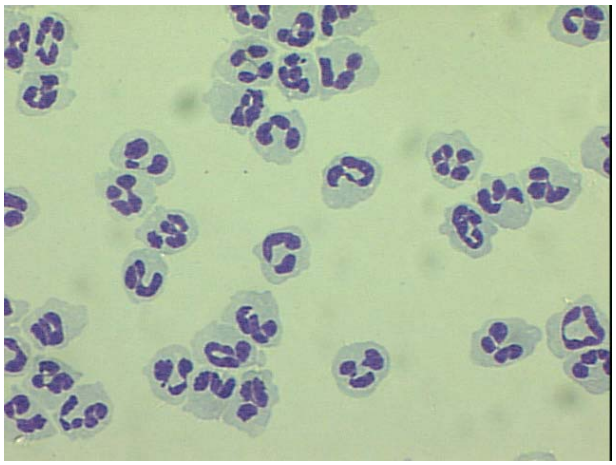


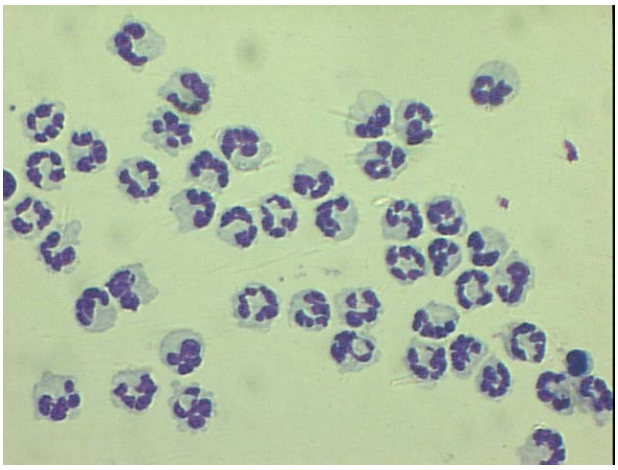
Figure 34: % apoptosis: difference (FC – LM) versus average of values (FM + LM) with 95% limits of agreement

