Expression of pro-inflammatory proteins in the lung epithelial cell line A549, in response to cytokine and environmental particle exposure.

Lindsay Ramage

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Declaration

I declare that this thesis was written by myself and that all the work contained within it was carried out by me, unless otherwise stated.

Acknowledgements

Firstly I would like to thank Dr Keith Guy for his supervision and support over the past few years. I would also like to thank the whole of the Biomedicine Research Group for their friendship, particularly Drs David Brown and Janet Lightbody who provided invaluable help in the lab. Thanks also to the rest of the School of Life Sciences who supported and entertained me over the course of this project.

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Summary

This thesis investigates the effects of various inflammatory stimuli, including cytokines and air pollution particles, on the expression and secretion of various pro-inflammatory proteins in the lung epithelial cell line A549. The pro-inflammatory proteins investigated were C-reactive protein (CRP), fibrinogen, and heat shock protein 70 (Hsp70), all of which are known to be produced during inflammation and are also known risk factors for cardiovascular disease. These proteins were investigated because inhalation of particulate air pollution has been associated with increased cardiovascular disease and mortality. The production of these proteins in the lung may be involved in a systemic inflammatory response which increases the plasma levels of these proteins and other cardiovascular risk factors therefore increasing the susceptibility for cardiovascular events. Alternatively, localised CRP functions as an opsonin, while fibrinogen and its degradation products are involved in the recruitment of neutrophils and leukocytes to the lung.

Both CRP and fibrinogen are known to be produced in hepatocytes in response to cytokines. Chapter 3 investigates the effect of cytokine treatment of the A549 cells which shows that CRP and fibrinogen can be induced in this cell line by various cytokines. CRP was found to be induced by the cytokines IL1β, IL6, IL8, TNFα and IFNγ with increased effects shown by simultaneous treatment with two cytokines. Fibrinogen was found to be induced mainly by IL6, but fibrinogen levels were also slightly increased by IL1β; simultaneous treatments of IL6 with either IL1β or TNFα reduced the effect of IL6 treatment. Treatment of A549 cells with IL6 and IL8 simultaneously induced a synergistic effect.

After establishing that cytokines induce the expression of CRP in A549 cells, the effects of particulate air pollution on pro-inflammatory protein expression were then investigated (chapter 4). The cells were treated with carbon black (CB), ultrafine CB (ufCB), and iron chloride ($FeCl_3$) to find out what effect these air pollution components and PM10 would have on the expression of CRP, fibrinogen, Hsp70 and the transcription factor NFκB and its inhibitor IκB. NFκB is known be activated by PM10 and is involved in the signalling of pro-inflammatory responses. All the

particulate treatments induced a pro-inflammatory response with expression and secretion of CRP, fibrinogen, and Hsp70, whereas the soluble metal treatment had little effect. The metal salt $FeCl_3$ was used to treat the cells since it has been suggested that PM10 may mediate its effect through the presence of transition metals which are implicated in oxidant generation. The particulate exposures were also associated with the activation and nuclear translocation of NFκB indicating the involvement of NFκB and IκB in the induction of the pro-inflammatory response.

Investigations into the mechanisms by which the particles induced the pro-inflammatory response are discussed in chapter 5. Firstly, investigations into the effect of particles on the secretion of the pro-inflammatory proteins were carried out; these indicated that particles were capable of inducing the secretion of CRP, fibrinogen and Hsp70. Investigations into the transcriptional mechanisms of pro-inflammatory protein expression were carried out using specific inhibitors. CRP and fibrinogen were induced in an NFκB dependent manner, while Hsp70 was produced as a result of activation of the JAK/STAT pathway. The effect of oxidative stress being induced as a result of particle exposure was investigated using antioxidants. This showed a reduction in the amount of intracellular and secreted CRP, fibrinogen and Hsp70 in the presence of antioxidants, indicating that oxidative stress was involved. This correlated with a reduction in the levels of intracellular ATP.

Finally in chapter 6 the effects of proteins secreted from A549 cells on the monocyte-like cell line MM6 were examined. This was carried out as a model of the effects of secreted pro-inflammatory proteins from the lung epithelium on other cells. It was found that CRP and fibrinogen were able to induce the activation of NFκB in MM6 cells, indicating that secretion of these proteins during lung inflammation could have an effect on other resident lung cells. Conditioned medium from A549 cells was also found to induce the expression of pro-inflammatory proteins including CRP, fibrinogen, Hsp70, IL6, IL8 and TNFα. The A549 conditioned medium also appeared to induce a stress response and cellular damage in MM6 cells, thereby potentially exacerbating the inflammatory response.

These results indicate that pro-inflammatory proteins can be produced in lung epithelial cells in response to particle exposure as a result of oxidative stress. They may be involved in the progression of localised lung inflammation and in the systemic response to particulate air pollution.

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Abbreviations

AM – alveolar macrophages

APP – acute phase proteins

APR – acute phase response

ATP – adenosine tri-phosphate

CB – fine carbon black particles

CRP – C-reactive protein

HSF – heat shock transcription factor

Hsp – heat shock protein

HSP – heat shock family of stress proteins

IFN-γ – interferon γ

IL – interleukin (e.g. IL1, IL6, IL8)

IκB – inhibitor of NFκB

JAK –Janus kinase

MM6 – Monomac 6 cell line

NAL - nacystelin

NFκB – nuclear factor κB

NLS – nuclear localisation sequence.

PM10 – environmental air pollution particles with diameter of 10 microns

ROS – reactive oxygen species

STATS - signal transducers and activators of transcription.

tBHP – tert- butyrl hydrogen peroxide

TLR – Toll-like receptors

TNFα - Tumour necrosis factor α

ufCB – ultrafine carbon black particles

Chapter 1

Introduction.

1.1.0 The lung

The main function of the lung is to supply oxygen to the body and to remove the waste product carbon dioxide. The airways consist of branching tubes which become shorter, narrower and more numerous as they penetrate deeper into the lung. The upper areas of the lung known as the conducting zone, consist of the trachea, bronchi and terminal bronchioles. The function of these is to draw the inhaled air into the respiratory region of the lung. The respiratory zone consists of the respiratory bronchioles and the alveolar region where gas exchange occurs. The alveoli wall contains a dense network of capillaries (with a diameter of around 10 microns) and a thin blood-gas barrier (0.5 microns) which allow efficient gas exchange (West, 1985).

The lung is constantly exposed to foreign particles and invading organisms. This can result in lung inflammation and can cause tissue injury. To deal with this problem the cells of the lung and respiratory tract have developed several defence mechanisms. The upper respiratory tract removes the vast majority of the foreign particles and invading organisms deposited on the thin mucous coating of the nasopharynx which is then removed by ciliated epithelial cells to the epiglottis where it is swallowed. Some of these particles can reach the lower airways where they require processing by the immune system. Every day infectious agents can enter the alveolar region of the lung and yet do not normally cause a threat to health. The alveolar region consists of $5x10^8$ alveoli which have a total surface area of $100m^2$ (Stone *et. al.,* 1992). The alveolar epithelium lines the alveoli and is the first point of contact for inhaled agents. The epithelium is capable of playing an active role in airway inflammation by the production and release of cytokines (Adler *et. al.*, 1997). The alveolar epithelium is composed of many different cell types including type I epithelial cells, type II epithelial cells, ciliated epithelial cells, Clara cells, fibroblasts and alveolar macrophages. In this study the type II-like cell line A549 cells and the monocyte- like cell line MM6 were used.

1.1.1 A549 cells.

The A549 epithelial cell line has been used in many studies as an experimental model of alveolar type II epithelial cells. The major advantage of these cells is that large numbers of the cells can be grown and controlled in reproducible conditions. There has been some controversy over the similarity of A549 cells to type II epithelial cells

(Mason and Williams, 1980). Type II cells represent 60% of the alveolar cells by number and like A549 cells are spherical in shape and approximately 9 μm in diameter. There are several major functions of type II cells including synthesis and secretion of surfactant, xenobiotic metabolism, transepithelial movement of water and regeneration of the alveolar epithelium following lung injury (Reviewed by Castranova *et. al.,* 1988).

1.1.2 MM6 cells

Many studies have used blood derived monocytes to represent the alveolar macrophage. In this study we have used a monocyte-like cell line, MM6, to represent alveolar macrophages (AM). AM are the first cells to encounter foreign material due to their situation at the air–tissue interface in the alveoli. AM have an important role in phagocytic, microbicidal and secretory functions, and play a role in lung immunity by regulating immune and inflammatory processes. Failure of the AM to clear potentially hazardous inhaled matter can represent the first step in the initiation of lung disease (Reviewed by Thepen *et. al.*, 1994; Twigg, 1998)

1.2.0 Lung inflammation

When foreign particles and invading organisms breach the defence mechanisms of the lungs inflammation can occur. The lung epithelium is involved in the inflammatory process either directly by contact with the foreign material or indirectly by interaction with various species released from AM. These can include cytokines and ROS produced by the AM during phagocytosis of the foreign material (reviewed by Adler *et. al.*,1997; Twigg, 1998). This results in the signalling of an inflammatory response in the epithelial cells which is often via the activation of transcription factors (review on cytokines and inflammation by Hanada *et. al.,* 2002). This can lead to the production of many pro-inflammatory proteins by epithelial cells, including cytokines and acute phase proteins which can interact with other cells in the lung or may be involved in a systemic inflammatory response. Many of these proteins produced in the lung during inflammation have important effects on the body as a whole and will now be discussed.

1.3.0 Fibrinogen

Fibrinogen is involved in the coagulation cascade and is produced rapidly during tissue injury and is therefore termed an acute phase protein. Acute phase proteins are produced in the acute phase response (APR) which occurs within the first 24 – 48 hours of inflammatory stimulation. The APR is the innate response to tissue damage or infection and is described as a "large number of systemic changes, distant from the site or sites of inflammation and involving many organ systems, which may accompany inflammation" (Gabay and Kushner, 1999). Physiological responses to the APR include the activation of monocytes and macrophages, and the induction of the release of pro-inflammatory cytokines from damaged and activated cells.

The coagulation cascade is usually initiated upon damage or inflammation of the tissue endothelium. Products of damaged cells can then stimulate endothelial cell retraction and the release of fibrinogen and other plasma constituents from the vasculature. Fibrinogen is the precursor of fibrin, an insoluble polymer which forms the basis of the blood clot. The primary function of fibrinogen is to control blood loss by platelet aggregation and the formation of fibrin clots. As well as its role in haemostasis, fibrin also provides a matrix for adhesion, migration and proliferation of cells during repair processes after tissue damage.

1.3.1 Fibrinogen in the liver.

Fibrinogen is a 340kDa glycoprotein produced constitutively by hepatocytes. The protein is produced as three separate polypeptide chains Aα, Bβ, and γ (Herrick *et. al.*, 1999). The constitutive production of fibrinogen results in secretion into the plasma and therefore a basal level of fibrinogen exists in all individuals. During inflammation the plasma levels of fibrinogen dramatically rise due to the function of fibrinogen as an acute phase protein. Extrahepatic synthesis of fibrinogen has been found in several tissues. Molmenti *et. al.* (1993) found that intestinal cells could produce fibrinogen and proposed that it functioned as part of a repair mechanism. Simpson-Haidaris (1997) reported that the human lung epithelial cell line A549, was able to synthesise and secrete fibrinogen, and that this secreted fibrinogen in response to inflammation plays a role in wound repair.

1.3.2 Fibrinogen transcription

The three human fibrinogen genes are clustered within a 65kb region on chromosome 4 and are transcribed in a coordinated manner allowing all three subunits to be increased to the same extent in response to an upregulation signal (reviewed by Fuller and Zhang, 2001). The promoters for each fibrinogen gene contain IL6 responsive elements which enable IL6 to be the major inducer for fibrinogen transcription. Signalling by IL6 requires the transcription factors STAT3 (signal transducer and activator of transcription) and C/EBP (CCAAT- enhancer binding proteins). In hepatocytes the hepatic nuclear factor -1 (HNF1) is also required for the expression of Aα and Bβ proteins. The cytokines TNF α and IL1β have also been found to inhibit the IL6 response in hepatocytes and in animals when co-administered (Zhang and Fuller, 2000; Nguyen and Simpson-Haidaris, 2000). IL1β and TNF α activate fibrinogen production by NFκB signalling (Siebenlist *et. al.*, 1994) and in the fibrinogen gene the binding sites of STAT3 and NFκB overlap. Therefore if IL1β is present it induces NFκB, which when bound to the DNA prevents the binding of STAT3.

1.3.3 Function of fibrinogen.

One of the main functions of fibrinogen is in the coagulation cascade. Circulatory fibrinogen also can act as an acute phase protein and is involved in the inflammatory response. In particular fibrinogen has been found to promote leukocyte adhesion and cytokine secretion at inflammatory sites. Leukocyte and neutrophil migration is an important event during an immune or inflammatory response.

Fibrinogen participates in leukocyte and neutrophil movement and adherence to endothelium by binding to the leukocyte integrin $\alpha_M\beta_2$ (CD11b/CD18) (Altier *et. al.*, 1993). This allows the leukocyte (Forsyth *et. al.*, 2001) or neutrophil (Gross *et. al.*, 1997) to migrate across the endothelium to the site of inflammation. Fibrinogen can also stimulate the production of cytokines in leukocytes and neutrophils. Degraded fibrinogen molecules known as fibrin degradation products are also able to influence neutrophil movement indicating that the fibrinogen molecule is not required to be intact for these effects to occur (Leavell *et. al.*, 1996).

Fibrinogen has been shown to induce the activation of NFκB in monocytic U937 cells (Sitrin *et. al.*, 1998) which could result in the production of cytokines. Fibrinogen can also stimulate chemokine secretion through Toll-like receptor 4 (TLR4) in U937 and THP1 cells (Smiley *et. al.*, 2001). Fibrinogen was also found to increase cytokine gene expression in neutrophils when bound to the β_2 integrin (CD11/CD18).

1.3.4 Fibrinogen in the lung.

Fibrinogen has been found to be produced in many extrahepatic locations including the lung. Fibrinogen can be induced in A549 lung epithelial cells when treated with IL6 and dexamethasone suggesting that fibrinogen could induce a local response to lung inflammation (Simpson-Haidaris, 1997; Anderson *et. al.*, 2003). Fibrinogen was found to be secreted from these cells in a polarised manner with 80% being secreted basolaterally, toward the basement membrane in a microtubule dependent manner (Gaudiz *et. al.*, 1997).

Fibrinogen was also found to be induced in response to parasitic infection by *Pneumonocystis carnii* in an animal model (Simpson-Haidaris *et. al.*, 1998). Fibrinogen was found associated with the parasites aggregated along the alveolar membrane suggesting that it played a role in the adherence of the parasite to the lung epithelium similarly to the adhesive glycoproteins fibronectin and vitronectin.

Recently fibrinogen was found to be endocytosed by A549 cells in an integrin, $\alpha_v\beta_3$, dependent manner (Odrljin *et. al.*, 2001). It was suggested that the endocytosis of matrix-associated fibrinogen may be involved in the process of alveolar tissue repair and matrix remodelling.

1.4.0. C-reactive protein

C-reactive protein (CRP) is the prototypic human acute phase protein (APP) and is a member of the pentraxin family of proteins. This is a highly conserved protein family and has evolved since the horseshoe crab (*Limulus polyphemus*), (Shrive *et. al.*, 1999) and is thought to have been present before the evolution of immunoglobulins. The pentraxins are characterised by a ring-like structure consisting of 5 identical monomers (Szalai *et. al.*, 1999). CRP was originally thought to be produced only in

the liver in response to cytokine induction (Tonatti *et. al.*, 1990). Synthesis in hepatocytes occurs at low rates and CRP is retained in the endoplasmaic reticulum via interaction with two carboxylesterases (gp60a and gp60b) until the APR occurs, at which time the CRP is released into the circulation (Yue *et. al.*, 1996). Normal serum levels of CRP are <1μg/ml and during and APR there can be up to a 1000 fold increase within several hours of stimulus (Dowton and Colton, 1988). In recent years there have been reports of extrahepatic synthesis of CRP in many areas of the body including the brain (Yasojima *et. al.*, 2000), atherosclerotic plaques (Yasojima *et. al.*, 2001), respiratory tract (Gould and Weiser, 2001), blood lymphocytes (Murphy *et. al.*, 1991; Kuta and Baum, 1986), and alveolar macrophages (Dong and Wright 1996). CRP in the respiratory tract was found to be expressed and the epithelial cells and secreted from these cells which was detectable in sputum and nasal airway surface fluid.

1.4.1 CRP transcription

Transcription of CRP in hepatocytes during the APR is known to be induced by cytokines. The main translational activator of CRP is interleukin 6 (IL6) which is produced by T-cells, macrophages and endothelial cells (Baumann and Gauldie, 1994). However, although IL6 is essential for CRP transcription it is not sufficient for the induction of the CRP gene *in vivo* (Weinhold and Ruther, 1997). In the liver IL1β must also be present for the transcription of CRP (Weinhold *et. al.*, 1997; Zhang *et. al.*, 1995). However in human cell lines IL6 is able to induce CRP transcription without the need for IL1β to be present, although the presence of both cytokines increases the rate of CRP production. This occurs because the cytokines interact with the CRP gene at loci known as acute phase response elements (APRE) and induce the production of CRP (Zhang *et. al.*, 1996). Each APRE is thought to increase the production of CRP by 10-fold, and the CRP gene contains many of these APRE allowing more than one cytokine to induce the transcription of CRP (Tonatti *et. al.*, 1990). Other cytokines known to increase the production of CRP during inflammation include tumour necrosis factor α (TNF α), interferon γ (IFN γ), and transforming growth factor β (TGF β).

1.4.2 CRP function

The function of CRP is still not fully understood although CRP was discovered in the 1930s and has been widely studied since. It is known that CRP is dramatically increased during APR and has been highly conserved throughout evolution suggesting that it performs an important biological role. Unlike antibodies, CRP is present at the onset of infection and is rapidly reduced in expression when infection subsides (Du Clos, 2000). CRP is found at most sites of inflammation associated with neutrophils and other inflammatory cells (Heuertz *et. al.*, 1993). The main biological function of CRP is determined by its ability to bind to pathogens and damaged cells and mediate their elimination by recruitment of the complement system and phagocytic cells (Volanakis, 2001).

CRP acts as an opsonin by binding to C-polysaccharide on bacteria and phosphatidylcholine on damaged cell membranes and signals for their removal by phagocytosis. Phagocytosis occurs by the binding of CRP to receptors on neutrophils, monocytes and macrophages similar to the IgG receptor Fcγ (Marnell *et. al.*, 1995; Bharadwaj *et. al.*, 1999; Mold *et. al.*, 2001).

CRP can also activate the early classical complement cascade (Kaplan and Volanakis, 1974). CRP has also been shown to inhibit the alternative pathway activation from C3 (Heurtz, 1993, 1994). Complement is an important factor in clearance of soluble immune complexes, damaged, transformed and apoptotic cells (Jarva *et. al.*, 1999). Complement allows these complexes to bind to erythrocytes (Mold, 1996) so that they can be transported to the liver and spleen to be degraded, thus minimising inflammation.

CRP is also known to enter the nucleus of apoptotic and necrotic cells. This is dependent on a nuclear localisation sequence (NLS) within the CRP gene (Du Clos *et. al.*, 1990). Once in the nucleus CRP can bind to chromatin (Robey *et. al.*, 1984; Du Clos, 1991; Pepys *et. al.*, 1994) and the small nuclear ribonuclear proteins (snRNPs), (Du Clos *et. al.*, 1989; Jewell *et. al.*, 1993; Pepys *et. al.*, 1994). It is believed that this facilitates phagocytosis of the nuclear material and reduces the chances of developing autoimmuninty (Du Clos, 1996). Shepherd *et. al.* (1986) also suggested that CRP binding to chromatin promotes its degradation by endogenous nucleases.

1.5.0 Heat shock protein 70.

The heat shock response is one of the most primitive and highly conserved cellular defence systems known in biology. Heat shock proteins (Hsps) are consitututively expressed in all cells and are essential in many cellular processes including protein folding, protection of proteins from denaturation and aggregation and in the facilitation of protein transport through membrane channels (Hartl, 1996). A wide variety of stressful stimuli including heat, radiation, and infection can induce increases in intracellular heat shock proteins. Heat shock proteins are a family of stress proteins which are divided in to subfamilies according to molecular weight and are generally classified as Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock proteins. Hsp70 is found in the nucleus and cytoplasm of mammalian cells whereas Hsp60 is located in the cell mitochondria and membrane and Hsp27 is found only in the cytoplasm of cells (reviewed by Garrido *et. al.*, 2001).

1.5.1 Transcriptional regulation of Hsp70.

The heat shock response is regulated by a transcription factor known as heat shock factor -1 (HSF1). In unstressed cells HSF1 is present in the nucleus and cytoplasm of cells in a monomeric form bound to Hsp70 and other chaperones. During stress the monomers trimerise and undergo conformational changes which allow HSF1 to bind to the responsive elements in the promoters of stress inducible genes. During this process HSF1 becomes phosphorylated which correlates with DNA binding (Kim *et. al.*, 1995). Hsp70 and Hsp90 belong to a subset of HSPs which can be transactivated by signal transducers and activators of transcription (STATS) (Stephanou *et. al.*, 1999). This occurs in the presence of IFNγ which induces STAT1. STAT1 can then bind to the DNA which is in an area around the Hsp70 and Hsp90 promoters. This region of the DNA also contains binding sites for NF-IL6, STAT3 and HSF1. STAT1 and HSF1 can also interact with each other and produce strong activation of transcription, while STAT3 and HSF1 antagonise each other (Stephanou *et. al.*, 1999).

1.5.2 Function of Hsp70 under 'normal' conditions.

During normal (unstressed) conditions Hsp70 functions as an ATP-dependent molecular chaperone assisting the folding of newly synthesised polypeptides. Hsp70 is an ATP binding protein and has ATPase activity. The ATP-bound form of the protein has a low binding affinity for substrate whereas the ADP-bound form can bind substrate. Inhibitors of the Hsp70 ATPase have been found which can inhibit Hsp70 chaperone activity (Raynes and Guerriero, 1998). Hsp70 also assists in the assembly of multi-protein complexes and the transport of proteins across cellular membranes (Beckmann *et. al.*, 1990; Shi *et. al.*, 1992; Murakami *et. al.*, 1988).

1.5.3 Function of Hsp70 during stressed conditions.

Cellular stress arises from many sources including heavy metals, inflammation, oxidative damage, ischemic stresses all of which can stimulate the expression of HSP genes. Hsp70 is produced rapidly in response to cellular stress and has been shown to protect against reperfusion lung injury, stress- and TNFα-induced apoptosis, radiation-induced apoptosis, and necrosis (Stuhlheimer, 2000). Hsp70 has also been shown to block the activation of the transcription factor, nuclear factor -κB (NFκB), therefore blocking the inflammatory process (Feinstein *et. al.*, 1996; Yoo *et. al.*, 2000; Malhotra and Wong, 2002). During a stress response Hsp70 is removed from the HSF1 allowing HSF1 to enter the nucleus and induce production of HSPs. The Hsp70 proteins then are involved in ensuring the correct folding of polypeptides in response to stress stimuli. Once the level of Hsp70 in the cells is high enough Hsp70 travels to the nucleus and can bind to the HSF1 and repress HSP gene transcription. Hsp70 is known to be involved in inhibition of apoptosis and therefore increasing the ability of cells to survive when exposed to lethal stimuli. Overexpression of Hsp70 protects cells from stress-induced apoptosis both upstream and downstream of the caspase cascade. Hsp70 can reduce or block caspase activation, suppressing mitochondrial damage and nuclear fragmentation (Buzzard *et. al.,* 1998). Hsp70 has also been suggested to have neutralising interactions with several pro-apoptotic effectors including Apaf1, AIF and signalling molecules such as JNK-1, p53 or c-myc (Garrido *et. al.*, 2001).

1.5.4 Hsp70 in the lung.

Hsp70 has been studied extensively in lung inflammation. Hsp70 has been found increased in the lung epithelium in response to hyperoxia (Wong *et. al.*, 1998), heavy metals (Timblin *et. al.*, 1998; Gaubin *et. al.*, 2000), asbestos (Timblin *et. al.*, 1998), hydrogen peroxide (Timblin *et. al.*, 1998) and cytokines (TNFα) (Ayad *et al.*, 1998). Hsp70 is induced by hyperoxia which can produce the generation of oxygen radicals such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH). These free radicals can cause cellular injury which can lead to mitochondrial damage, ATP depletion, oxidation of membrane lipids and intracellular proteins and DNA damage (Wispe and Roberts, 1987). A study was carried out which investigated the effect of hyperoxia on A549 cells transfected with human Hsp70 cDNA. This showed that the cells with higher levels of Hsp70 had greater survival rates than untransfected A549 cells suggesting Hsp70 expression was protective (Wong *et. al.*, 1998). Rat lung epithelial cells were used to examine the effects of environmental agents which are known to cause oxidative injury (Timblin *et. al.*, 1998). This study found that Hsp72/73 was not increased greatly by asbestos or H_2O_2, but was highly increased in response to cadmium. Another study, using cadmium found that in A549 cells Hsp70 was produced in response to oxidative stress within the cell, as a result of the formation of active oxygen species and inhibition of peroxide detoxification (Gaubin *et. al.*, 2000). Hsp70 is also known to inhibit the inflammatory response during lung injury. In A549 cells Hsp70 was found to inhibit the production of the chemokine RANTES (Ayad *et. al.*, 1998). It was found that RANTES expression and secretion was dependent on NFκB activation and that Hsp70 prevented NFκB nuclear translocation by preventing phosphorylation of its inhibitor protein, IκB. It has since been found that Hsp70 prevents the activation of IκB kinase (Yoo *et. al.*, 2000).

1.6.0 Nuclear factor κB (NFκB) and its inhibitor (IκB).

The transcription factor NFκB plays an important role in the inducible expression of many cellular genes particularly those involved in the immune system and inflammation. Activated NFκB can lead to the rapid induction of genes encoding defence or signalling proteins suggesting that NFκB has specialised during evolution as an immediate early mediator of immune and inflammatory responses. Human NFκB is composed of homo- or heterodimers of proteins belonging to the Rel family. In unstimulated cells NFκB is found in the cytoplasm in an inactive complex with its

inhibitor IκB. IκB is a term for a family of proteins which have the ability to interact with NFκB and prevent its activation thus keeping NFκB in the cell cytoplasm.

1.6.1 Rel protein family

The Rel protein family all share a very conserved region, the Rel homology domain, which contains sequences for the dimerisation, nuclear localisation and DNA binding of NFκB. The members of the Rel family include p50, p52, p65/RelA, c-Rel, and RelB. The Rel proteins comprise two groups classified on their structure, function and mode of synthesis. Group 1 Rel proteins include p50 and p52 which are synthesised as precursor proteins of 105 (p105) and 100kDa (p100) respectively. Group 1 mature proteins are characterised by the presence of the Rel homology domain which includes a DNA binding domain and nuclear localisation signal (NLS). Group 2 Rel proteins comprises p65, Rel or c-Rel , RelB. Group 2 mature proteins possess the Rel homology domain as well as one or more transcriptional activator domains (Sen and Packer, 1996). Dimerisation is functionally important since it allows DNA binding and then activation for transcription (Bauerele and Henkel, 1994). The differences between functions of the two groups of Rel proteins arise in the N-terminal DNA–binding domains where a sequence motif R(F/G)(R/K) YXCE determines the binding ability (Tolenado *et. al.,* 1993). Commonly the p50-p65 dimer is known as NFκB.

1.6.2 IκB protein family

In unstimulated cells, NFκB DNA binding activity is not detectable in nuclear, cytosolic or membrane fractions. This is due to the factors known as inhibitors of NFκB (IκB). In mammalian cells the most common members of this family are IκBα and IκBβ. IκB functions by binding to NFκB masking the NLS and DNA binding activity of NFκB (Beg *et. al.,* 1992). IκBα is composed of a central, protease-resistant domain containing five ankarin repeats and flanked by a surface exposed N-terminal extension and connected to the core by a flexible flanker (Jaffray *et. al.*, 1995). IκBβ is similar to IκBα but has a larger molecular size and is thought to be the product of a different gene. The main differences between IκBα and IκBβ are that IκBα is specific for NFκB (p65, Ito *et. al.*, 1995) while IκBβ can also inhibit the

DNA binding of c-Rel. Another difference is that *in vitro* phosphatase treatment interfered with the inhibitory activity of IκBβ but not IκBα (reviewed by Grimm and Baeuerele, 1993).

Upon cell stimulation IκBα is phosphorlyated and degraded allowing active NFκB to translocate to the nucleus (Henkel *et. al.,* 1993). Levels of IκB are found to increase due partly to an increase in mRNA transcription through the interaction of NFκB with NFκB-binding sites in the IκB promoter (Le Bail *et. al.,* 1993; Sun *et. al.,* 1993; Malek *et. al.,* 1998). IκB has also been found in the nucleus as well as the cell cytoplasm. This is due to the accumulation of newly synthesised IκB localising to the nucleus to bind to the NFκB complexes. At this time a progressive reduction of both NFκB-DNA binding and NFκB-dependent transcription are found in the nucleus (Arenzana-Seisdedos *et. al.,* 1995; Chiao *et. al.,* 1994). It was then found that this was due to IκB binding with NFκB and exporting it out of the nucleus by use of nuclear export signal located in C-terminal domain of IκB (Arenzana-Seisdedos *et. al.,* 1997).

1.6.3 Activation of NFκB.

Activation of NFκB occurs in response to a wide range of stimuli including LPS, viruses, cytokines, physical and oxidative stress (reviewed by Wang *et. al.,* 2002). Activation of NFκB leads to the transcription of genes encoding cytokines, cell surface receptors, and acute phase proteins as well as transcription factors including IκB, p50 precursor and c-Rel. The signals that lead to modification of IκBα presumably involve the generation of intracellular reactive oxygen species (ROS) (Schreck *et. al.,* 1991; Baeurele and Henkel, 1994). The main ROS involved are likely to be hydrogen peroxide and its derivatives (Reviewed by Sen and Packer, 1996). Antioxidants have been shown to inhibit the activation of NFκB confirming the involvement in the activation process (Reviewed by Sen and Packer, 1996).

1.6.4 Role of activated NFκB in disease.

As mentioned previously activation of NFκB can lead to the transcription of various cytokines. In particular NFκB activation is associated with increased production of the pro-inflammatory cytokines IL1, IL8, IL6, TNFα and IFN-γ. Over-activation of

NFκB can lead to the progression of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, psoriasis and asthma (reviewed by Hanada and Yoshmuri, 2002; Li and Verma, 2002). NFκB has also a role in the development of adaptive immunity by regulating lymphocyte development through effects on proliferation, protection from TNF-induced apoptosis and expression of anti-apoptotic genes (Li and Verma, 2002). NFκB has another effect on immunity through the rapid induction of acute phase proteins in response to microbial pathogens.

1.6.4 NFκB activation in lung inflammation

ROS have been implicated in the pathogenesis of a wide variety of lung diseases. It has been suggested that ROS may act as signal transduction messengers, inducing new gene expression in response to injury (Schreck *et. al.*, 1991). The body is continually exposed to oxidants produced endogenously by metabolic reactions or exogenously by air pollutants, cigarette smoke and in the diet. NFκB can also be activated by pro-inflammatory cytokines, bacterial and viral products in the lung (Reviewed by Fan *et. al.*, 2001; Christman *et. al.*, 2000). NFκB has been shown to be involved in the progression of lung inflammation associated with particulate air pollution. Several reports have indicated that various air pollution particles can induce the activation of NFκB which then lead to the production of pro-inflammatory cytokines such as IL6 and IL8. These studies have identified that NFκB activation was mediated by the generation of free-radicals by transition metals on the particle surface (Quay *et. al.*, 1998; Kennedy *et. al.*, 1998; Jiminez *et. al.*, 2000; Shukla *et. al.*, 2000). One study (Jiminez *et. al.*, 2000) identified NFκB activation in response to PM10 treatment occurred by a transition metal-mediated mechanism in the absence of IκB degradation. In these studies it was determined that ROS were responsible for NFκB activation by using antioxidant treatments which mop-up the ROS and prevent NFκB activation. Inhibition of NFκB activation can also occur during lung inflammation. One study showed that overexpression of IκBα in A549 cells prevented the nuclear translocation of NFκB (Park *et. al.*, 2001). During lung inflammation neutrophils secrete a protein known as secretory leucocyte protease inhibitor (SLPI). This protein was also found to be expressed by pulmonary vascular epithelial cells during lung inflammation. SLPI was found to reduce the levels of

NFκB activation during the inflammatory process; this reduction was the result of increased IκB within the cell (Lentsch *et. al.*, 1999).

1.6.0 Particulate air pollution.

Elevated levels of air pollution have been associated with adverse health effects for a long time. In London in 1952, smog covered the city for several days and led to an excess of 4000 deaths due to the high concentration of air pollution. Today particulate air pollution is still associated with increases in morbidity and mortality from respiratory and cardiovascular diseases (Pope *et. al.*, 1992; Schwartz and Dockery, 1992; Dockery *et. al.*, 1993; Schwartz and Morris, 1995). The concentrations of particulate air pollution today are much lower in the UK than in the 1950s resulting from changes in the source of particles. During the 1950s the main source of air pollution particles was from coal burning which is not present now in UK cities, whereas the majority of particles today are generated from vehicle exhaust emissions. Particulate air pollution is also associated with asthma exacerbations, increased respiratory symptoms, decreased lung function and increased hospital admissions (discussed by Wilson and Spengler, 1996).

Particulate air pollution is derived from many sources including industrial processes and vehicle exhausts. Particulate air pollution can be classified by its size in microns e.g. PM10 or PM2.5 etc., or by composition or source e.g. ROFA (residual oil fly ash), DEP (diesel exhaust particles) or mineral dusts. These particles can be collected on filters for chemical analysis and biological effects. Air pollution particles are a complex mixture of different components including aggregates of organic and elemental carbon, metals, sulphates, nitrates, course wind blown material, pollen and microbial contaminants. PM10 is composed of airborne particles which are less than or equal to 10 microns in size. The particles in a PM10 sample can also be sub-categorised by size into ultrafine, fine and course particles. The ultrafine particles have a mean aerodynamic diameter of 0.1 microns or less, and typically arise from combustion sources such as diesel engines. The fine particles have a diameter of 2.5 microns or less and can arise from aggregation of ultrafine particles or can be produced by the combustion of fossil fuels. The course particles have a diameter

between 2.5 and 10 microns and arise from natural sources such as course wind blown material and pollen (Harrison *et. al.,* 1997). The different components of PM10 result in differences in the composition of PM10 samples depending on the source e.g. rural PM10 samples will contain mainly biological particles whereas urban PM10 will contain mainly combustion derived particles. Many studies have found that particulates can cause not only localised inflammation but also systemic effects which may lead to the morbidity and mortality associated with episodes of air pollution.

1.6.1 Effect of air pollution particles on the lung.

Inhaled air pollution particles can be deposited in different areas of the lung depending on the size of the particle. Larger particles can be deposited in the mucous of the upper airways and are cleared by the ciliated epithelial cells. Smaller particles in the ultrafine range (diameter less than 100nm) can enter the alveoli of the lung where they are likely to undergo phagocytosis by AM. After phagocytosis the AM leave the lung on the mucocilliary escalator. However there tends to be very high numbers of these ultrafine particles and this process is not always efficient in removing them from the lung and this can lead to macrophage damage, known as particle overload (reviewed by Oberdorster, 1995). This leads to the presence of high numbers of ultrafine particles in the lung where they can cause damage and inflammation at sub-overload doses (Ferin *et. al.*, 1992; Peters *et. al.*, 1997).

1.6.2 Particle induced inflammation

Many studies have focused on the inflammatory potential of air pollution particles. Particles from many sources have been shown to induce the production of cytokines in human and rat lung cells. Several studies have shown that instillation of air pollution particles induces lung inflammation, one of these was carried out on the effects of PM10 from Utah valley, USA on human non-smoking volunteers (Ghio *et. al.*, 2000; Ghio and Devlin, 2001) and another using a rat model was on the effects of PM10 from sites around the UK (Lightbody *et. al.,* 2002).

Other studies have been carried out on cellular models of inflammation. Several studies have identified the effect of particulate air pollution on macrophage and monocyte cells. These studies have found that these cells produced pro-inflammatory

cytokines and reactive oxygen species in response to particle treatments (Monn and Becker, 1999; Dorger and Krombach, 2000; Becker *et. al.*, 1996; Schins *et. al.*, 2002). One study showed that PM10 exposed macrophages could stimulate a pro-inflammatory response in lung epithelial cells via the production of TNFα (Jimenez *et. al.*, 2002).

Studies on the effects of particulate matter on epithelial cells have also been carried out. Cytokine production has been demonstrated with particulate matter treatment of human bronchial and alveolar epithelial cells (Fujii *et. al.*, 2001; Veronesi, *et. al.*, 1999; Schins *et. al.*, 2002). Another study using human bronchial epithelial cells found that the cytokine production in response to ROFA particle treatment was metal dependent (Carter *et. al.*, 1997).

1.6.3 Effect of particle derived oxidants on lung inflammation

Oxidative stress has been shown to be involved in the pathogenesis of air pollution particles in the lung. This occurs when there is an imbalance between oxidising agents (ROS, NOS) and antioxidants. Production of oxidant species has been attributed to reactions on the surface of ultrafine particles with transition metals and surface radicals (reviewed by Donaldson, *et. al.*, 1998; Donaldson and MacNee, 1998; Dorger and Krombach, 2000). Free radicals in the lung can also be produced by activated macrophages, auto-oxidation of small molecules, enzyme activity, mitochondrial electron transport and other intracellular mechanisms.

Several studies have looked at the toxicity of particles with respect to free radical production. One study on the generation of free-radicals by PM2.5 (combustion derived particles) found that stable radicals were found on the particle surface but in a biological system redox cycling occurred resulting in the production of the DNA – damaging hydroxyl radical (Dellinger *et. al.*, 2001). Another study looked at the free-radical activity of PM10 *in vivo* and *in vitro* and found that lung and epithelial injury was the result of hydroxyl activity (Li *et. al.*, 1996). A study by Stone *et. al.* (1998) looked at the effect of carbon particles and their ability to produce free radicals and it was found that the ultrafine carbon particles produced greater oxidative stress than

larger carbon particles and once again the hydroxyl radical was generated by redox cycling.

During oxidative stress there is an imbalance between oxidative and anti-oxidative agents. Several studies have looked at the effect of antioxidants in lung lining fluid on the particle induced oxidation. One of these studies looked at the oxidative interactions of synthetic epithelial lining fluid with ROFA and found that this particle could initiate oxidation of the lining fluid (Sun *et. al.,* 2001). Another study using synthetic lining fluid found that the antioxidants present in the fluid were able to reduce the effects of oxidative damage induced by PM10 and PM2.5 (Greenwell *et. al.,* 2002).

1.7.0. Systemic effects of particulate inhalation.

Particulate air pollution has been associated with systemic effects as well as localised inflammation. This is a result of the thin blood-gas barrier (less than 0.5 microns) in the lung to allow efficient gas exchange. The alveolar wall has a dense network of capillaries which are in close proximity to the alveolar epithelium (West, 1985). This allows the efficient movement of gas, and during inflammation the epithelium can have increased permeability allowing movement of proteins between the circulation and the lung. Systemic effects associated with inhaled particles include an increased incidence of cardiovascular diseases. It is believed that cytokines are responsible for the systemic response to inhaled particles (van Eeden *et. al.,* 2001). Instillation of particles into the lung has been shown to cause increases in the number of circulation leukocytes arising from bone marrow stimulation (Mukae *et. al.,* 2001). Increases in the number of circulating neutrophils and platelets were also found in response to inhalation of diesel exhaust particles (Salvi *et. al.,* 1999). Other effects on the cardiovascular system associated with inhaled particles can include changes in heart rate variability and the autonomic nervous system (Utell *et. al.,* 2002; Holguin *et. al.,* 2003).

Increases in circulating inflammatory proteins are also found in response to inhalation of particulate matter. Several studies have found increases in the plasma level of fibrinogen, one in humans exposed to PM10 (Pekkanen *et. al.,* 2000) and another in rats exposed to ROFA (Gardner, *et. al.,* 2000). However one study (Seaton *et. al.,*

1999) found that there was a decrease in fibrinogen levels in the blood associated with PM10 inhalation but there was an increase in the level of CRP. Increase in plasma CRP has also been associated with an acute phase reaction in response to particulate air pollution (Peters *et. al.*, 2001).

1.7.1 Epidemiological studies on effects of air pollution.

Many epidemiological studies have been carried out on the effects of air pollution. Increases in air pollution levels have been associated with increased hospital admissions and mortality due to respiratory conditions (Fusco *et. al.*, 2001). Even at very low levels of PM10 respiratory health has been shown to be decreased (reviewed by Brunekreef *et. al.*, 1995). Many studies have found that exposure to air pollution can lead not only to respiratory disease but also cardiovascular disease. The level of risk for cardiovascular disease varies depending on the study carried out. One study found that air pollution increased the number of hospital admissions for cardiovascular admissions (Schwartz, 1999), while another found that air pollution increased mortality due to cardiovascular causes (Dockery *et. al.*, 1993) in various areas in the USA. In Dublin it was found that a ban on coal burning reduced the levels of death due to respiratory causes by 15.5% and cardiovascular causes by 10.3% (Clancy *et. al.*, 2002). The cardiovascular disease responsible for mortality or hospital admission in most of these studies is not determined. However the incidence of atherosclerosis (Suwa *et. al.*, 2002) and myocardial ischemia (Pekannen *et. al.*, 2002) has been found to be increased by particulate air pollution. Many reviews also exist on the morbidity and mortality associated with particulate air pollution (Pope *et. al.*, 1995; Pope and Dockery, 1999; Hoek *et. al.*, 2002; Brunekreeef and Holgate, 2002).

1.7.2 Cardiovascular disease and particulate matter.

It has been suggested that the systemic response to inhalation of particulate matter is responsible for the increase in cardiovascular disease. As discussed earlier the systemic effects are associated with increases in the levels of circulating proteins such as cytokines, CRP and fibrinogen. It has been suggested that the oxidative stress in the lung may also be able to reach the blood and have an effect on the systemic response (Donaldson *et. al.*, 2000). Several reviews exist which discuss the effect of exposure

to particulate air pollution on cardiovascular disease (Costa, 2000; Donaldson *et. al.*, 2001; Donaldson and MacNee, 2001).

1.8.0 Role of pro-inflammatory proteins in cardiovascular disease.

Cardiovascular disease has been associated with systemic effects resulting from particle induced lung inflammation. The APR and oxidative stress have been implicated in the progression of cardiovascular disease. Therefore the effects of CRP, fibrinogen and Hsp70 on cardiovascular diseases will be discussed.

1.8.1 CRP and cardiovascular disease

A high level of expression of CRP in plasma has been found to be associated with an increased risk of heart disease, particularly atherosclerosis. This is due to heart and blood vessel inflammation in the damaged tissue. CRP has been found localised around altherosclerotic vessels and infarcted myocardium (Lagrand *et. al.*, 1997). CRP binds to any damaged cells within the cardiovascular system. Monocytes can infiltrate the arterial wall at site of CRP deposition. The monocytes then differentiate into macrophages and, along with accumulation of lipids, foam cells are generated (Zwaka *et. al.*, 2001). Further recruitment of inflammatory cells and cell proliferation leads to the development of altherosclerotic plaques. The level of CRP present in the plasma is thought to reflect the extent of inflammation in atherosclerosis and is also associated with increased risk of myocardial infarction and stroke (Ridker *et. al.*, 1998; Harris *et. al.*, 1999; Cleland *et. al.*, 2000; Danesh *et. al.*, 2000; Blake and Ridker, 2001; Ferranti *et. al.*, 2002,). However several reports show that CRP only indicates the presence of cardiovascular disease and is unreliable as a marker of extent or severity (Azar *et. al.*, 2000; Hunt *et. al.*, 2000). IL6 and TNF α play a central role in the amplification of the inflammatory cascade (Blake and Ridker, 2001) involved in atherosclerotic development and may induce further production of CRP.