Fig 35 – Control cells

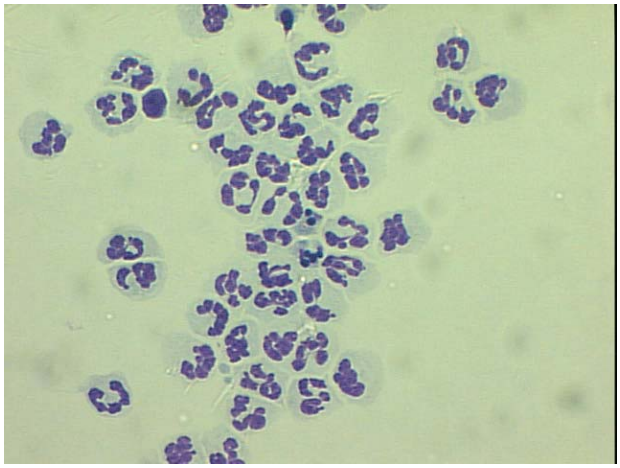
A. 30 minutes



B. 3 hours



C. 6 hours



D. 12 hours

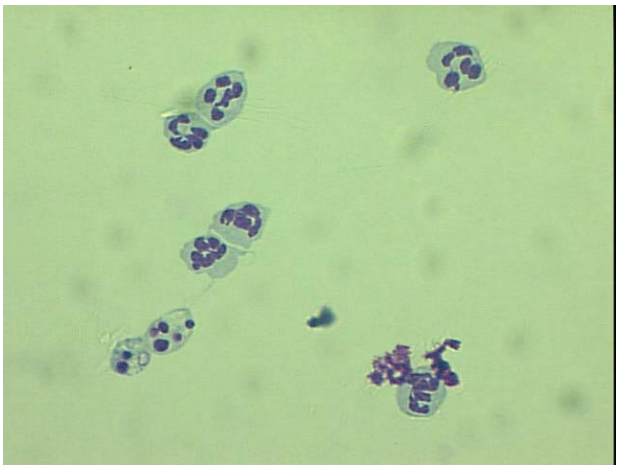
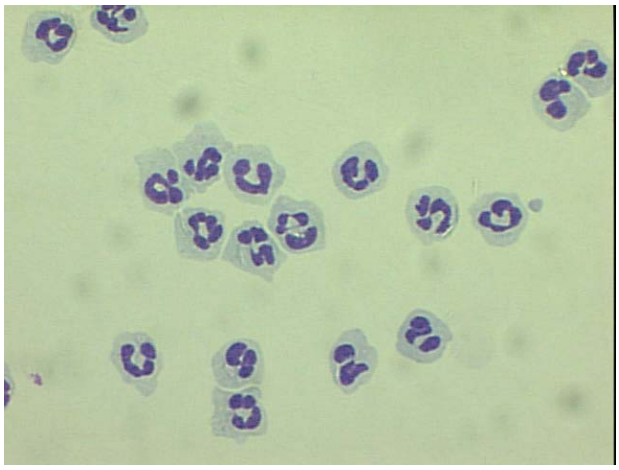
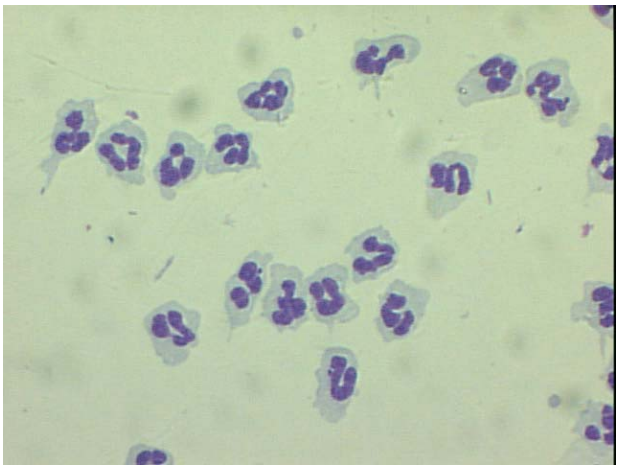