1.8.2 Fibrinogen and cardiovascular disease.

Fibrinogen and many other clotting factors are associated with increased frequency of coronary heart disease (reviewed by Tracy, 1999). Increased levels of fibrinogen are associated with traditional cardiovascular risk factors such as age, diet and smoking. Fibrinogen can bind to the arterial wall and bind low density lipoproteins and this can

lead to atherogenesis. Raised levels of fibrinogen can increase the viscosity of the blood which results in reduced blood flow and increased platelet aggregation which are associated with atherogenesis, thrombogenesis and ischemia (reviewed by Montalescot *et. al.*, 1998). These findings indicate that fibrinogen itself is a predictive marker of cardiovascular disease.

1.8.3 Hsp70 in cardiovascular disease.

Several heat shock proteins have been associated with increased incidence of cardiovascular diseases. Both Hsp70 and Hsp60 have been associated with increases in myocardial ischemia and atherosclerosis. Myocardial ischemia can induce a stress response with results in the production of Hsp70. Hsp70 has also been found increased in atrial tissue of patients with unstable angina (Yellon and Marber, 1994). It has been found that coronary artery bypass grafting can induce a rapid release of Hsp70 into the circulation followed by modulation of monocyte TLR2 and TLR4 which then leads to an inflammatory response (Dybdahl *et. al.*, 2002). During ischemia and reperfusion there is the generation of free radicals, hydrogen peroxide and cellular calcium overload in myocytes (Yellon and Marber, 1994) which is likely to induce the production of Hsp70. Hsp70 can also be produced by various tissues during inflammation and may contribute to high levels of Hsp70 in the circulation with a potential effect on cardiovascular disease.

Chapter 2

Materials and Methods

2.0.0 Materials

All reagents used were purchased from Sigma Aldrich Chemicals, London unless otherwise stated. Phosphate buffer saline solution (PBS) (1 tablet to 200ml dH_2O), acetone, 5% sheep serum from Scottish Antibody Production Unit (SAPU) in PBS. Primary antibodies in ascites fluid were used in these experiments : mouse anti-human fibrinogen, mouse anti-phycoerythrin (2A4), mouse anti-human CD44, mouse anti-human C-reactive protein (CRP), and mouse anti-NF-κB(subunit p65) (Santa Cruz) (all diluted in sheep or donkey serum). Polyclonal antibodies: sheep anti-human Hsp70 (1/200 in 5% donkey serum), and rabbit anti-human IκB (both Santa-Cruz, and diluted 1/200 in 5% sheep serum). Secondary antibodies: biotinylated anti-mouse Ig, anti-rabbit Ig, anti sheep/goat Ig, anti-mouse Ig FITC and anti sheep- FITC conjugated antibody (diluted 1/100 in serum). The FITC antibody is $F(ab')_2$ AMIg-FITC.

2.1.0 Cells and cell culture

A549 cells (ECACC cell no. 86012804) are adherent carcinoma cells and were cultured in sterile RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum, penicillin (50μg/ml), streptomycin (50μg/ml) and L-glutamate (2mM) (Gibco). The cells were kept at 37°C and in a 5% CO_2 concentration in humid conditions. Cell culture density was controlled by subculture (1/10) when confluence was reached.

Before being used in any experiment the A549 cells were recovered from the tissue culture flask by addition of 1% trypsin- EDTA (Gibco) for 10 minutes to break adherence of the cells to the flask. The cells were washed twice in sterile medium. This was achieved by centrifuging at 500 x g for two minutes followed by removal of supernatant and re-suspension in RPMI-FCS. After completion of washing steps the cells were counted and assessed for viability using nigrosin dye and a Neubauer counting chamber. Adjustments were made to the cell suspension to produce a suspension of $2x10^5$ cells/ml.

2.2.0 Cell treatments

2.2.1 Cytokine treatment

Cells grown at a density of 10^6 cells/ml were treated with 1ml sterile medium containing cytokines which was added to each well in a 24 well plate, for immunofluoresence or in 25 cm^3 flasks for cell lysate preparation. Untreated controls were incubated with fresh medium. Cells were incubated at 37°C (5% CO_2) overnight. Medium was removed and cells washed with PBS.

2.2.2 Carbon black (CB) or ultrafine carbon black (ufCB) treatment

Particle suspensions were prepared by addition of 1 ml culture medium to 5mg particles, and this solution was sonicated for 10 minutes prior to dilution, to 100μg/ml, and addition to cells. Cells were treated for 0, ½, 1, 2, 3, 6, or 18 hours in conditions as for cytokine treatment. The fine carbon black used was of 320nm diameter (Huber 900, H Haeffner, Chepstow, UK) and ultrafine carbon black 14nm diameter (Printex 90, Degussa, Germany).

2.2.3 Metal salt treatment

A solution of 100μM $FeCl_3$ was prepared directly into culture medium and cells were treated for 0, ½, 3, 6 or 18 hrs. These time points were used to investigate the effects on fibrinogen, CRP, and Hsp70 expression. To investigate the effects of $FeCl_3$ on NFκB and IκB expression the time points 0, ¼, ½, 1, 2, and 4 were used.

2.2.4 PM10 treatment

PM10 TEOM filters were place in 1ml culture medium and sonicated for 10 minutes to remove particles from the filter. Turbidity assay of the resulting particle suspension was then carried out using known concentrations of CB. This allowed the determination of PM10 concentration removed from the filter. The PM10 suspension was then diluted to 80 μg/ml before cell treatment. Cells were treated for 0, ½, 3, 6, 18 hrs.

2.2.5 Brefeldin A treatment.

Brefeldin A was used to treat cells at a final concentration of 10μg/ml. This was prepared directly into cell culture medium and used to treat cells for 6 hours.

2.2.6 t-butyrl hydrogen peroxide (tBHP) treatment.

Cells were treated with 5mM tHBP added directly into culture medium for 0, ½, and 6 hours.

2.2.7 Transcription factor inhibitor treatments.

Cells were treated with 50μg/ml sn50 (Calbiochem), a peptide inhibitor of NFκB nuclear translocation. Sn50 was diluted directly into culture medium and treated simultaneously with ufCB. Cells were also treated with 50 μM Ag490 (Calbiochem) an inhibitor of the JAK/STAT transcription complex. Ag490 was diluted in DMSO prior to further dilution to working concentration in culture medium. Ag490 was used to treat cells 12 hours prior to ufCB treatment.

2.2.8 Antioxidant treatments.

Cells were treated with 200μM Nacystelin (NAL) which was diluted directly into culture medium prior to treatment simultaneously with ufCB. Cells were also treated with 25μM Trolox (Fluka) which was initially dissolved in DMSO prior to dilution in culture medium prior to treatment simultaneously with ufCB.

2.2.9 Conditioned medium treatments.

A549 conditioned medium was prepared by treating A549 cells overnight with ufCB. Medium was removed and particles were centrifuged out and the supernatant was used to treat MM6 cells. CRP-conditioned medium was prepared by diluting recombinant human CRP in culture medium to a final concentration of 4 μg/ml. Fibrinogen-conditioned medium was prepared by diluting recombinant human fibrinogen in culture medium to a final concentration of 2 μg/ml.

2.3.0 Diff-Quick staining.

A549 cells were grown in 24 well culture plates in which they were also treated. Cell culture medium was removed and cells washed in ice cold PBS. Cells were then stained using Diff-Quick staining kit. Cells were washed in distilled water and left to

dry. Cells were photographed in wells using a Zeiss inverted microscope and Sony digital camera.

2.4.0 Immunofluoresent staining

10mm glass cover slips (Raymond Lamb) sterilised by immersion in 70% IMS were added to 24 well plates. The cell suspension was added to give 10^5 cells/well on the plate. Cells were left to incubate at 37°C (5% CO_2) overnight to allow cells to adhere to cover slips. The next day the medium was removed and 1ml of fresh medium containing treatment was added to each well in the 24 well plate while untreated controls were incubated with 1ml of fresh medium. Cells were incubated at 37°C (5% CO_2) overnight. Medium was removed and cells washed with PBS.

2.4.1 Slide preparation and antibody staining.

Cold acetone was added to each well to fix cells to the coverslip for 10 minutes on ice. Cells were then washed three times with PBS. Cells were blocked with 5% sheep serum (or 5% BSA for Hsp70) in PBS for 45 minutes at room temperature to prevent non-specific binding. Cells were then washed three times with PBS. Parafilm was stretched over an empty 24 well plate and 50µl of 5% sheep serum was added to the first wells of treated and untreated rows on the plate. Each well thereafter had 50µl of primary antibody dispensed to appropriate wells (to correspond with original plate). Negative controls of 5% sheep serum and anti-2A4 antibody were set up to assess non-specific or irrelevant binding (2A4 binds to proteins found in red algae, which are not present in human cells). The positive control was set up to show the optimum fluorescence and reproducibility of the experiment. The positive control used was an antibody to CD44, the cell surface glycoprotein found on all human cells. Coverslips were then inverted onto antibody solutions and left to incubate in a humid chamber for 30 minutes at room temperature. Cover slips were retuned to 24 well plate and washed three times with PBS. Cells were incubated with secondary antibody, for 30 minutes in darkness, in a humid chamber. Three washes with PBS were carried out and coverslips mounted with Citifluor glycerol-PBS solution and examined using a UV microscope. Magnification of all images is X400 unless specified otherwise.

2.4.2 Analysis of photographs

Photographs of cells were analysed using Metamorph software. The intensities of 100 individual cells for each treatment were recorded and the mean intensity was calculated shown by Anderson (2003).

2.4.3 Propidium iodide staining

After cell treatment coverslips which were not acetone fixed were stained for immunofluoresence (2.4.1). Cells were then also stained using 10μg/ml propidium iodide for 20 seconds. After the cells were washed as before the coverslips were mounted and viewed using UV microscope. The percentage of cells which showed propidium iodide staining was then calculated.

2.5.0 Cell sample preparation

2.5.1 Lysate preparation

A549 cells were seeded at a density of 2 x 10^6 cells/ml in 25 cm^2 culture flasks to allow adherence. The cells were then treated with 10 ng/ml cytokines for 18 hrs. The medium (supernatant) was then removed, aliquoted and stored at -80°C. The cells were then removed using trypsin, washed firstly in fresh medium and then twice in ice cold PBS. The resulting pellet of cells was solubilised in 200ul 0.5% PBS-NP40 containing complete protease inhibitors (Roche) and incubated on ice for 30 minutes to allow lysis of the cells. The resulting lysate was centrifuged at 13,000 g for 20 minutes at 4°C. The supernatant was then aliquoted and stored at -80°C.

2.5.2 Protein concentration determination and equalisation

Before sample preparation the protein concentration of the lysates was determined. This was achieved using the Bioinchonic acid (BCA) protein determination method using BCA and copper II sulphate and the absorbance was read at 550nm (Dynex sample reader). Bovine serum albumin was used as the standard to determine the protein concentration.

2.6.0 Fibrinogen / CRP ELISA

Polyclonal anti-fibrinogen or anti-CRP (Sigma/Universal Biologicals, 1/100 in PBS) was added to wells of 96 well plate (Costar, high binding plate) and left overnight at 5°C. Antibody was removed and the plate washed in PBS. Samples and standards were added to wells in triplicate and incubated at room temperature for 2 hours. The samples were removed and the plate washed in PBS–Tween (0.05%). Monoclonal anti-fibrinogen or anti-CRP was added to the wells and left for 1 hour at room temperature. Plate was washed in PBS-Tween before addition of biotinylated anti-mouse Ig to wells before incubation for a further hour at room temperature. The plate was washed again in PBS-Tween before addition of streptavidin–HRP and incubation for 1 hour. After further washing in PBS–Tween, substrate was added (R & D systems) and incubated in the dark for 15 mins. The reaction was stopped by addition of 1M sulphuric acid. The plate was read at 550nm in a Dynex sample reader.

2.7.0 Western Blot – to detect Hsp70, NF-κB or IκB

2.7.1 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysate and supernatant samples were run on a 10% acrylamide gel in Hoefer minigel equipment (Hoefer Scientific Instruments) using the Laemelli (1970) method. All buffers and gels were prepared as per manufacturer's instructions. The samples were run under reducing conditions by adding dithiothreitol (DTT) to give a final concentration of 0.1M. Coloured and biotinylated (Biorad) standards were run as molecular weight markers. Gels were electrophoresed at 40mA for 45 minutes.

2.7.2 Transfer of protein to nitrocellulose membrane

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Protran, SLS) prior to detection by antibody staining using the Towbin (1979) method. All transfer equipment was equilibrated in transfer buffer for 10 minutes. Transfer was then carried out using Biorad equipment as per manufacturer's instructions at 100V for 1 hour. Resulting nitrocellulose blots were soaked in PBS-Marvel (5%)-Tween (0.1%)-NP40 (0.2%) overnight to block non-specific antibody binding sites.

2.7.3 Antibody detection of protein.

Proteins were visualised on the nitrocellulose blots using sheep anti-human Hsp70, mouse anti-human NF-κB, or rabbit anti-human IκB diluted 1/200 in blocking buffer, biotinylated secondary antibody and streptavidin–HRP (both Amersham-Pharmacia, diluted 1/500). Blots were then incubated with ECL Western Blotting detection substrate for 1 minute and exposed to radiographic film (Amersham-Pharmacia). The film was developed using AGFA hand developing solutions.

2.8.0 Cytokine ELISAs.

2.8.1 Interleukin-6 ELISA

Interleukin-6 ELISA was carried out using a Biosource kit. The manufacturer's instructions were followed. ELISA was carried out by incubation of capture antibody in 96 well plate (Costar) overnight. The plate was then blocked in BSA for 2 hours prior to washing (0.01% PBS-Tween) and incubation of samples and detection antibody simultaneously. The plate was then washed and incubated with streptavidin-HRP. The enzyme substrate (R+D systems) was added to achieve colour change before addition of stop solution (1M H_2SO_4). The plate was read at 550nm in Dynex plate reader

2.8.2 Interleukin-8 ELISA

Interleukin-8 ELISA was carried out using a Cytoset kit. The manufacturer's instructions were carried out. The capture antibody was incubated overnight in 96 well plate (Costar). The plate was blocked with BSA (1%) prior to washing (0.01% PBS-Tween) and addition of samples. The plate was washed and detection antibody was added. The plate was washed again and streptavidin-HRP was added and plate incubated in the dark. The plate was washed and enzyme substrate (R+D systems) was added to achieve colour change before addition of stop solution (1M H_2SO_4). The plate was read at 550nm in Dynex plate reader

2.8.3 Tumour necrosis factor α ELISA

TNFα ELISA was carried out using a Biosource kit. The manufacturer's instructions were carried out. The capture antibody was incubated overnight in 96 well plate

(Costar). The plate was blocked with BSA (1%) prior to washing (0.01% PBS-Tween) and addition of samples. The plate was washed and detection antibody was added. The plate was washed again and streptavidin-HRP was added and plate incubated in the dark. The plate was washed and enzyme substrate (R+D systems) was added to achieve colour change before addition of stop solution (1M H_2SO_4). The plate was read at 550nm in Dynex plate reader.

2.9.0 Reverse Transcriptase –polymerase chain reaction (RT-PCR).

2.9.1 Cell treatments.

Cells were seeded at a density of 10^6cell/ml and treated for 90 minutes in a 24 well plate. Medium was removed and cells washed in ice cold PBS before 300µl tri-reagent was added. Cells were then added to RNA free Eppendorf tubes and then either RNA extraction was carried out or samples were stored at -80°C until extraction at a later date.

2.9.2 RNA extraction.

If samples were frozen they were defrosted and left for 10 minutes. To cell/tri-reagent mixture 200 µl chloroform was added before vortexing and samples were left to stand for 10 minutes. Samples were centrifuged at 15,000g for 15 minutes at 4°C. The resulting upper phase was transferred to a fresh RNA free tube and 450µl isopropanol was added before centrifugation at 15,000g for 10 minutes. The supernatant was then removed and discarded and 50µl 70% ethanol was added to the remaining pellet prior to centrifugation at 7500g for 5 minutes. The supernatant was removed and the pellet left to dry for 1 minute before the addition of 50 µl nuclease free-water.

2.9.3 RNA quantification.

The amount of RNA in the samples was than quantified using absorbance readings at 260nm. The absorbance reading of the RNA was then used to calculate the dilution required to equalise 10 µl of RNA.

2.9.4 Polymerase chain reaction.

Reaction mixtures were made up using Access kit (Promega) and primers for CRP (forward 5' TCG TAT GCC ACC AAG AGA CAA GAC A- 3', reverse 5' AAC ACT TCG CCT TCG ACT TCA TAC T- 3'), fibrinogen (forward 5' GGG CAC ATT TGA AGA GGT GT-3', reverse 5' GCT ATC CCA GGG TGA TGA GA-3'), Hsp70 (forward 5' ATC GAC CTG GGC ACC ACC TA-3', reverse 5' CAG CAC CAT GGA CGA GAT CT-3') and housekeeping gene GAPDH (forward 5' TCT AGA CGG CAG GTC AGG TCA ACC-3', reverse 5' CCA CCC ATG GCA ATT TCC ATG GCA -3') (all MWG Biotech). Samples for fibrinogen, Hsp70 and GAPDH were run for 25 cycles, and CRP was run for 35 cycles.

2.9.5 Agarose gel electrophoresis.

On completion of PCR reactions 6μl of product was added to 40μl BJ/OJ loading dye (Promega). A 1.5% agarose gel was prepared in 200 ml 1x TBE buffer (Promega) with 5μl ethidium bromide added. This gel solution was poured into a horizontal Biorad GT electrophoresis tank and left to set for 1 hour. Once the gel was set 10μl of sample was added to the gel with 10μl molecular marker (2μl DNA marker (Roche), 2μl BJ/OJ dye and 6ml nuclease free water). The gel was then electrophoresed at 100V for 90 minutes. PCR products were visualised under UV light using Gene Snap software (Syngene).

2.9.6 Quantification of product.

Quantification of PCR product bands were carried out using Gene Snap and Gene tools software (Syngene).

2.10.0 ATP Assay.

2.10.1 Cell extraction.

Cells were seeded at a density of 1.3 x 10^5 cells/well in a 24 well plate and treated for various time points (0, ½ , 3 ,6 18 hours). Medium was removed and cells were treated with 30μl 1% sulphosalycylic acid (SSA) for 5 minutes at room temperature. Cells were than removed from the plate and resuspended in the acid solution. Cells were centrifuged at 15,000 g for 3 minutes and stored at -80°C.

2.10.2 Measurement of samples.

Reagent buffer was prepared (0.05M Tris, 2mM EDTA, 2mM $MgCl_2$, 0.4g/lBSA) and 2mM dithiothreitol (DTT), luciferase (1mg/ml) and luciferin (1.5mM) were added. The buffer was left at room temperature for 2 hours. ATP standards were made up in water (0, 0.5, 1, 2.5, 5, and 10μM). In a polypropylene cuvette 200μl reagent and 20μl sample were mixed and luminescence was recorded immediately over a 30 second integral using a BioOrbit 1250 luminometer (Labsystems, UK).

2.11.0 LDH Assay

Samples were collected as supernatants from treated cells. Standards were made up of NADH in sodium pyruvate (0.75mM) to concentrations of 2000, 1530, 1040, 640, 280 and 0 U/ml. 60μl of each was added in triplicate to wells of 96 well plate. The rest of the plate was filled with 50μl sodium pyruvate and the plate was incubated for 5 minutes at 37°C. 10μl of each sample was added to the plate before further incubation at 37°C for 30 minutes. 50μl of 2,4-dinitrophenylhydrazine was added and the plate was incubated at room temp for 20 minutes in dark. 50μl 4M sodium hydroxide was added and the plate was allowed to stand for 5 minutes at room temperature. The plate was read at 550 nm in Dynex plate reader.

2.12.0 Data handling

2.12.1 Experimental results

All experiments were repeated three times giving n = 3 for statistical analysis. All graphs are expressed as mean of all three experiments +/- SEM

2.12.2 Statistical Analysis

Normality test of the data was carried out using Kolmogorov-Smirnov test. Statistical significance of data was carried out using one way ANOVA and Tukey's multiple comparisons.

Chapter 3

Effect of cytokine treatment on acute phase protein (APP) expression in lung epithelial cells.

3.1.0 Effect of cytokine treatment on fibrinogen expression.

3.1.1 Treatment of A549 cells with IL6, TNFα and IFNγ.

The aim of this experiment was to identify if A549 cells could express fibrinogen with cytokine treatment. It was expected that there would be expression of fibrinogen within these cells as shown by Simpson-Haidaris (1997) and Anderson (2003).

Immunofluorescent staining was used to show expression of fibrinogen in the treated cells (10ng/ml IL6, TNFα, or IFNγ). This technique is used to show the localisation and intensity of protein expression within the cells. Photographs taken show that fibrinogen is expressed in a sub-population of the untreated cells (Fig 1a), localised in the cytoplasm, and that this can be dramatically increased by IL6 treatment (Figs. 1b). Cells treated with TNF α showed little increase in fibrinogen expression when compared to the untreated cells (Fig.1c) which agrees with immunofluorescence carried out previously (Anderson, 2003).

The photographed cells were analysed using Metamorph software package to indicate the intensity of fluorescence in individual cells. One hundred cells from each treatment were analysed to indicate the levels of expression ($n = 3$). These results then enabled statistical analysis of any increase or decrease with treatment (Fig 2). The intensity analysis shows that there was a significant up-regulation of fibrinogen by IL6 ($p<0.05$). Although this method gives a semi-quantitative result for the immunofluorescence results it is limited by the fact that the expression of proteins in the cells is not uniform. This results in some under and over-estimation of the intensities and therefore can only be used as a guide to the actual level of fibrinogen in the cell at any given time.