Figure 36 – Actinomycin D

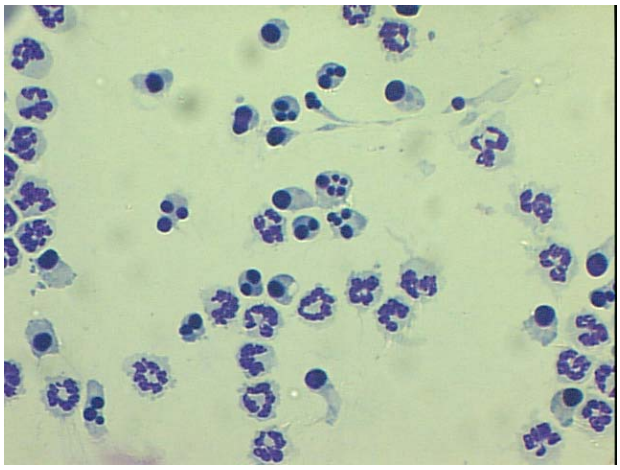
A. 30 minutes



B. 3 hours



C. 6 hours



D. 12 hours

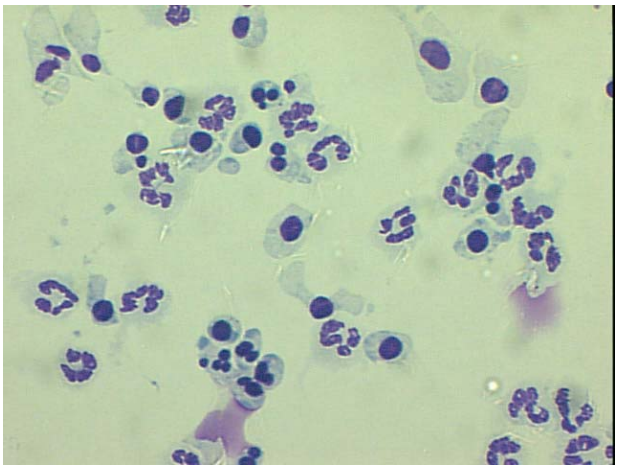
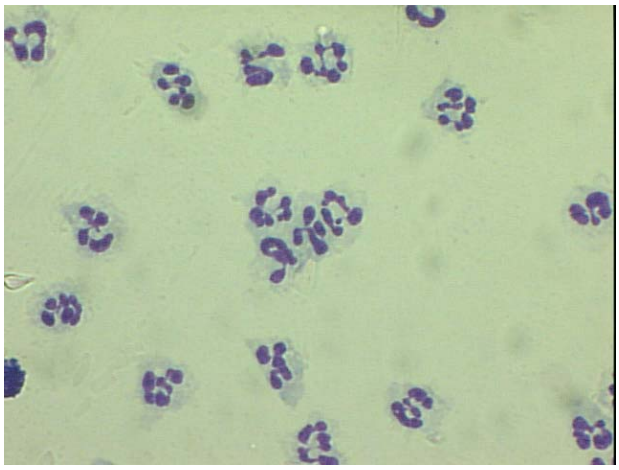
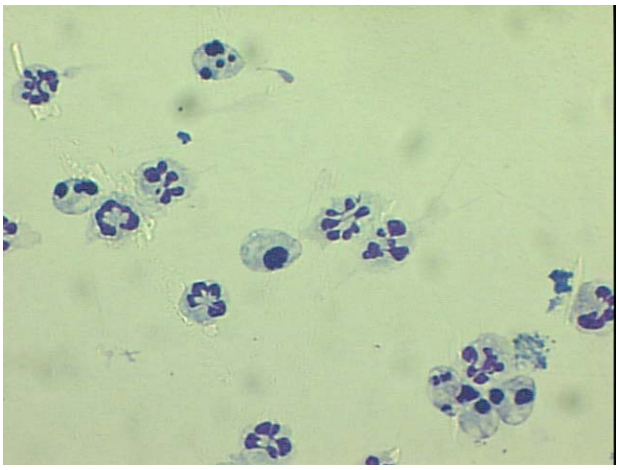