ELISA assays were performed to determine if there was any secretion of fibrinogen from the cells. This technique is more sensitive than the immunofluorescence intensites for indicating the level of protein expression within the cells. The cell supernatants (Fig 3) indicated that fibrinogen was being secreted from the cells with all treatments. TNF α treated cells have a lower level of secreted fibrinogen than the untreated cells, however this was not statistically significant when $n = 3$. This is due

to variation between the samples day-to day, although all assays showed the same trend. This reduction in the amount of secreted fibrinogen when cells are treated with TNFα is similar to that found by Anderson (2003) where treatments were carried out in serum free conditions.

3.1.2 Treatment of A549 cells with IL6, TNFα and both cytokines simultaneously

In the liver many cytokines are produced simultaneously during an APR and the combined effects of these control the levels of the acute phase proteins such as fibrinogen. Therefore the effect of more than one cytokine at a time was investigated.

Cells were treated with 10ng/ml of IL6, TNFα, 10ng/ml IL6 plus 10ng/ml TNFα, with untreated cells for comparison. Indirect immunofluorescence staining showed that IL6 plus TNFα treatment induced fibrinogen staining in the cytoplasm. The levels of staining were below the levels of IL6 treatment alone but above that of TNFα alone (Fig 4). Intensity analysis ($n=3$) of the images was then carried out using Metamorph software, giving an indication of the levels of fibrinogen within the cells (Fig 5). This showed that IL6 increased the levels of fibrinogen 5-fold and the IL6 plus TNFα treatment increased the levels 4-fold, with both these treatments being statistically significant ($p<0.001$) over that untreated cells.

ELISAs were then carried out on the cell supernatants to investigate whether fibrinogen was secreted from the cells. This showed that there was little secretion of fibrinogen from these cells (Fig 6). The IL6 plus TNFα treatment showed some increase of secretion from the cells, however this was not significant, but all the other treatments were similar to the untreated cells.

3.1.3 Treatment of A549 cells with IL6 and IL1β and both cytokines simultaneously.

Cells were treated with 10ng/ml of IL6 or IL1β, or 10ng/ml IL6 plus 10ng/ml IL1β, with untreated cells for comparison. Immunofluorescence showed that IL6 increased the levels of fibrinogen above the untreated control (Fig 7). All the cells in the IL6 treated cells showed cytoplasmic expression of fibrinogen but a small sub-population of cells in the other treatments showed low levels of fibrinogen expression.

Intensity analysis of the images shows that indeed IL6 was the only cytokine treatment which increased the expression of fibrinogen (Fig 8). The level of CRP expression in the IL6 treated cells was statistically significant from the untreated cells ($p<0.001$). The immunofluorescence intensites also indicated that the IL6 plus IL1β treatment resulted in less expression of fibrinogen than the untreated cells.

ELISAs were than carried out on both the cell supernatants. Analysis of the cell supernatant (Fig 9) indicated that all the cytokine treatments increased the expression of fibrinogen over that of the untreated cells. IL6 once again showed the greatest increase in expression with IL1β showing the next greatest increase with the combination of both cytokines being below the levels of either cytokine alone. Again there is no statistical significance between the treatments due to variations among the results.

3.1.4 Treatment of A549 cells and with IL6 and IL8 simultaneously.

Cells were treated with 10ng/ml of IL6 or IL8, or 10ng/ml IL6 plus 10ng/ml IL8, with untreated cells for comparison. Immunofluorescence shows that IL6 produces the greatest increase in fibrinogen, with IL6 plus IL8 showing an increase in expression over the untreated cells (Fig 10). Intensity analysis of the cells using Metamorph (Fig 11) shows that the greatest increase in expression is in the IL6 plus IL8 treated cells ($p>0.001$), which is not evident when looking the pictures. IL6 treatment alone is also increased above that of the untreated cells ($p<0.01$) with IL8 being very similar to that of the untreated cells.

ELISAs were carried out on the cell supernatants. Analysis of the cell supernatant (Fig 12) indicated that all the cytokine treatments increased the expression of fibrinogen over that of the untreated cells. IL6 once again showed the greatest increase in expression with IL8 showing the next greatest increase with the combination of both cytokines being below the levels of either cytokine alone. Neither treatment showed any statistical significance compared to the untreated cells due to large variations between the samples but all showed the same trends.

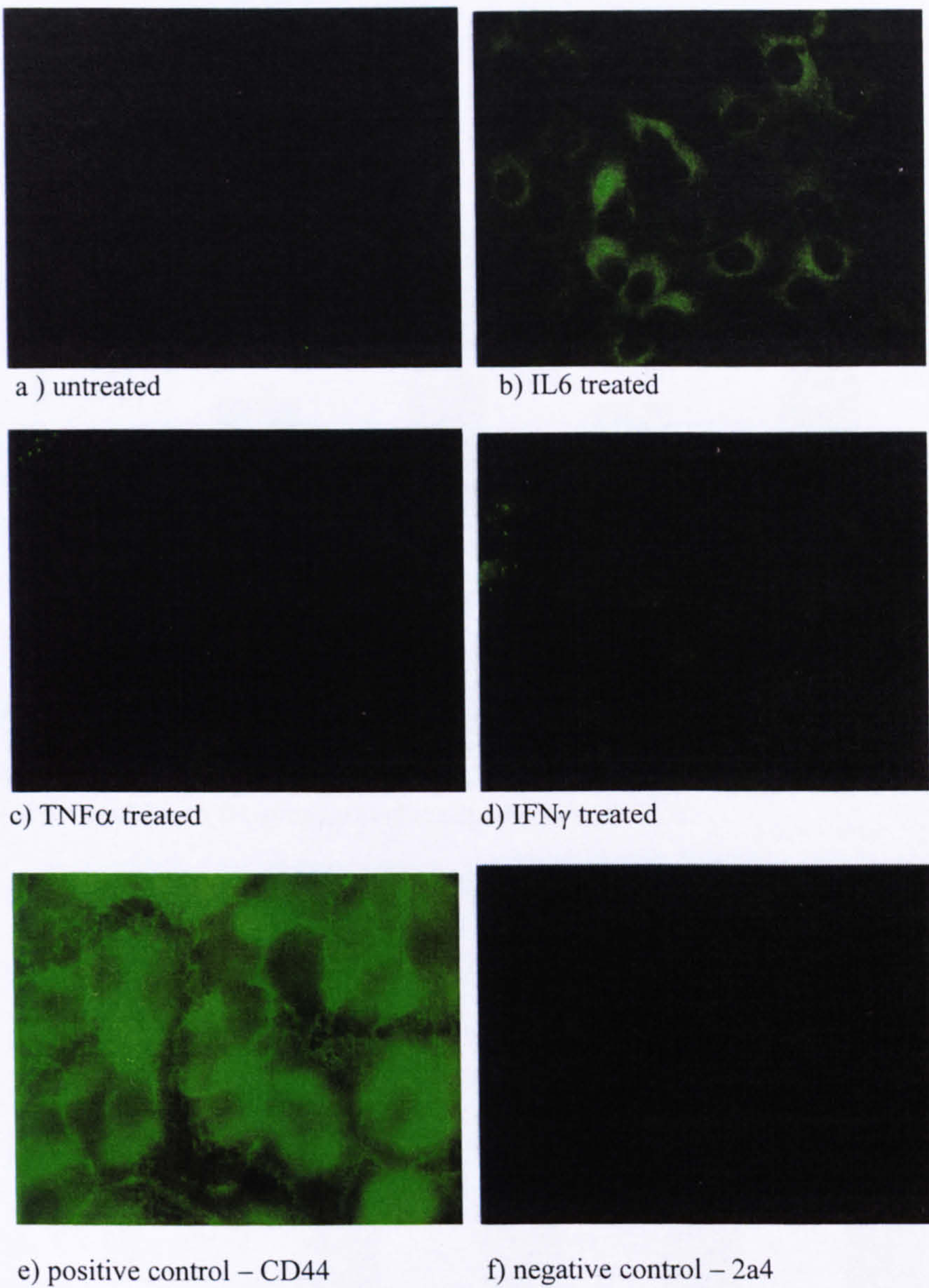


Figure 1: Expression of fibrinogen in A549 cells treated with 10ng/ml of each cytokine for 18 hours as shown by indirect immunofluorescence

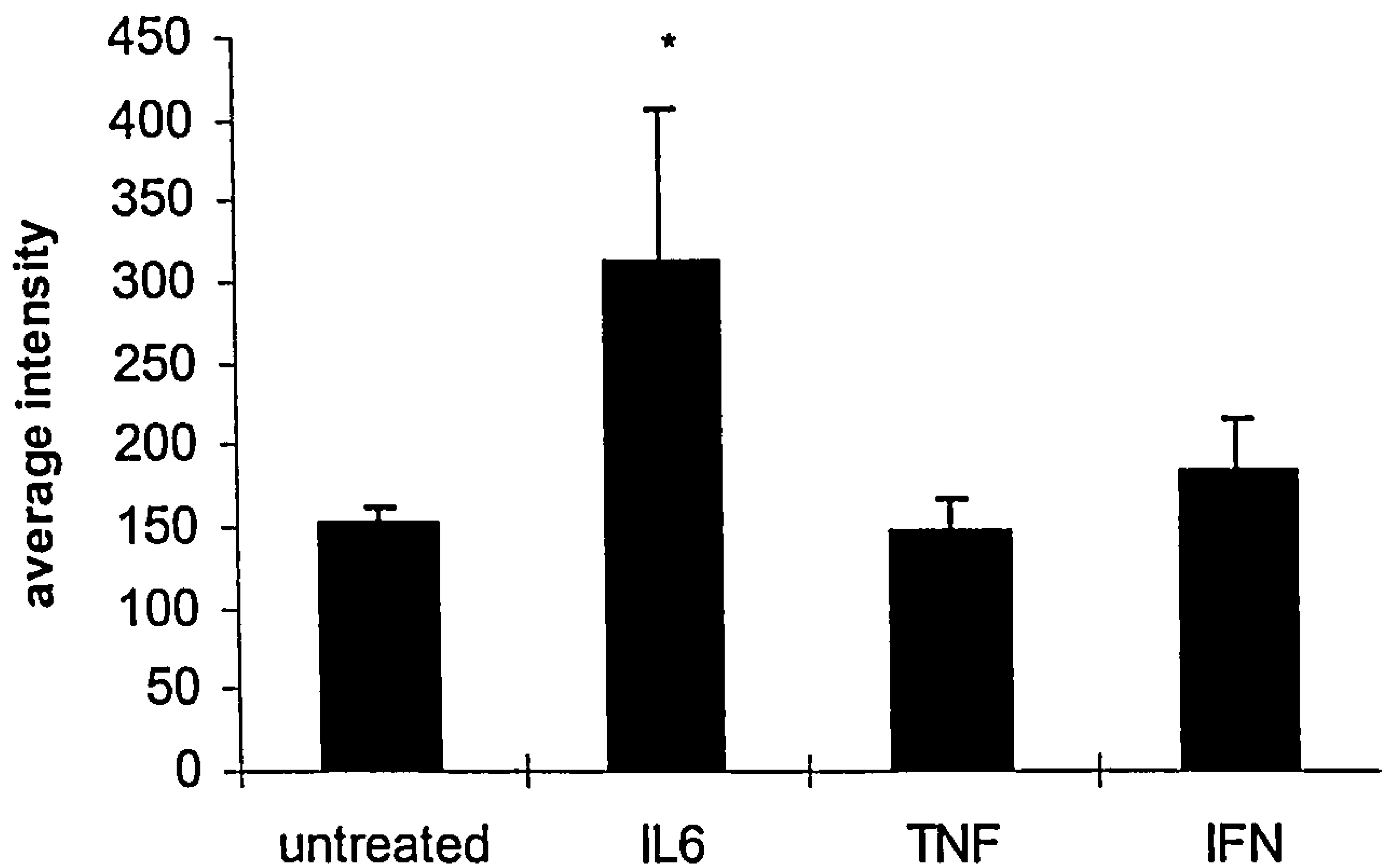


Figure 2: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of treatment with 10ng/ml of each cytokine. The average intensity is an arbitrary unit which is used in all figures. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated (control) cells, $p < 0.05$.

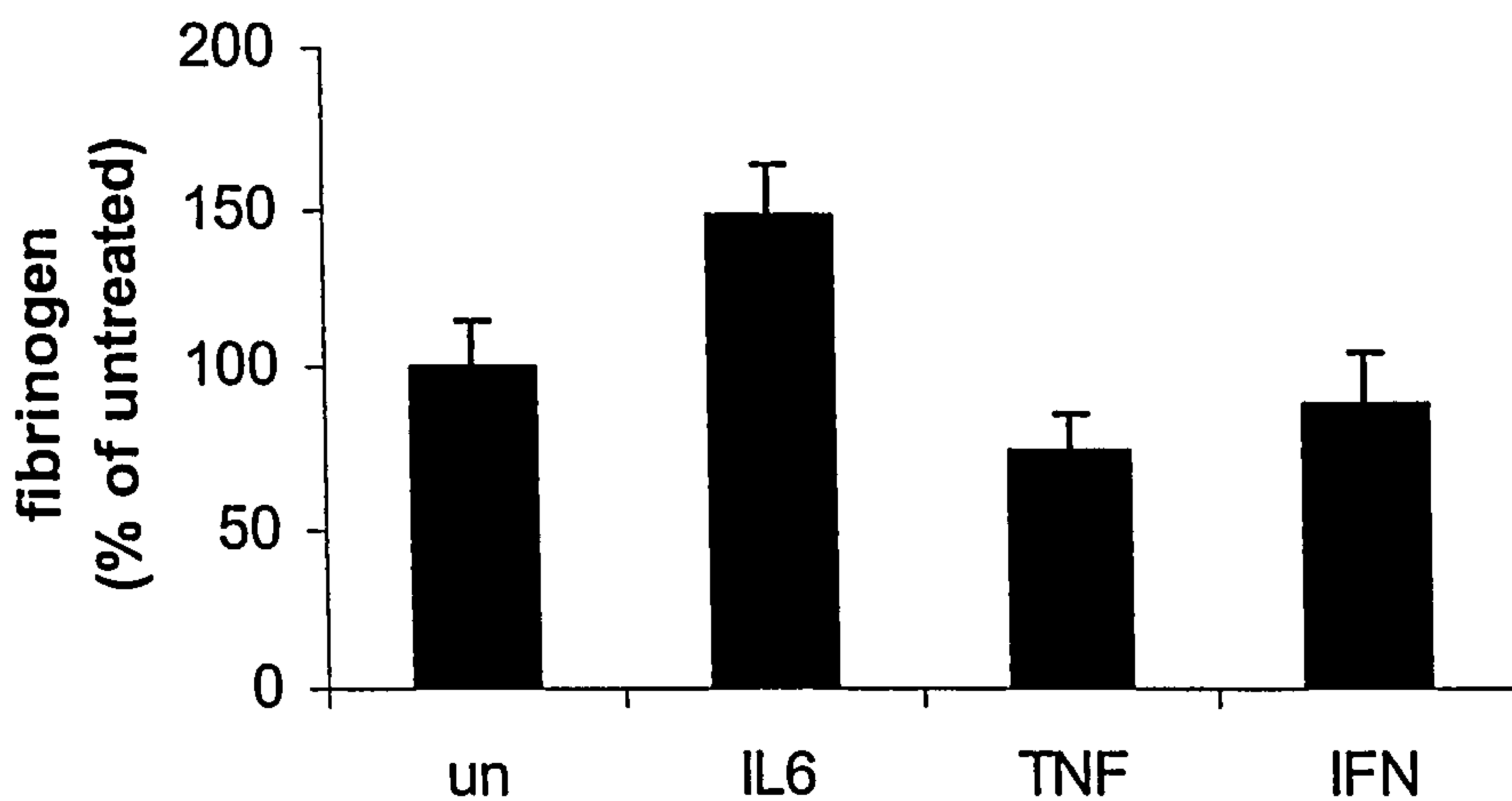
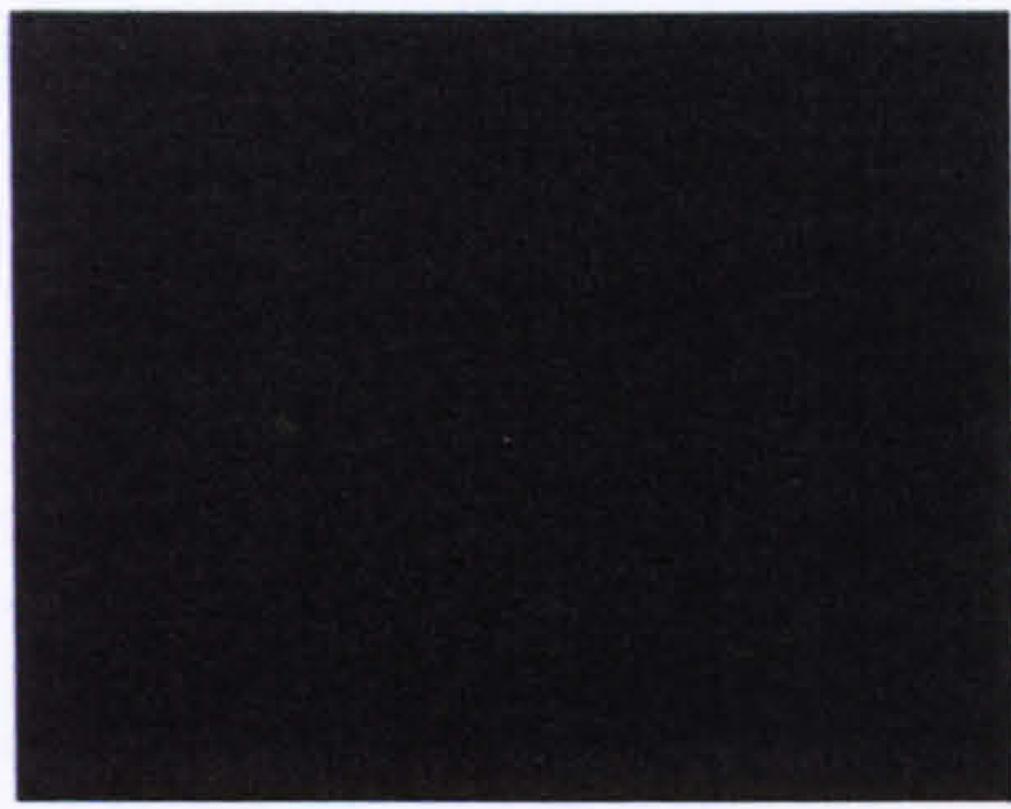
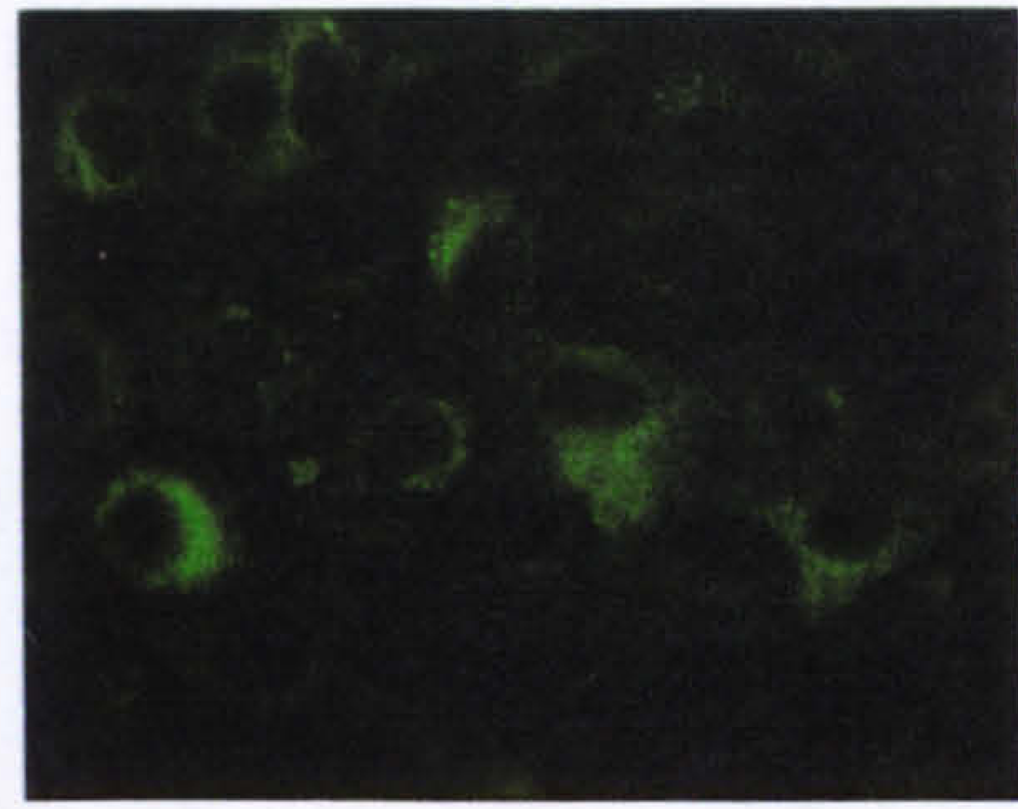


Figure 3: Concentration of fibrinogen in cell supernatant measured by ELISA after 18 hours of treatment with 10ng/ml of each cytokine. Fibrinogen is expressed as a percentage of the concentration present in the untreated cells. The results are the mean of triplicate results from 3 experiments ± SE.

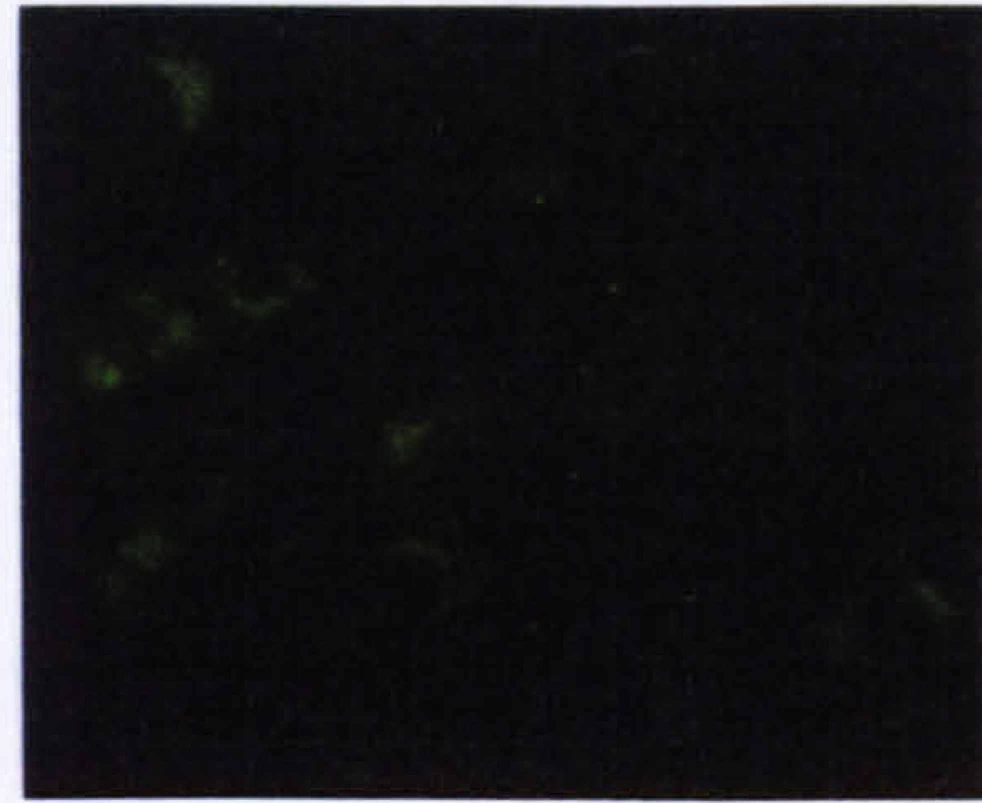


a) untreated

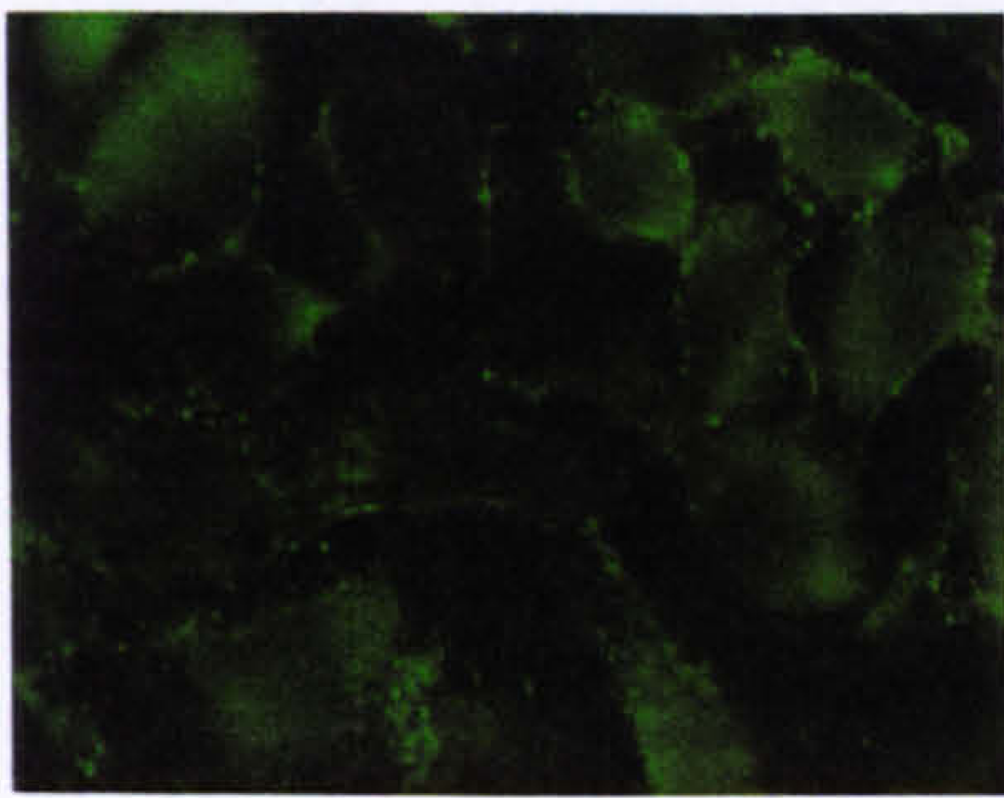


b) IL6 treated

c) TNF α treated



d) IL6 + TNF α treated.



e) positive control – CD44

f) negative control – 2a4

Figure 4: Immunofluorescent photographs of A549 cells stained for fibrinogen. Cells were treated with 10ng/ml of TNFα or IL6 for 18 hours.

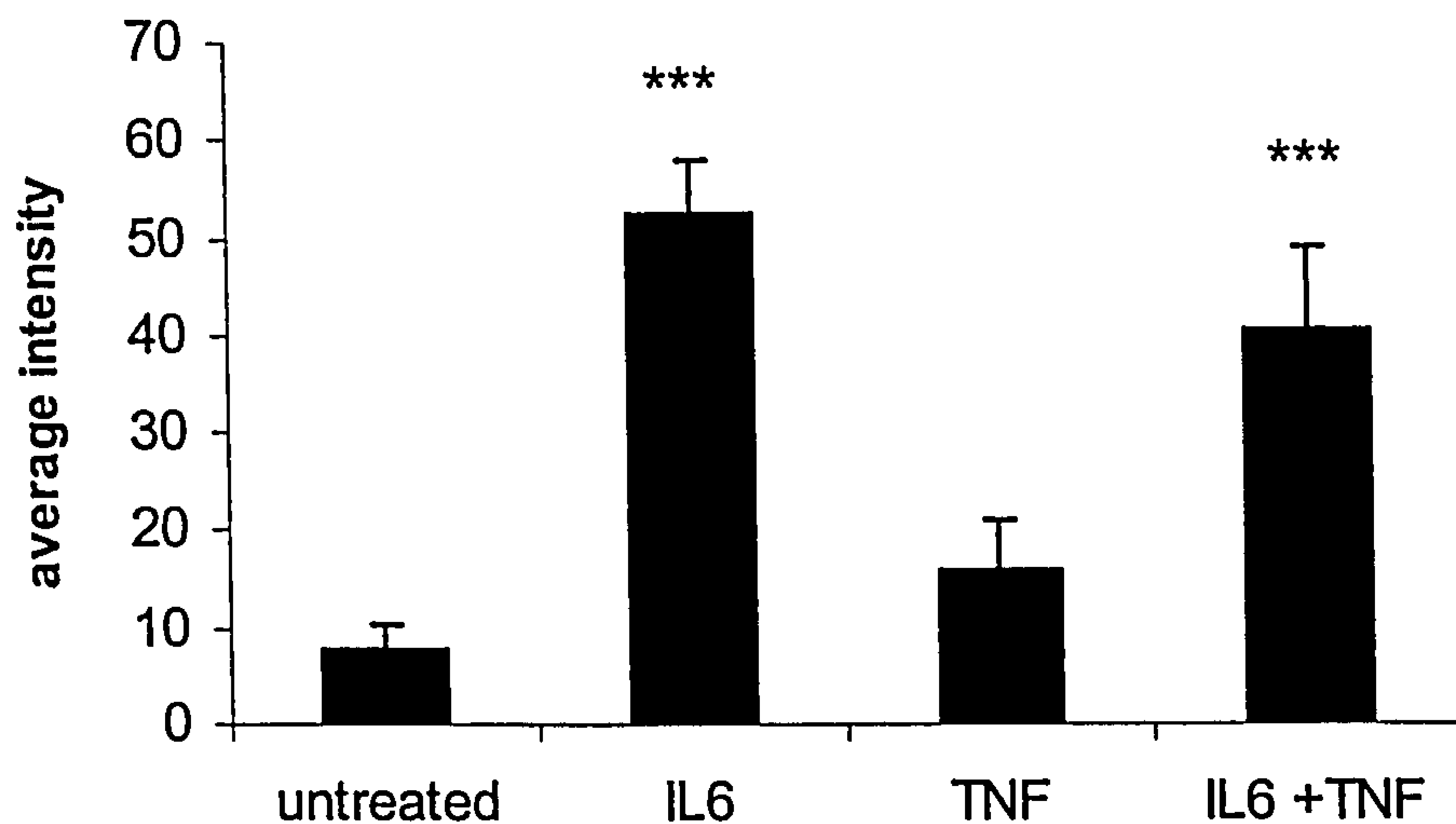


Figure 5: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 10ng/ml TNFα or IL6 treatments. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denotes significant changes from untreated (control) cells, p< 0.001.

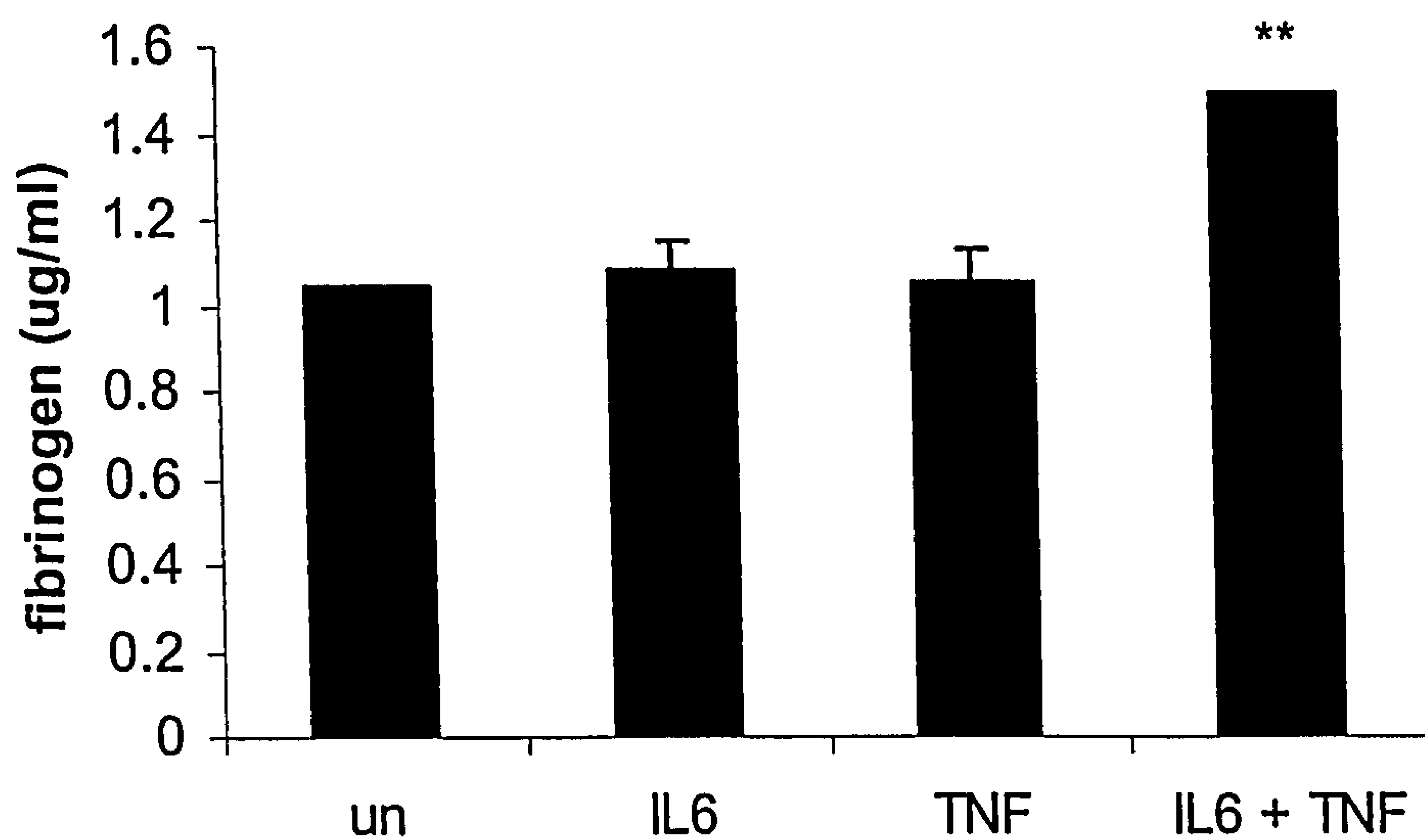


Figure 6: Concentration of fibrinogen in cell supernatant measured by ELISA after 18 hours of 10ng/ml TNFα or IL6 treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, p< 0.01.

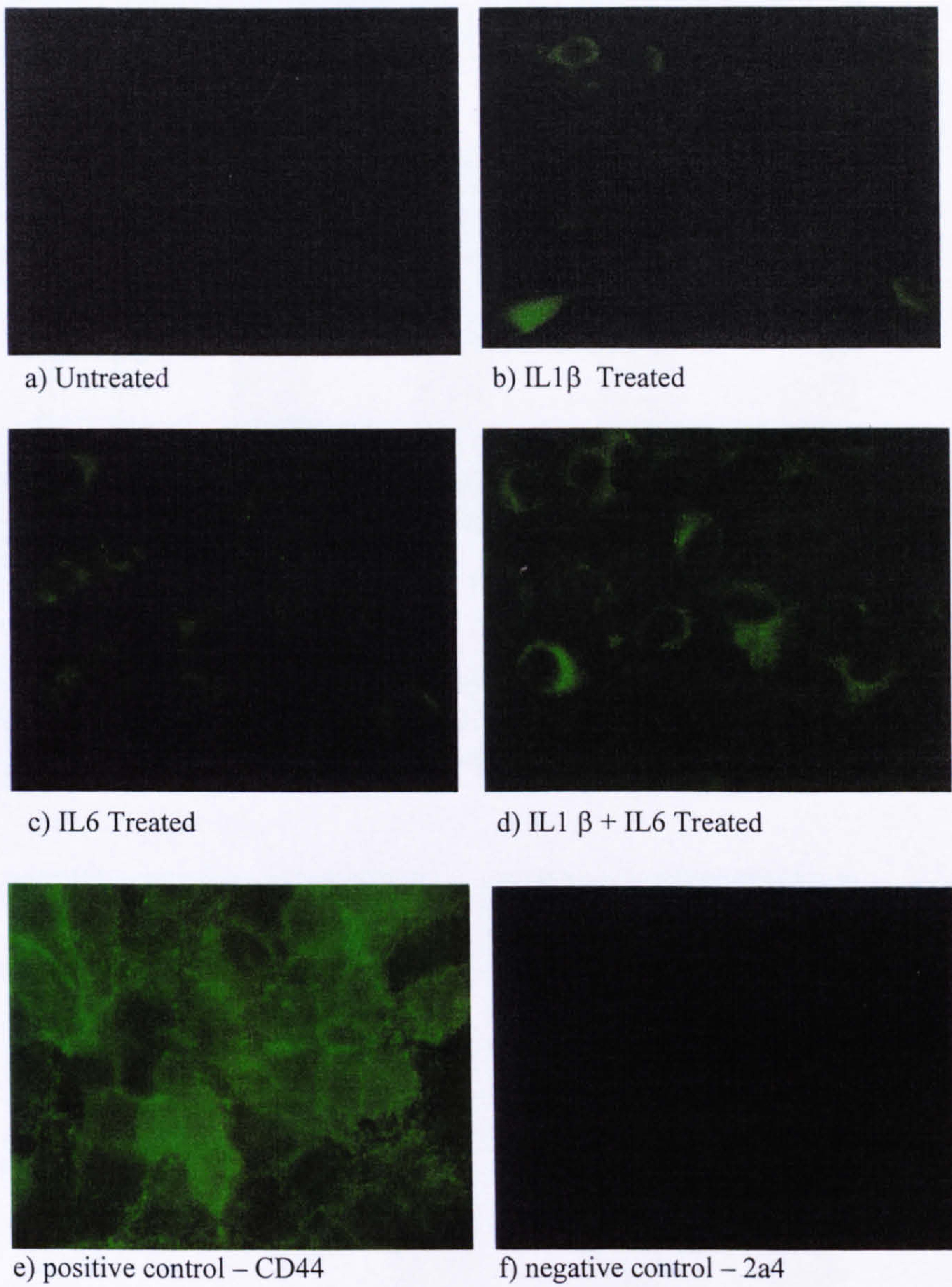


Figure 7: Expression of fibrinogen in A549 cells treated with 10ng/ml IL6 or IL1β for 18 hours shown by indirect immunofluorescence.

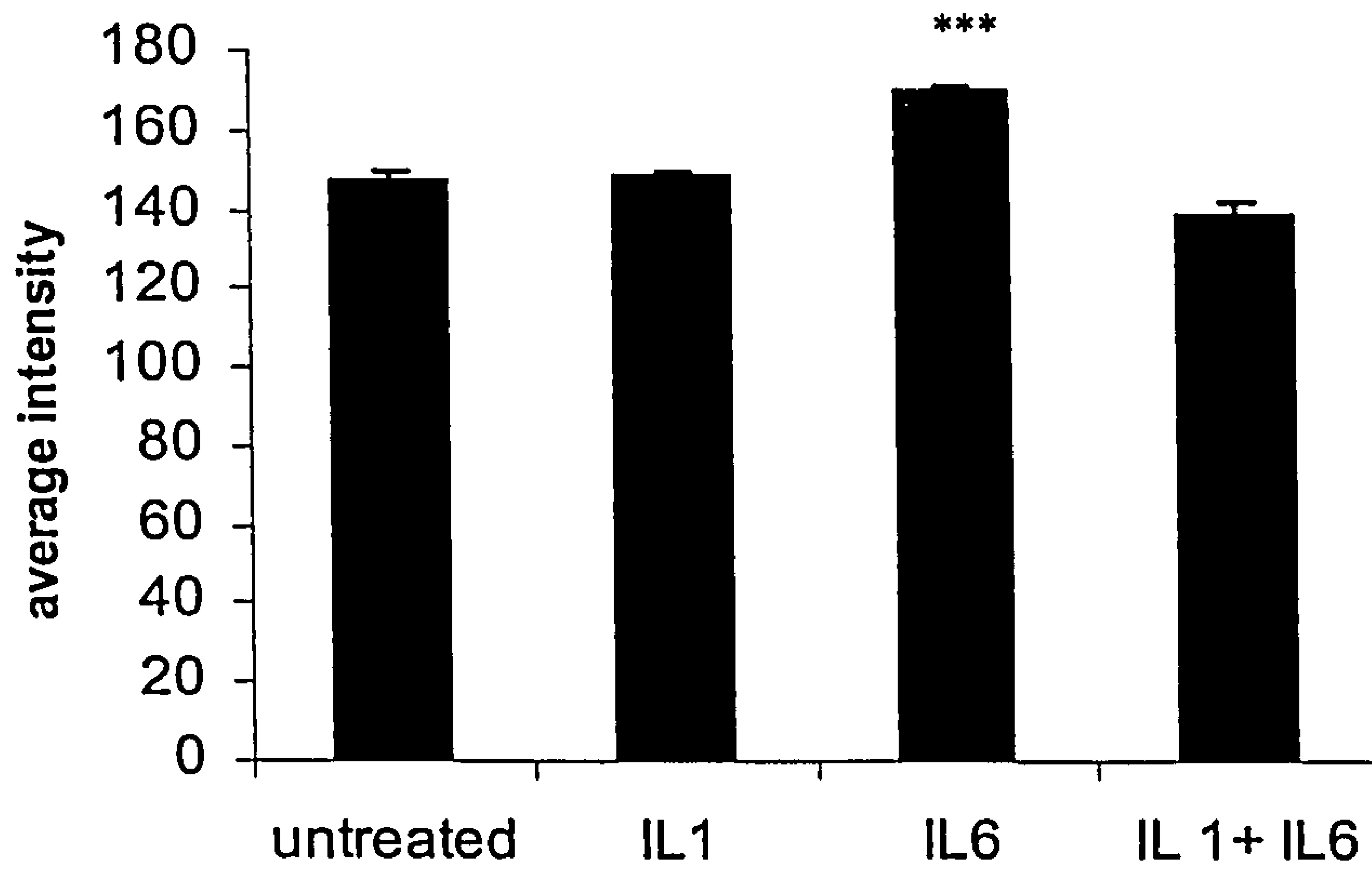


Figure 8: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 10ng/ml IL6 or IL1β treatments. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denotes significant changes from untreated (control) cells, $p < 0.001$.