Figure 37 – Staurosporin

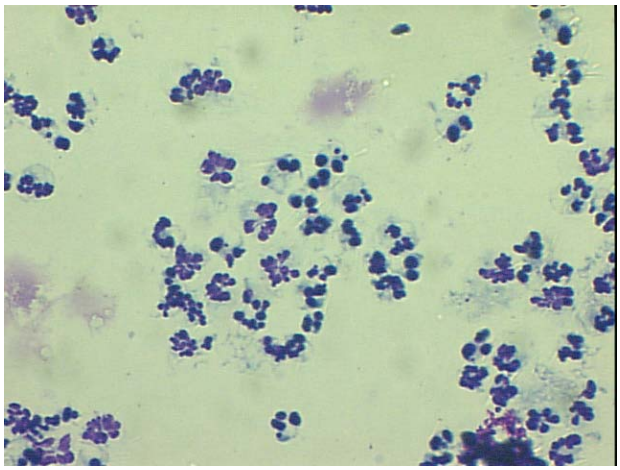
A. 30 minutes



B. 3 hours



C. 6 hours



D. 12 hours

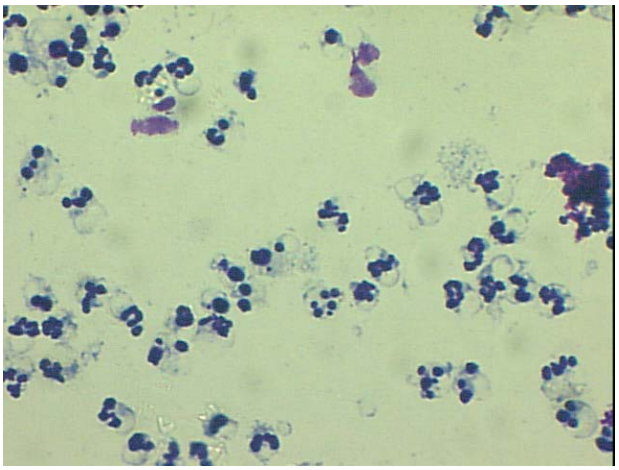
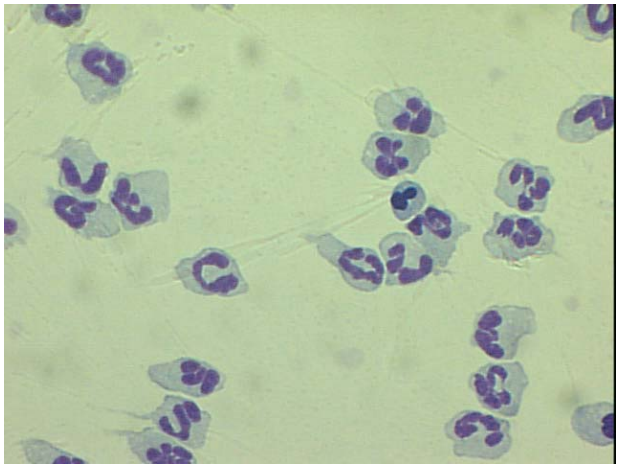
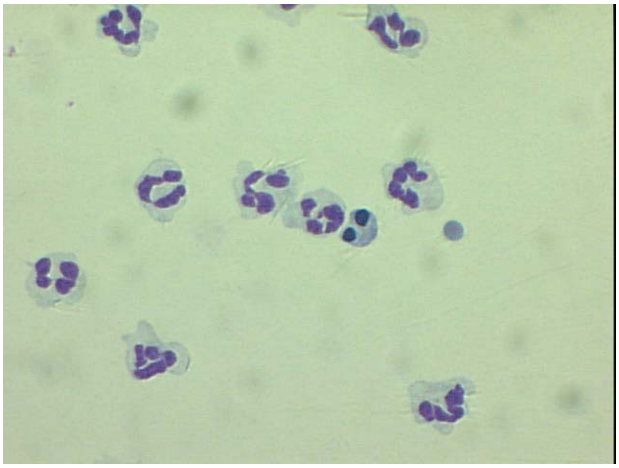


Figure 38 – Cyclohexamide

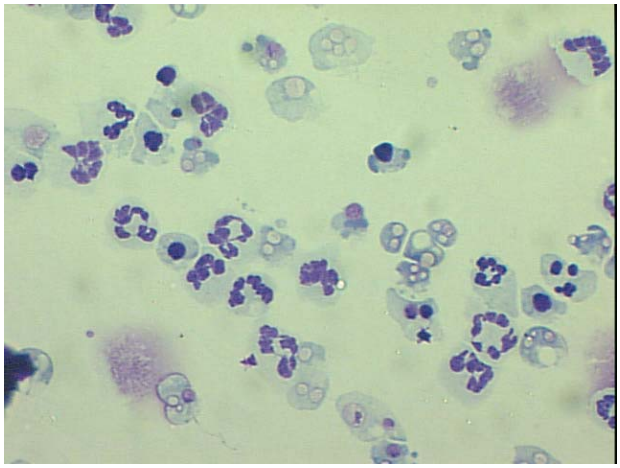
A. 30 minutes



B. 3 hours



C. 6 hours



D. 12 hours

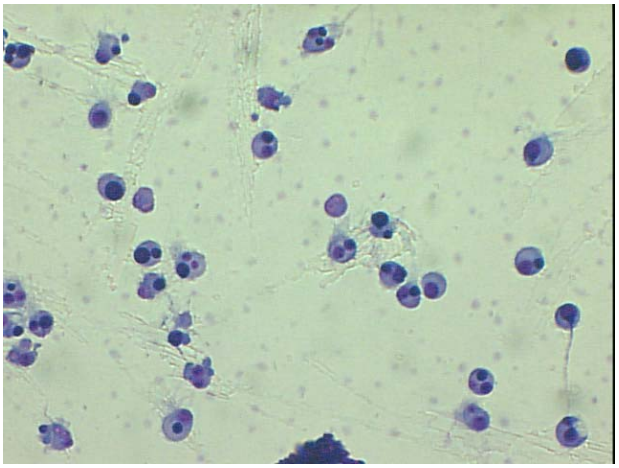
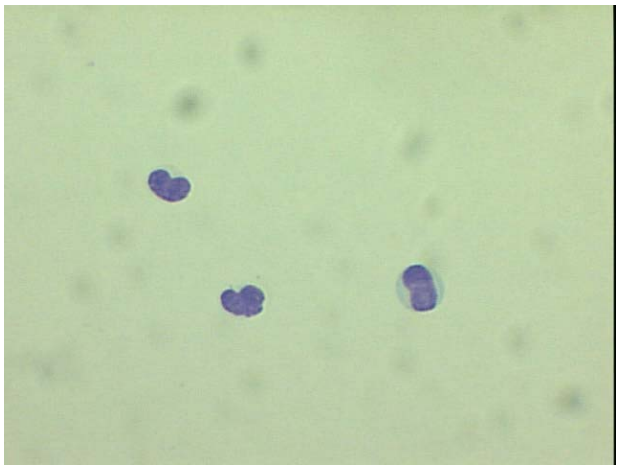
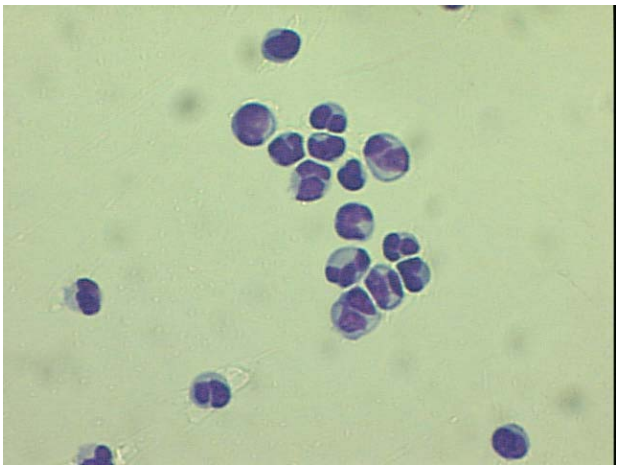