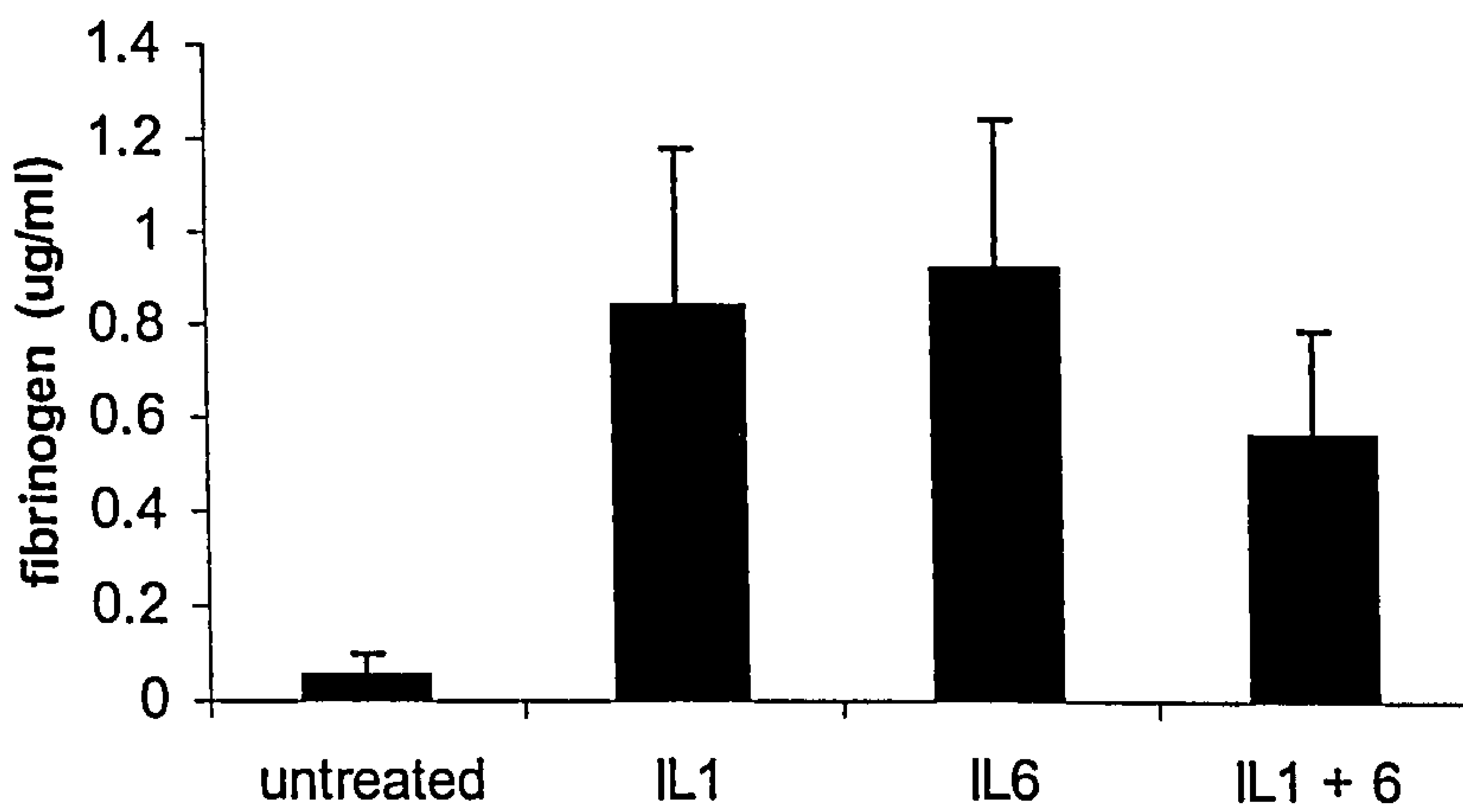


Figure 9: Concentration of fibrinogen in cell supernatant measured by ELISA after 18 hours of 10ng/ml IL6 or IL1β treatment. The results are the mean of triplicate results from 3 experiments ± SE.

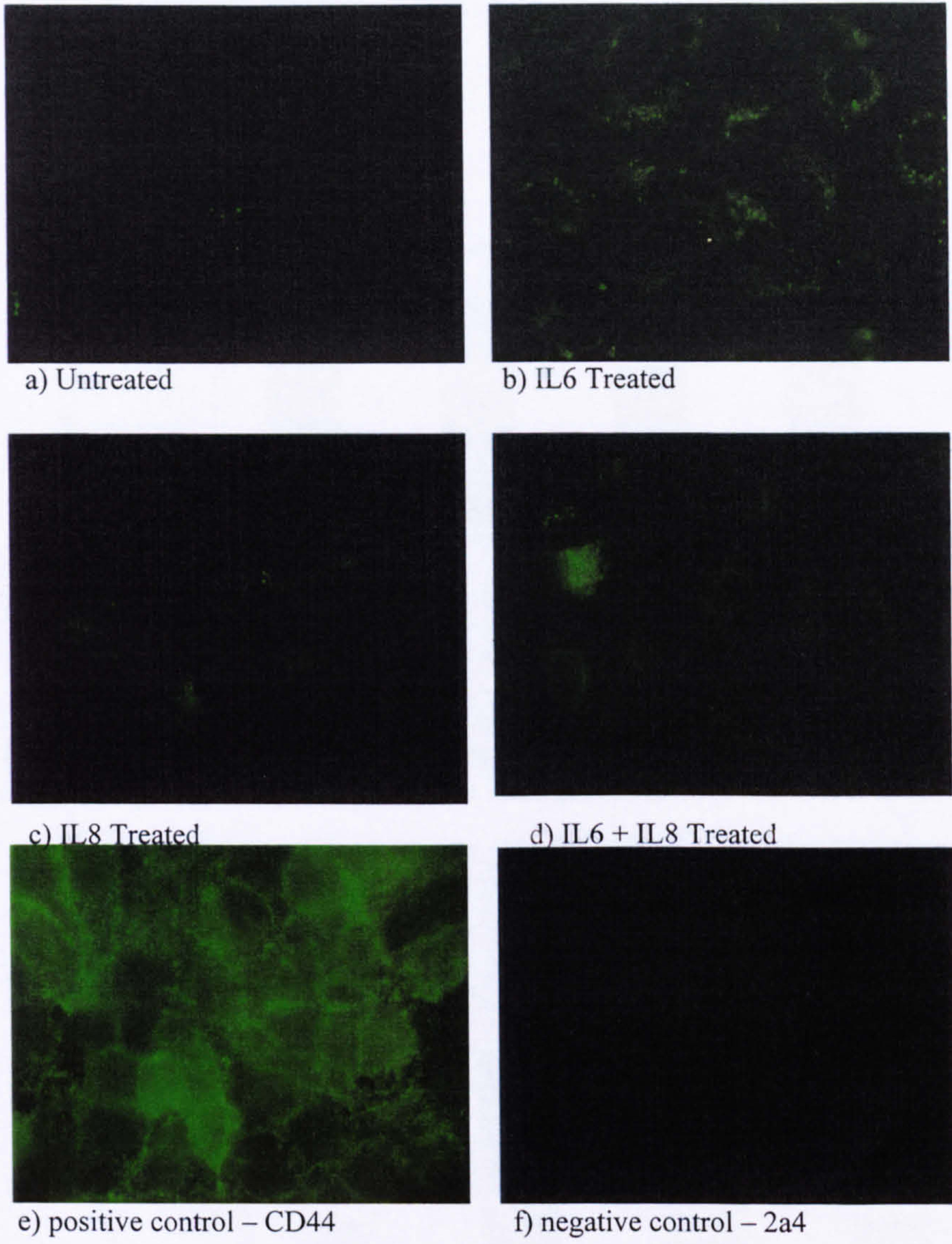


Figure 10: Expression of fibrinogen in A549 cells treated with 10ng/ml IL6 or IL8 for 18 hours as shown by indirect immunofluorescence.

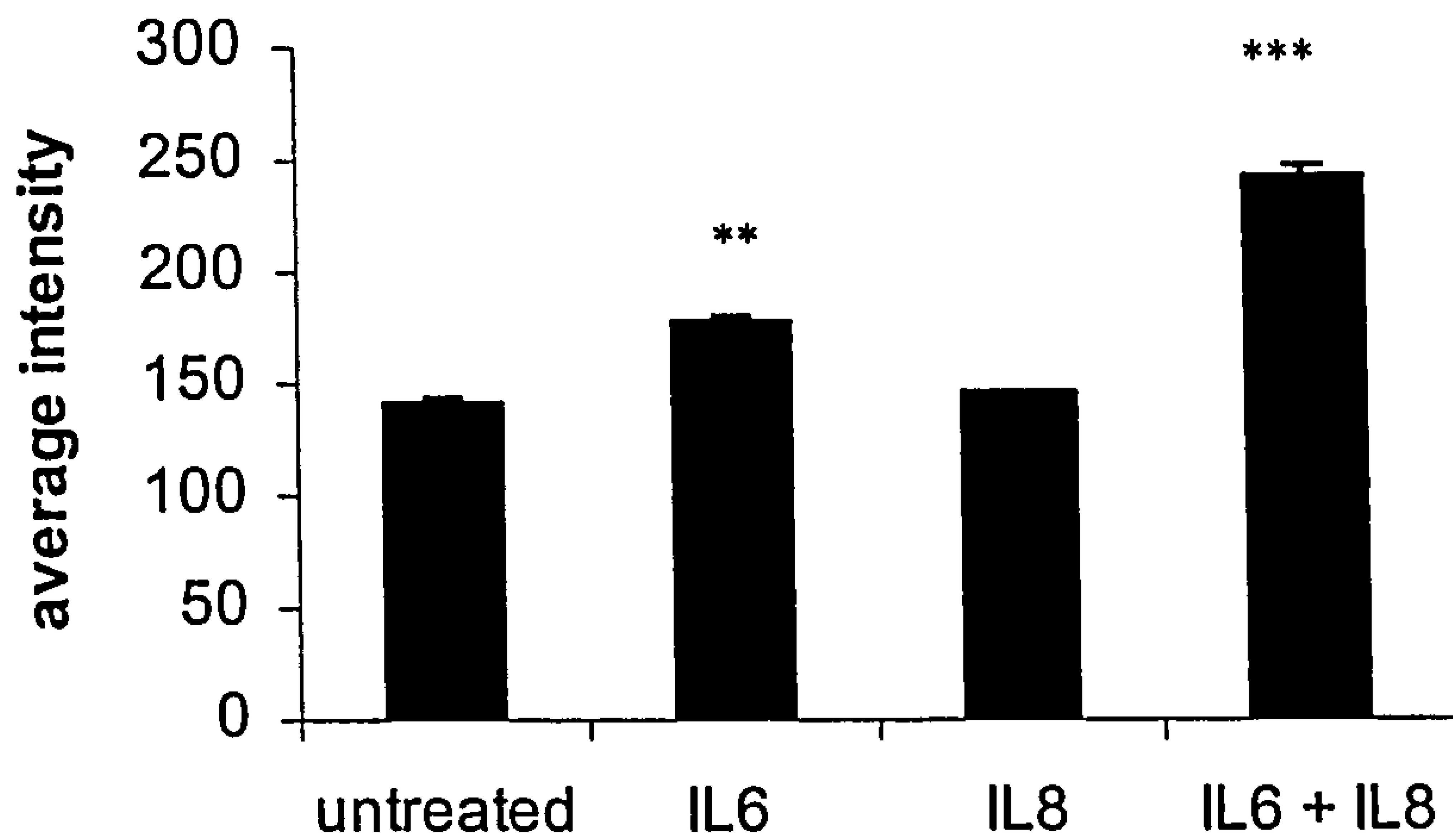


Figure 11: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 10ng/ml IL6 or IL8 treatments. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (, ***) denotes significant changes from untreated (control) cells, p< 0.01, p<0.001.**

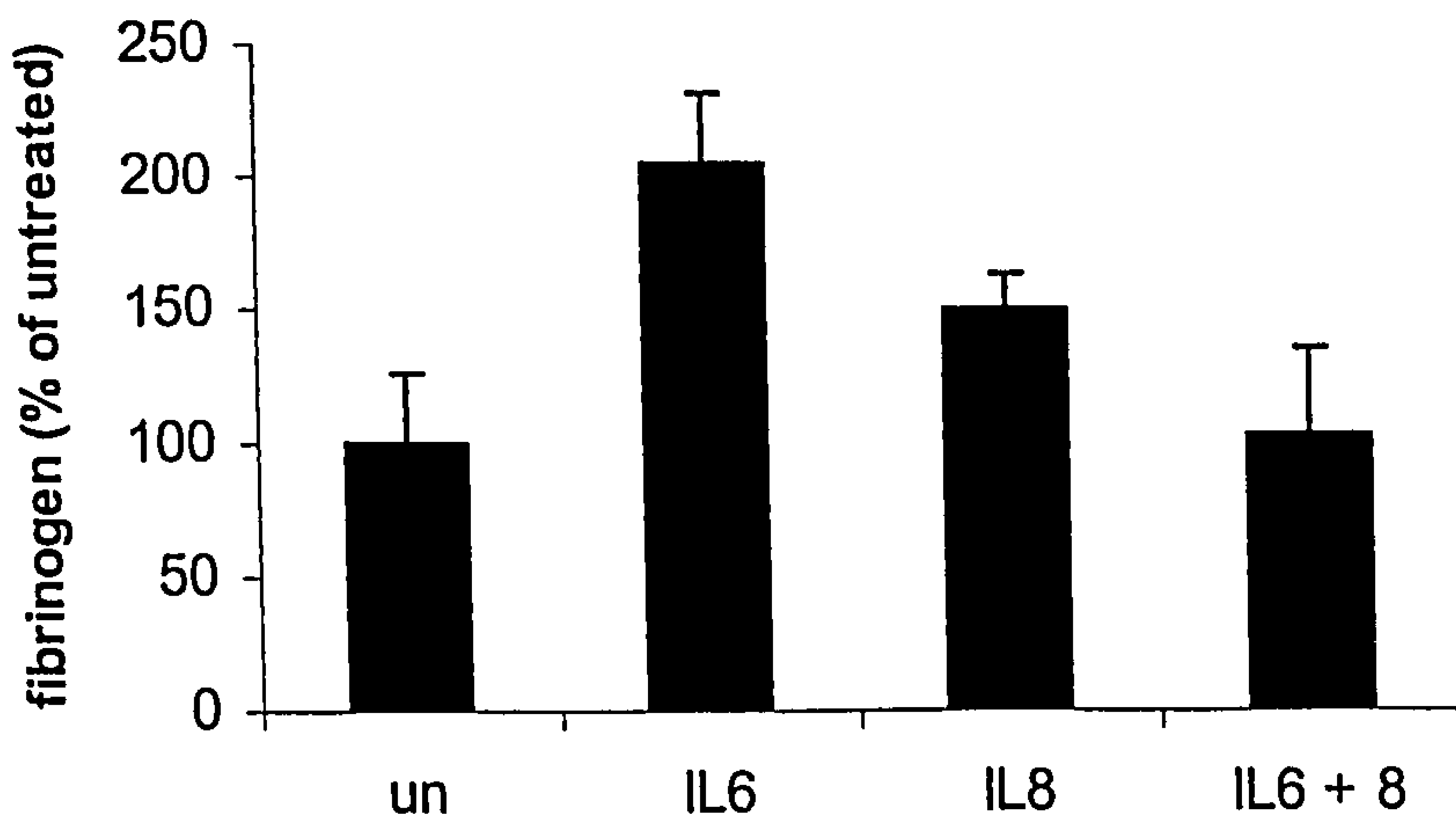


Figure 12: Concentration of fibrinogen in cell supernatant measured by ELISA after 18 hours of 10ng/ml IL6 or IL8 treatment. Fibrinogen is expressed as a percentage of the concentration present in the untreated cells. The results are the mean of triplicate results from 3 experiments ± SE.

3.2. Effect of cytokine treatment on C-reactive protein (CRP) expression.

3.2.1 Treatment of A549 cells with IL6, TNFα and IFNγ.

Since fibrinogen was expressed in A549 cells an investigation into the expression of CRP, the prototypic human APP in the lung epithelium was carried out. IL6 is a major regulator of CRP expression in hepatocytes and it was investigated as to whether it would have a similar effect in A549 cells.

Immunofluorescent staining was used to show CRP in the treated cells (10ng/ml IL6, TNF α or IFN γ). Photographs taken (Fig 13) show that there are very low levels of CRP in A549 cells, and this can be dramatically increased by treatment with IL6 and IFN γ. There also appears to be a smaller increase of CRP expression with TNF α treatment. CRP is located within the cytoplasm which would be expected since in hepatocytes CRP is found in the cytoplasm attached to the endoplasmic reticulum (Yue *et. al.*, 1996).

The photographed cells were also analysed using Metamorph software to indicate the intensity of fluorescence of the individual cells. One hundred cells from each treatment were analysed to indicate the level of expression from each treatment ($n = 3$). These results then enabled statistical analysis of the increase in CRP within the cells (Fig. 14). Intensity analysis shows that there is a significant up-regulation of CRP by IL6 and IFN γ ($p<0.001$)

ELISA assays were carried out on the cell supernatant to determine if there was any secretion of CRP from the cells (Fig 15). It is clear from this figure that the CRP produced from the TNF α treated cells is being secreted from the cells during the 18 hour incubation.

3.2.2 Treatment of A549 cells with IL6, TNFα and both cytokines simultaneously.

As for fibrinogen the effect of several cytokines on the expression of CRP was investigated since more than one cytokine is likely to be found during inflammatory stimuli.

Cells were treated with 10ng/ml of IL6, TNFα, 10ng/ml IL6 plus 10ng/ml TNFα, with untreated cells for comparison. Immunofluorescence showed that the cytokine treatments produced staining in the cytoplasm, above the untreated levels. TNF α showed the smallest increase, with the levels of IL6 treatment alone and in combination with TNFα appearing similar (Fig 16). Intensity analysis (n=3) of the images was then carried out using Metamorph software, giving an indication of the levels of CRP within the cells (Fig 17). This showed that IL6 increased the levels of fibrinogen 6-fold and the IL6 plus TNFα treatment increased the levels 7-fold, with both these treatments being statistically significant ($p<0.01$) over that untreated cells.

ELISAs were then carried out on the cell supernatants to investigate whether CRP was secreted from the cells. This showed that there was little secretion of CRP from the untreated and IL6 treated cells (Fig 18). The TNFα and IL6 plus TNFα treatment showed an increase in secretion from the cells, however these results were not statistically significant.

3.2.3 Treatment of A549 cells with IL1β, IL6 and both cytokines simultaneously.
It is known that IL1 β and IL6 are required for the maximal synthesis of CRP in hepatocytes. Therefore the effect of several cytokines administered at the same time was investigated for CRP in lung epithelial cells.

Cells were treated with 10ng/ml of either IL1 β , IL6, or 10ng/ml IL1β plus10ng/ml IL6, with untreated cells for comparison. Immunofluoresence revealed that IL1β upregulated expression of CRP in A549 cells (Fig 19). The levels of IL6 induced CRP appear similar to that of the IL1 β treated cells. The IL1 β and IL6 treated cells have slightly higher levels than either of the cytokines on their own. The distribution of CRP within these cells is mainly in the cytoplasm but also with clear nuclear staining probably associated with snRNP binding. The nuclear staining is particularly prominent in the cells treated simultaneously with IL1 β and IL6. Intensity analysis ($n = 3$) shows that there is increased CRP in the order IL1 β $<$ IL6 $<$ IL1 β plus IL6-treated cells (Fig 20). Statistically this is significant ($p< 0.01$) in the IL1 β plus IL6 treated cells over the control (untreated cells). ELISAs were performed to indicate the

presence of secreted CRP with IL6 or IL1β (Fig 21). It was found that CRP was secreted from the cells with all treatments and there was a slight increase over untreated cells with the cytokines treatments however none of there treatments was statistically significant from the untreated cells. Individual assays show the same trend, however the concentrations of CRP secreted from the cells varied from day-to-day and were therefore not statistically significant.

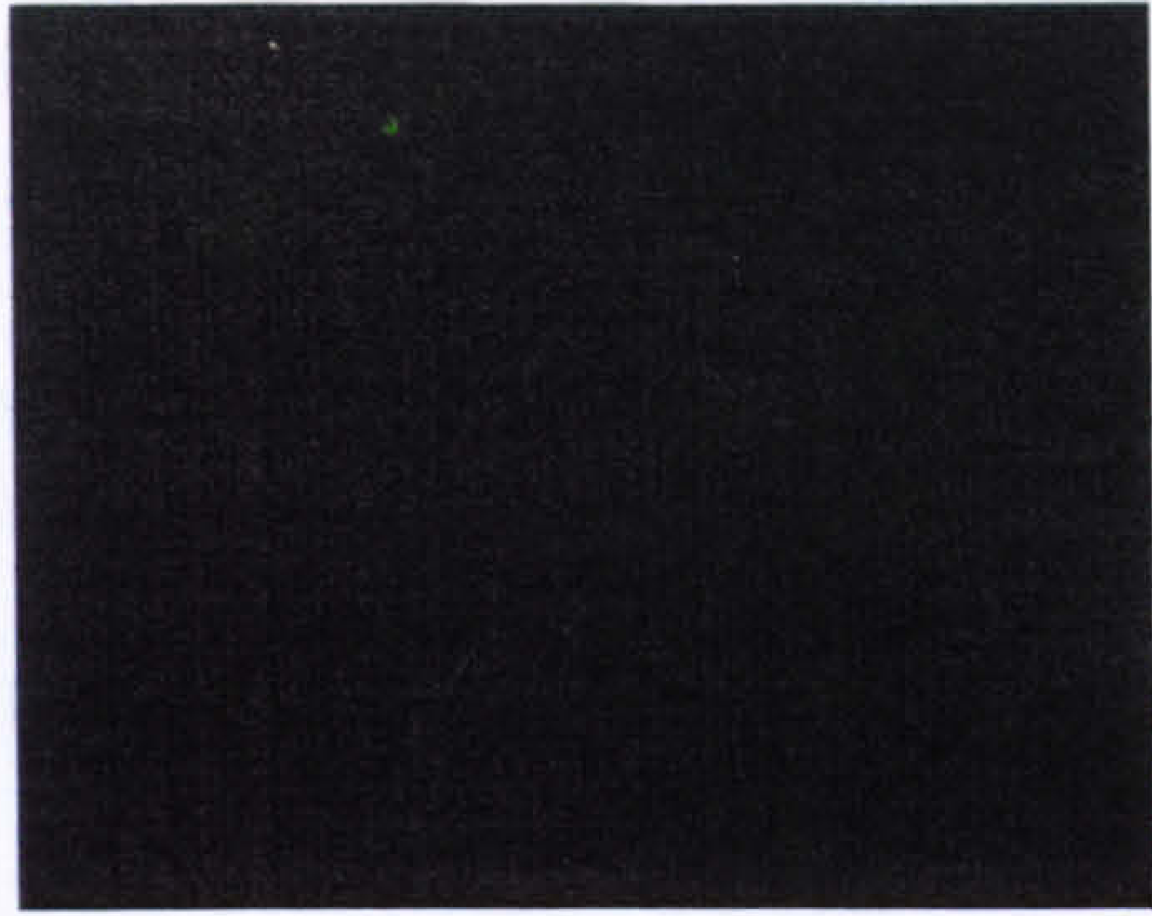
3.2.4 Treatment of A549 cells with IL6, IL8 and both cytokines simultaneously.

The addition of IL1β with IL6 had an increased effect on the expression of CRP, so simultaneous treatment with other cytokines and IL6 was investigated. This time the effect of IL8 was used to see if the effect was increased with addition of IL6.

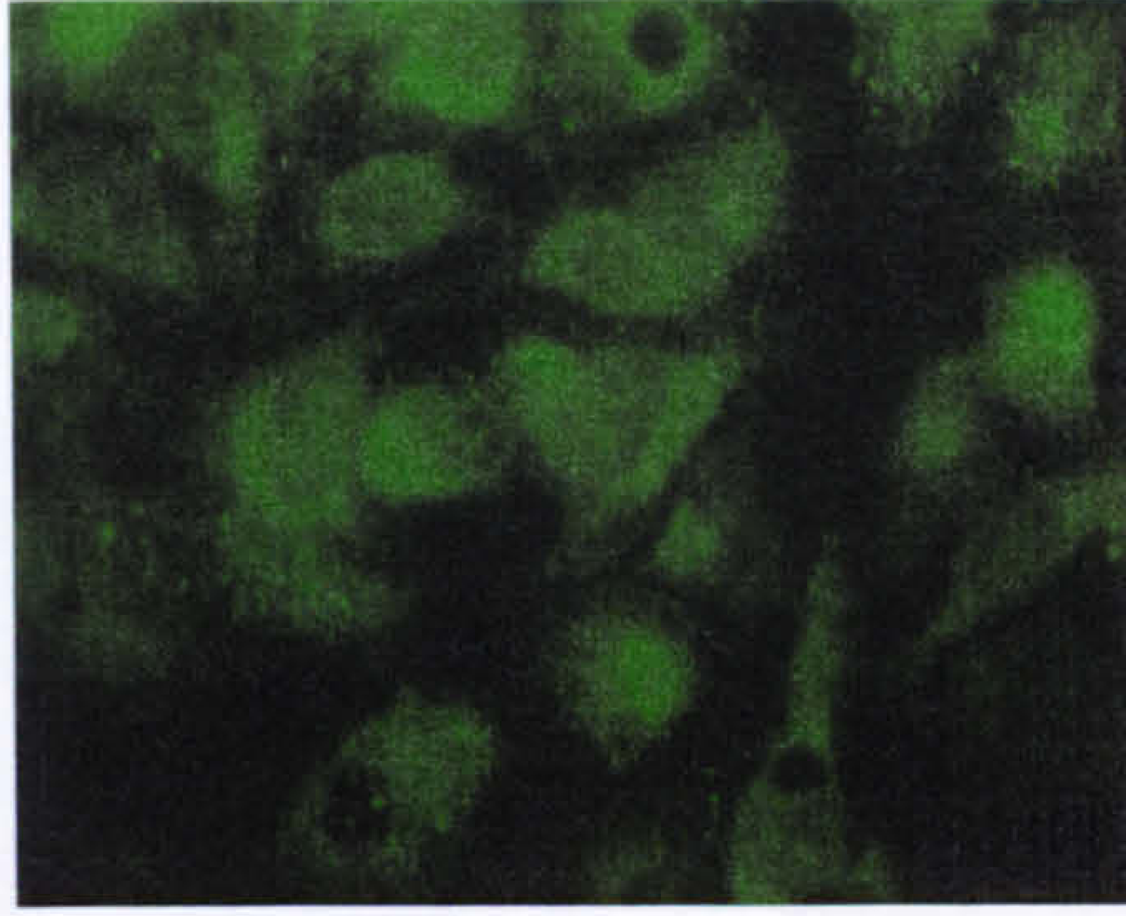
Cells were treated with 10ng/ml of either IL6 , IL8, or 10ng/ml IL6 plus10ng/ml IL8, with untreated cells for comparison. The result of immunofluoresence analysis revealed that IL8 up-regulated the expression of CRP in A549 cells (Figs. 22). The levels of IL6 induced CRP appear higher than that of the IL8 treated cells. The IL6 and IL8 treated cells have slightly higher levels than either of the cytokines on their own. The distribution of CRP within these cells appears mainly in the cytoplasm but also appeared to be expressed in the external cell membrane.

Intensity analysis ($n = 3$) shows that there is increased CRP in the order IL8 > IL6 > IL6 plus IL8 treated cells (Fig 23). Statistically this is significant ($p < 0.01$) in the IL6 plus IL8 treated cells over the control (untreated cells).

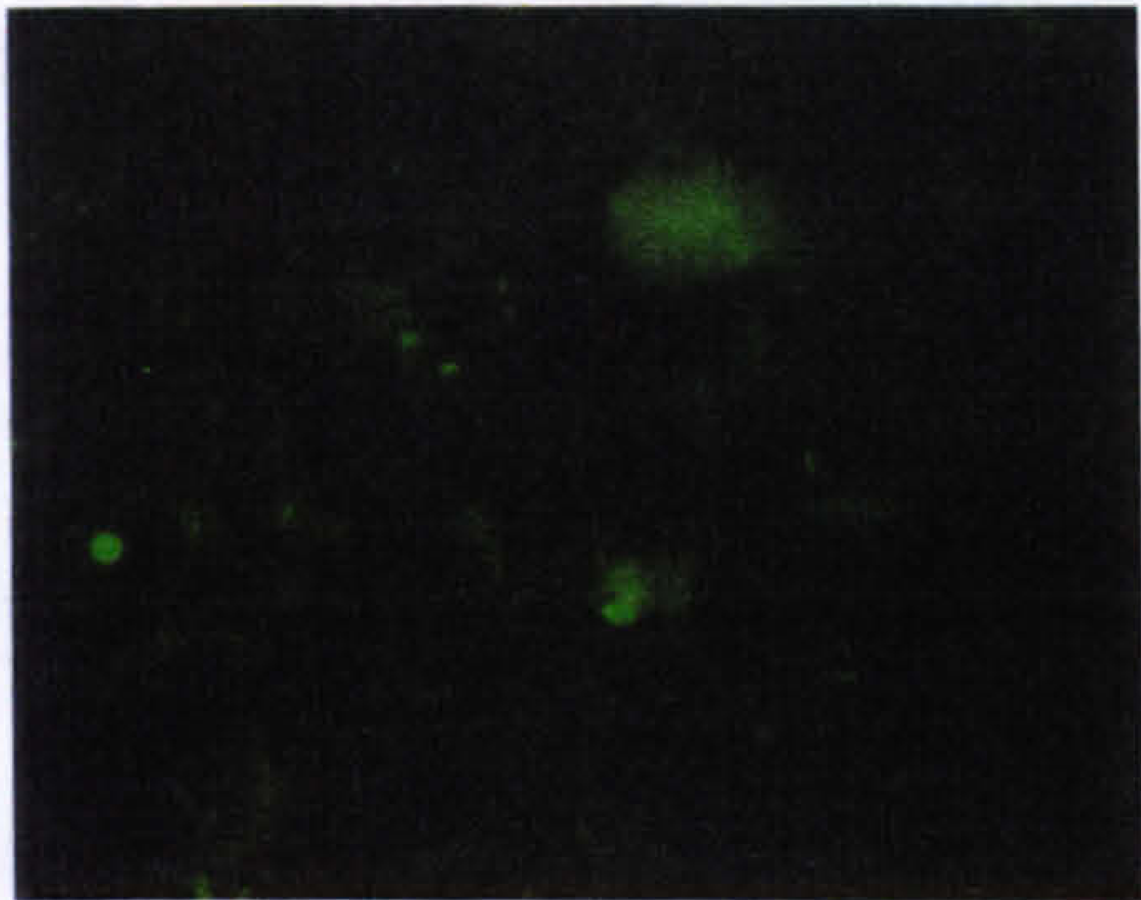
ELISAs were performed to indicate the presence of secreted CRP (Fig 24). Statistical analysis shows that the IL6 plus IL8 treatment results induces a significant increase in the amount of CRP ($p<0.05$).



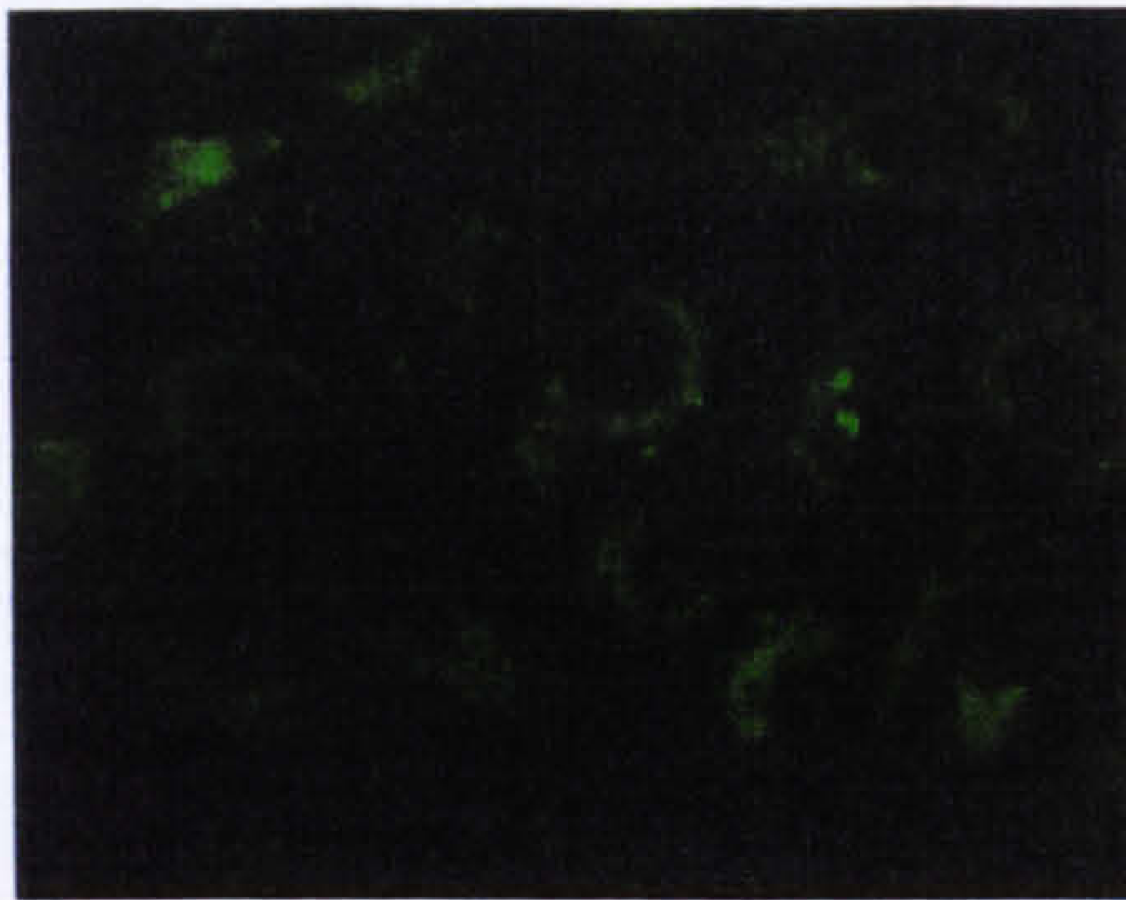
a) Untreated cells



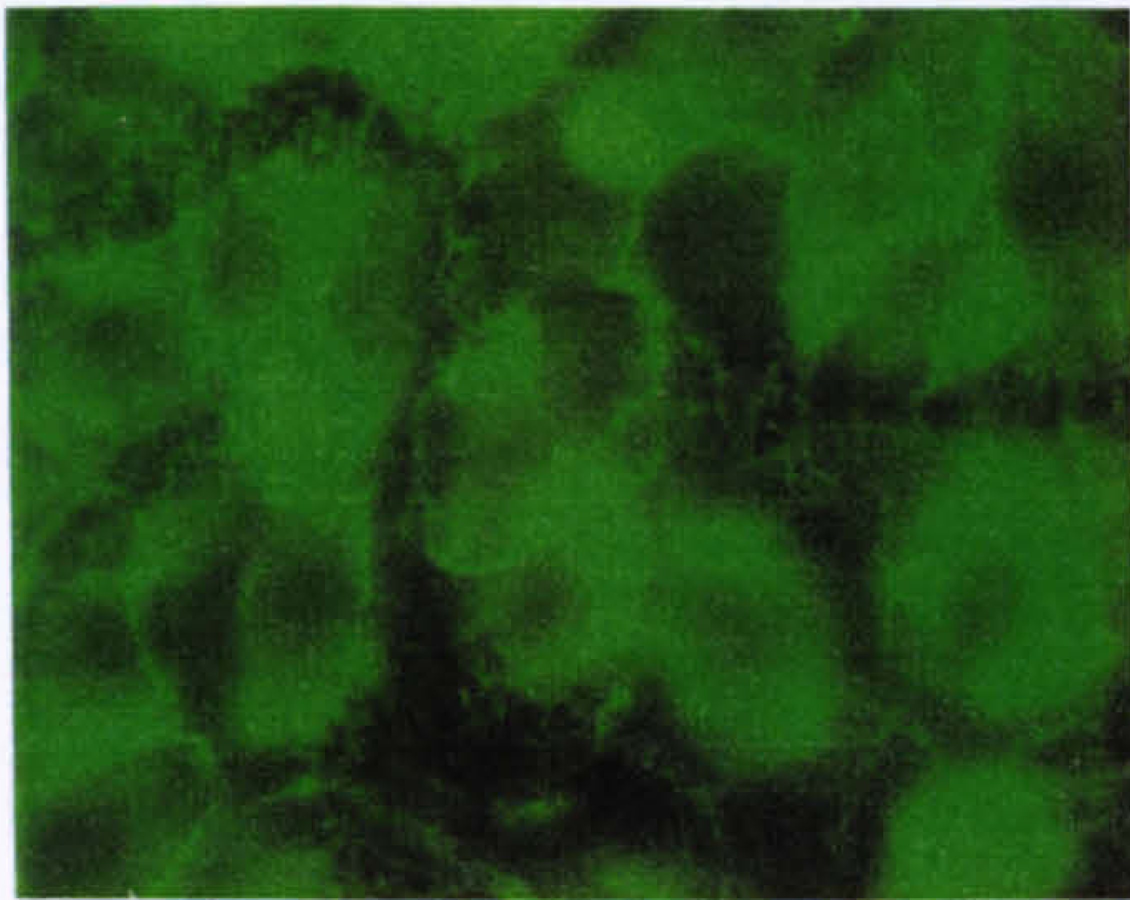
b) IL6 treated cells



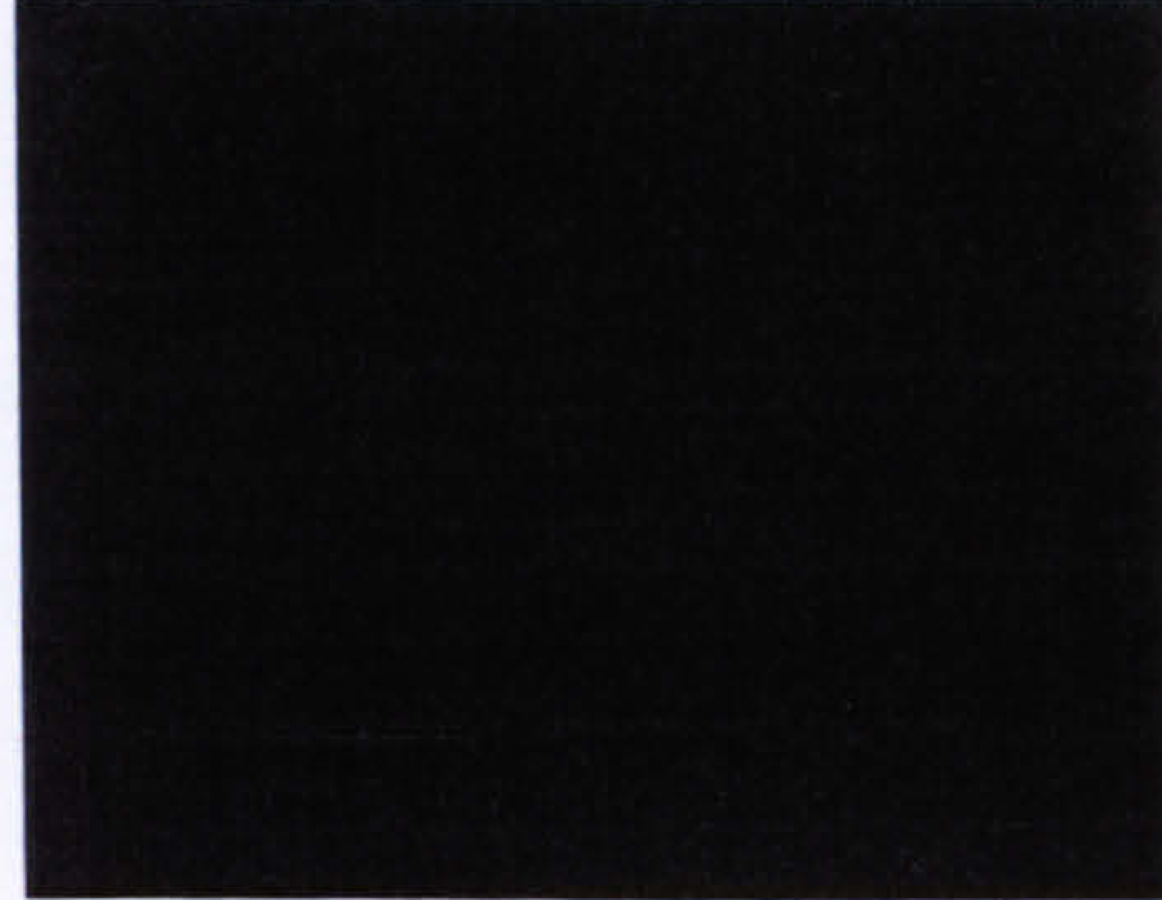
c) TNF α treated cells



d) IFN γ treated cells



e) positive control – CD44



f) negative control – 2a4

Figure 13: Expression of CRP in A549 cells treated with 10ng/ml of each cytokine for 18 hours as shown by indirect immunofluorescence

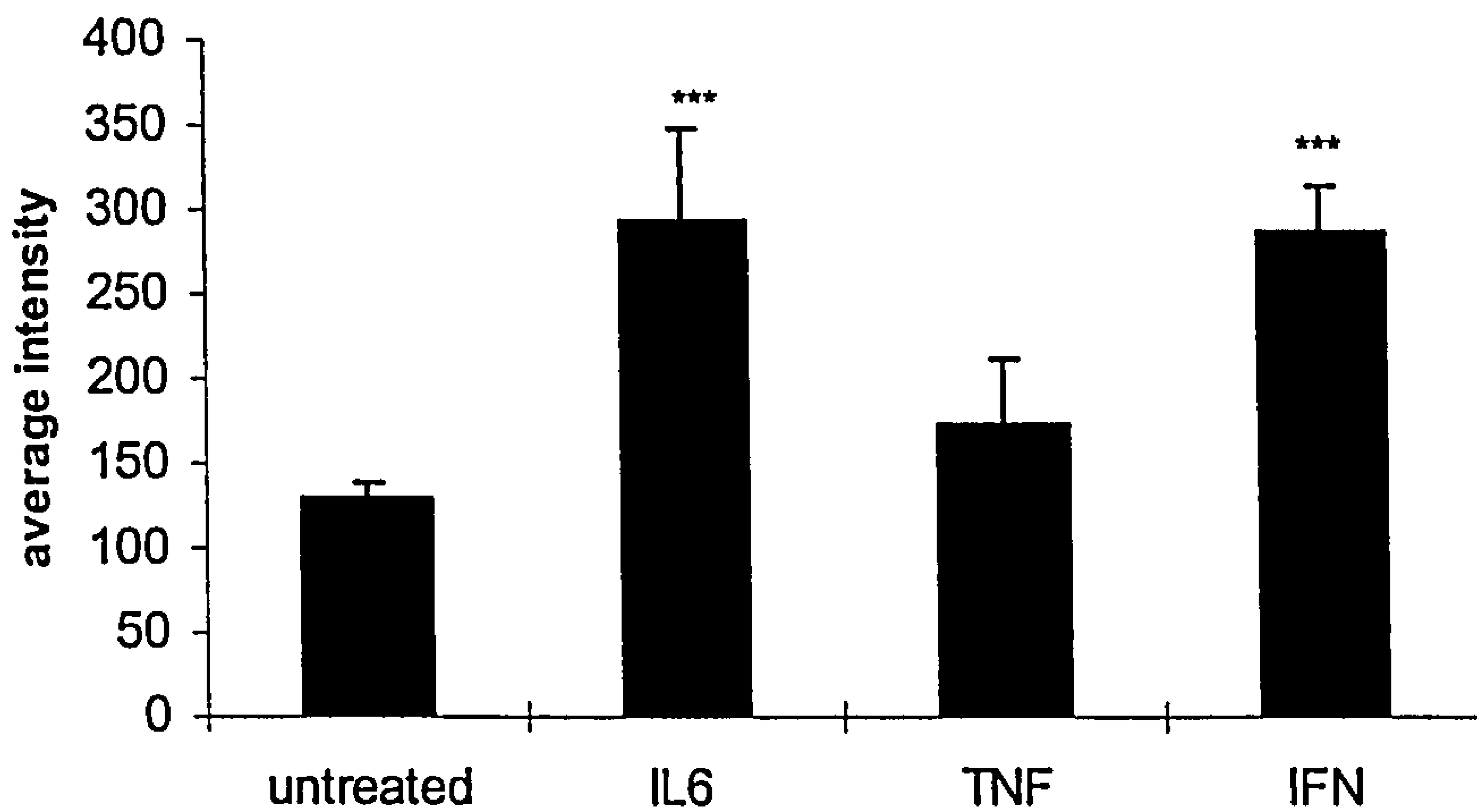


Figure 14: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of treatment with 10ng/ml of each cytokine. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denotes significant changes from untreated (control) cells, p< 0.001.