Figure 39 – Bleach

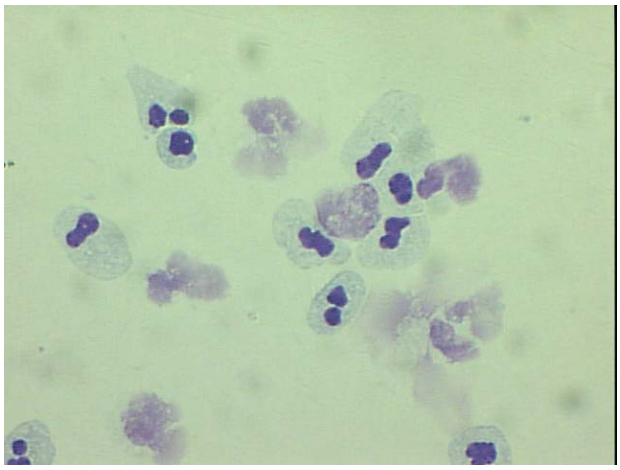
A. 30 minutes



B. 3 hours



C. 6 hours



D. 12 hours

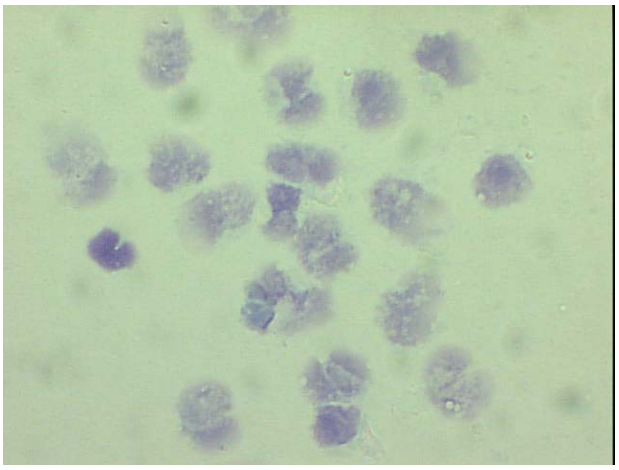
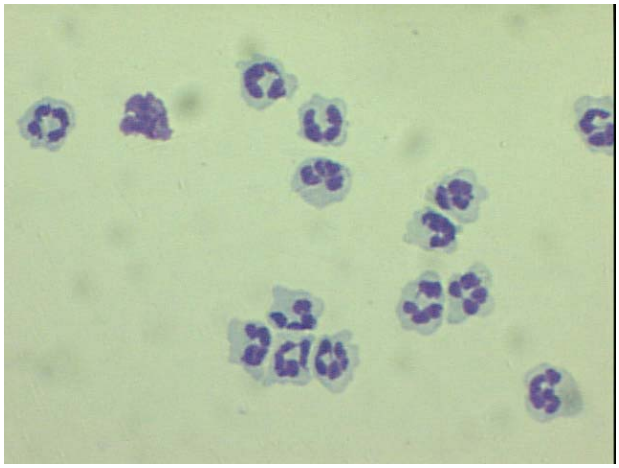
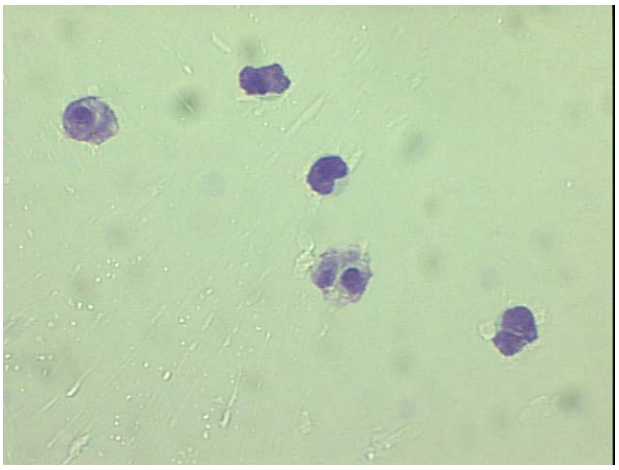


Figure 40 – Freeze-thaw

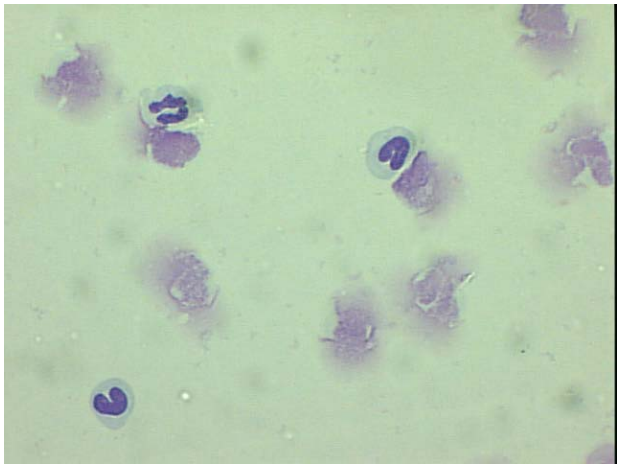
A. 30 minutes



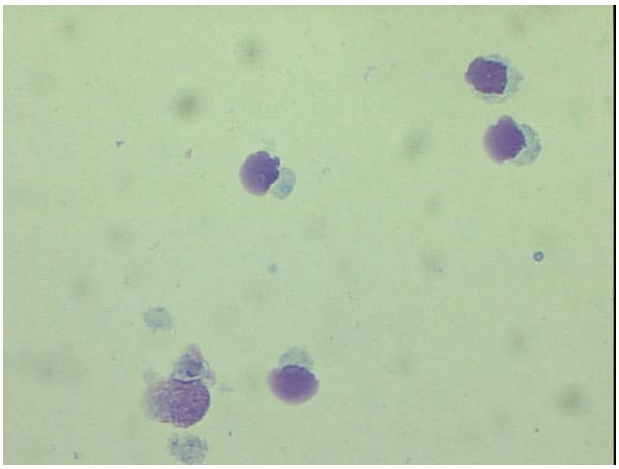
B. 3 hours



C. 6 hours



D. 12 hours



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