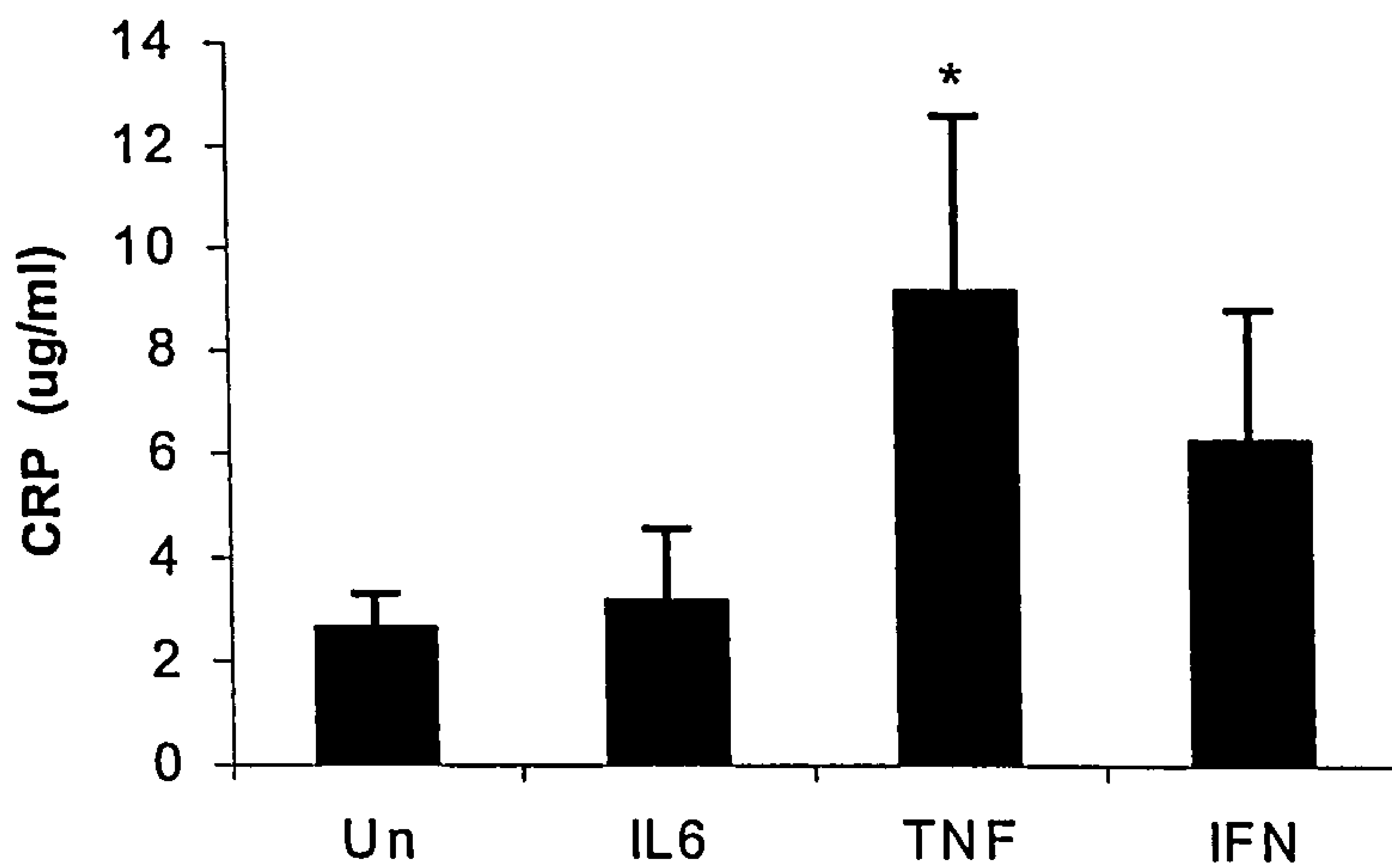
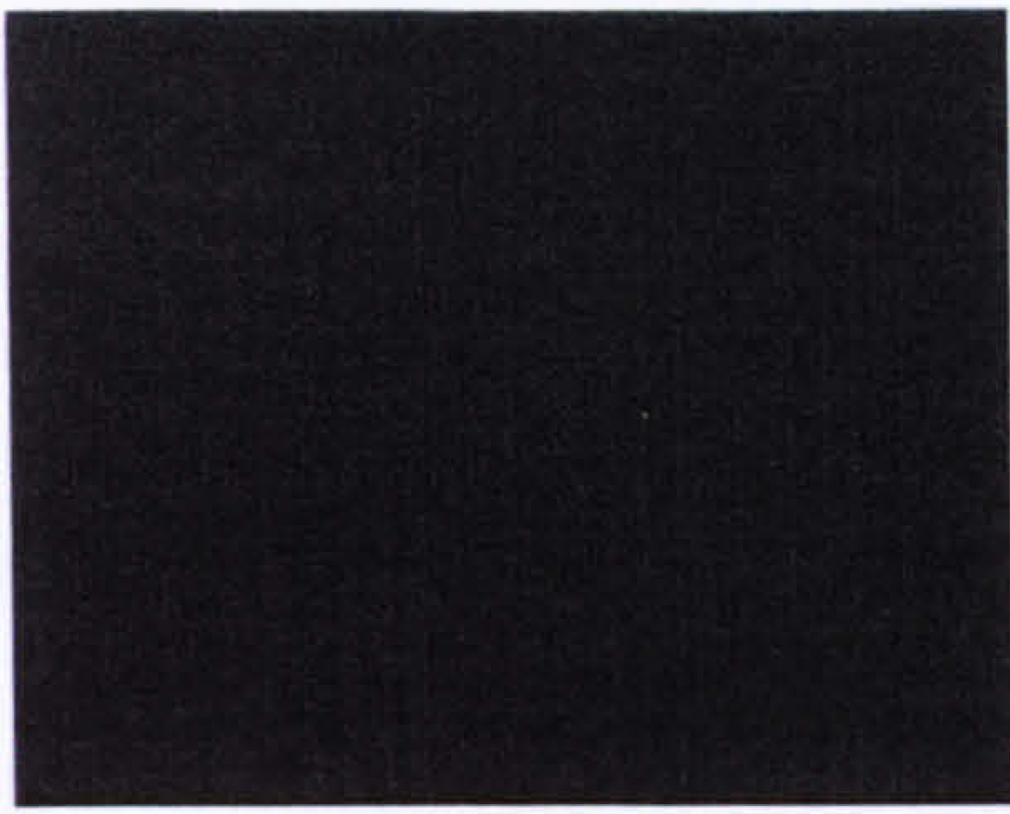
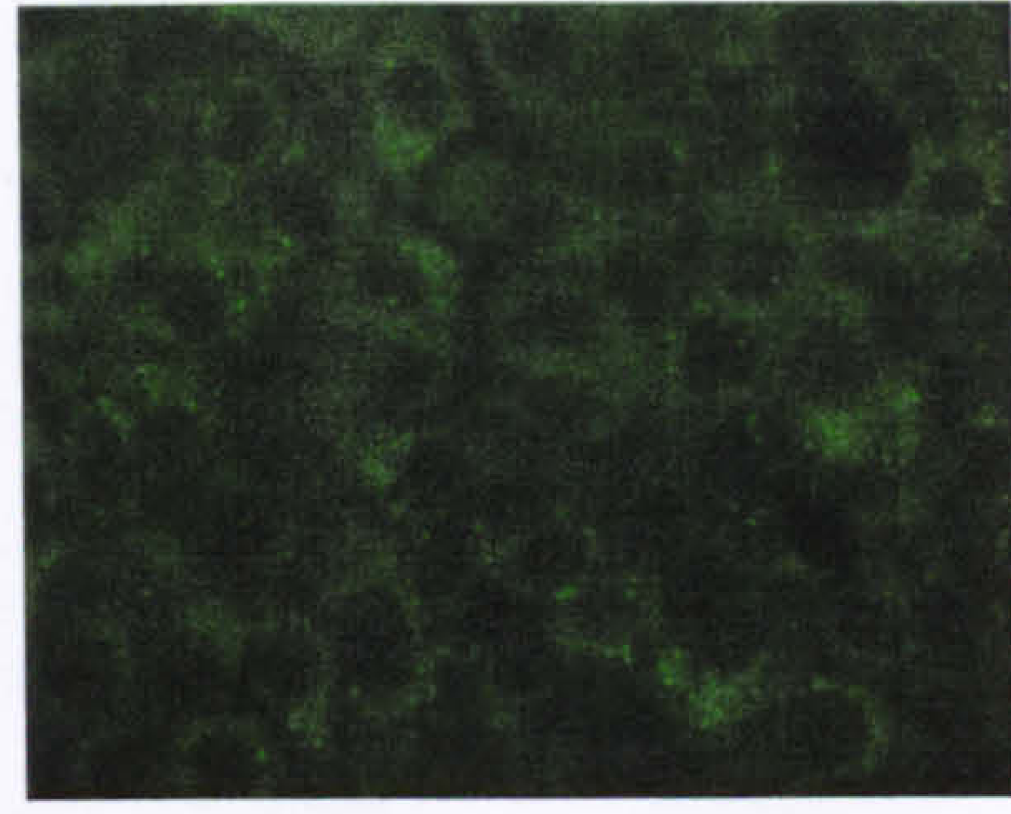


Figure 15: Concentration of CRP in cell supernatant measured by ELISA after 18 hours of treatment with 10ng/ml of each cytokine. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated (control) cells, p< 0.05.

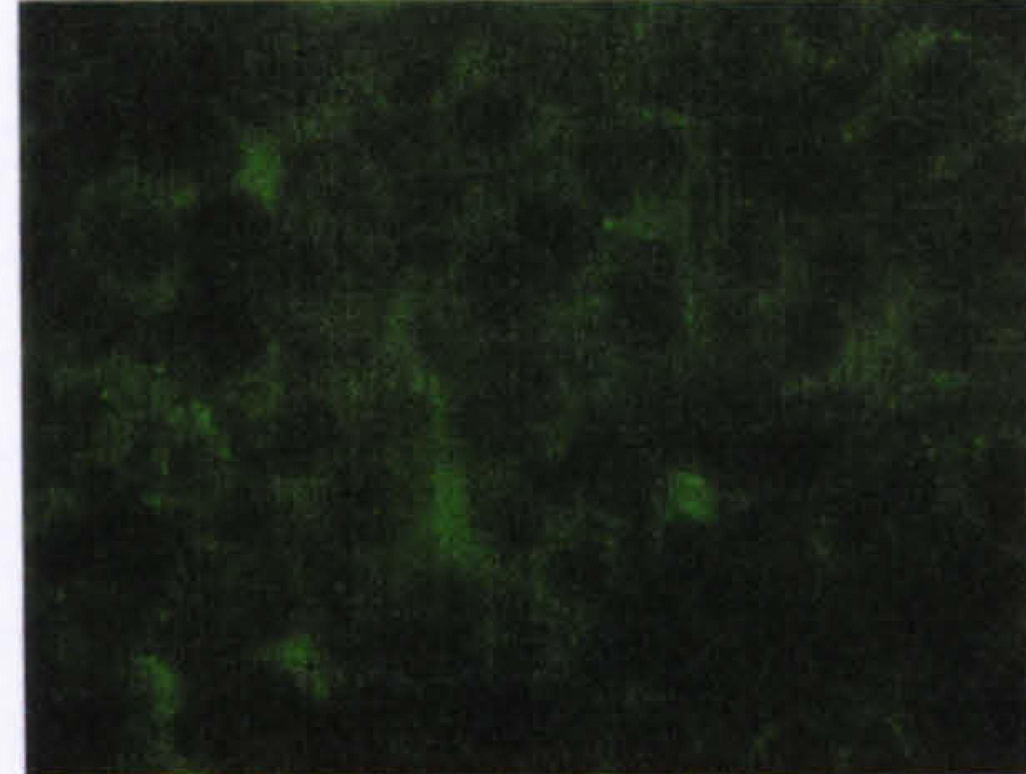


a) Untreated

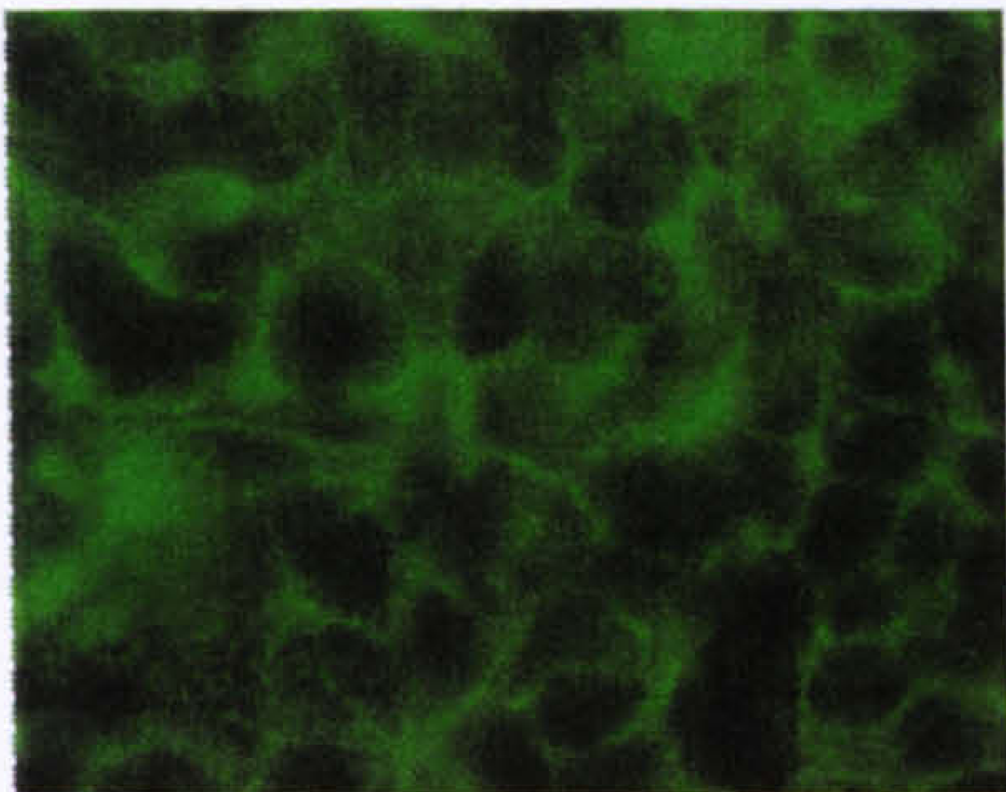


b) IL6 treated

c) TNFα treated



d) IL6 + TNFα treated



e) positive control – CD44

f) negative control – 2a4

Figure 16: Expression of CRP in A549 cells treated with 10ng/ml TNFα or IL6 for 18 hours as shown by indirect immunofluorescence.

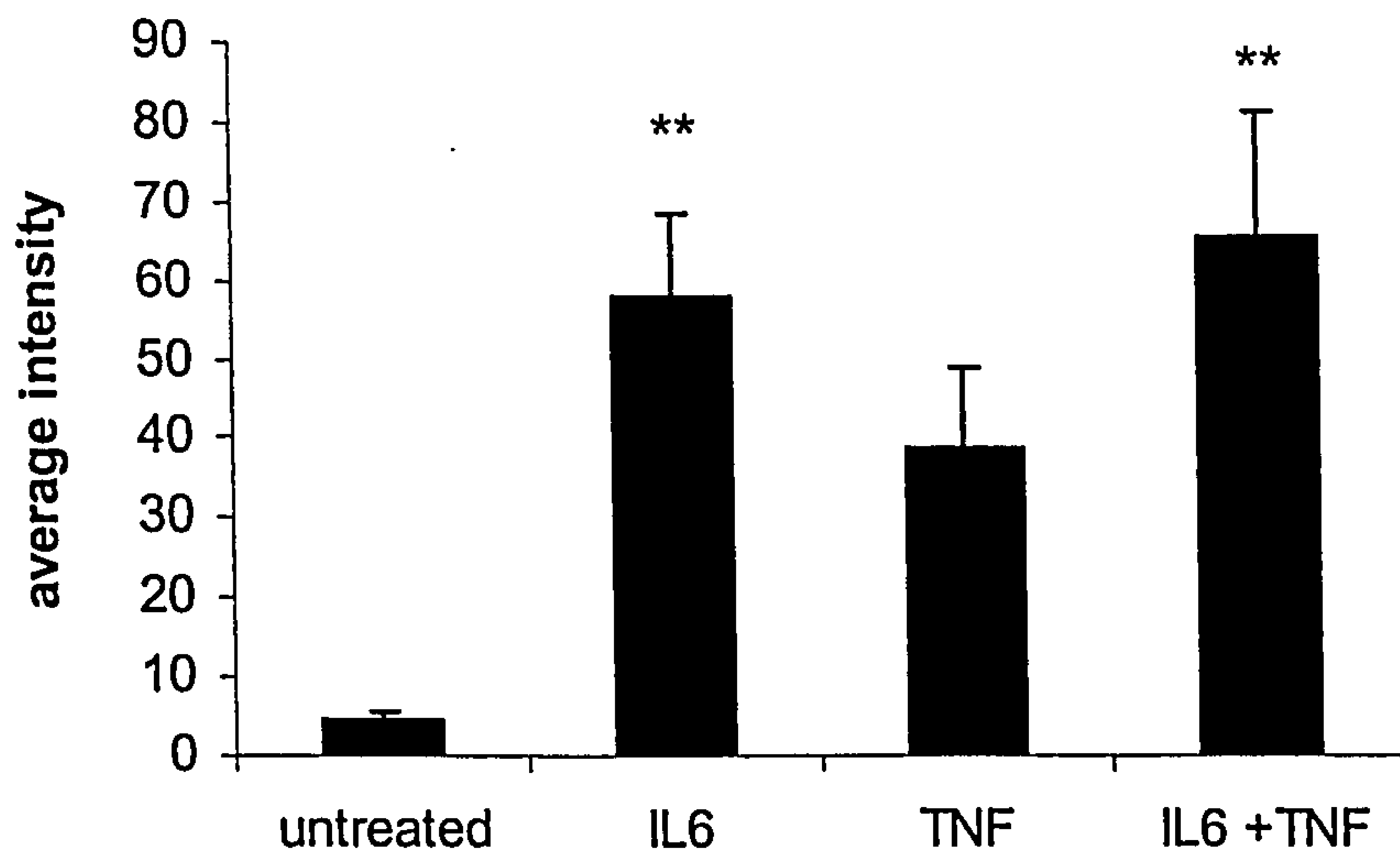


Figure 17: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of treatment with 10ng/ml of TNFα or IL6. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, p< 0.01.

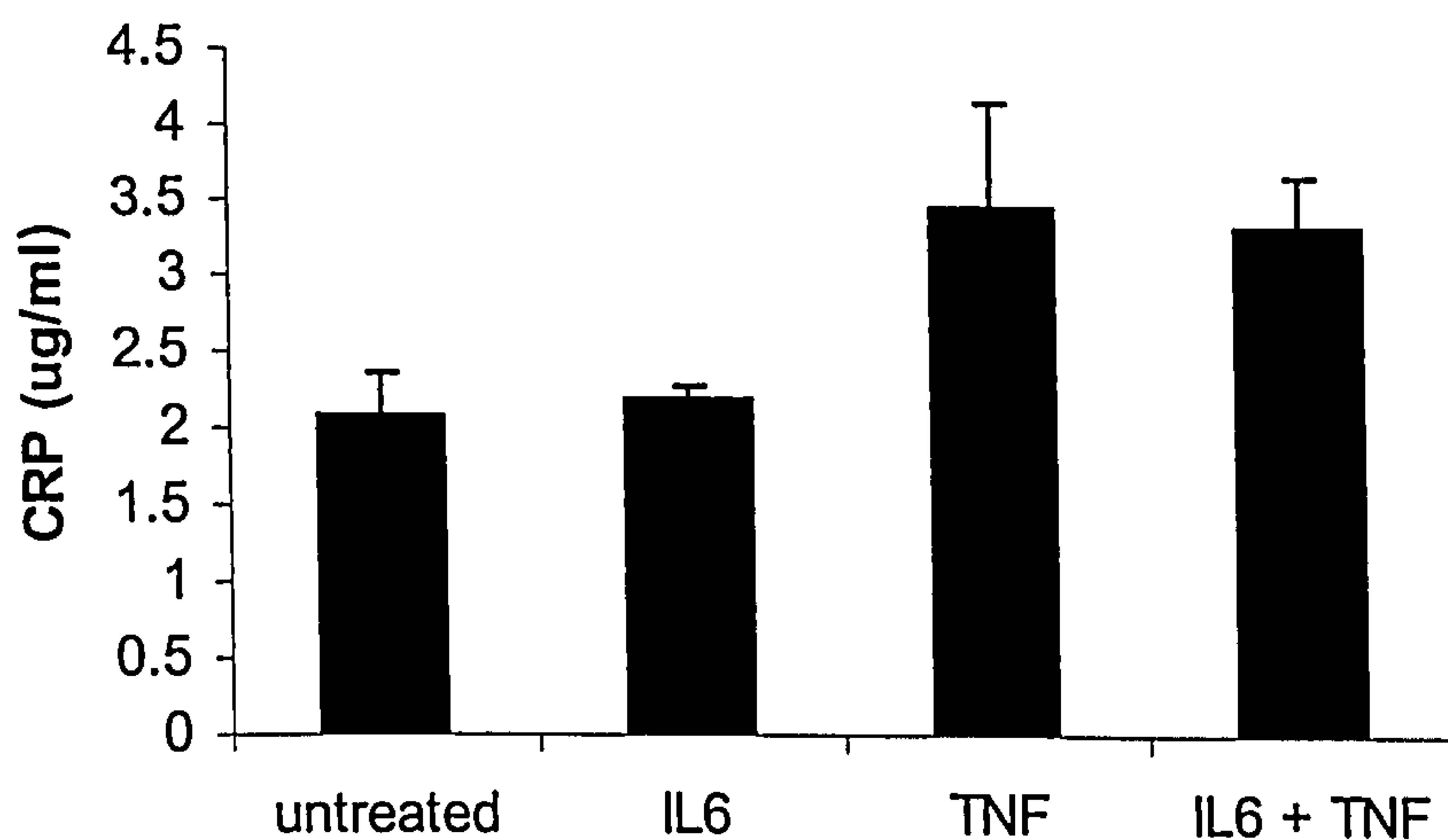


Figure 18: Concentration of CRP in cell supernatant measured by ELISA after 18 hours of treatment with 10ng/ml TNFα or IL6 treatment. The results are the mean of triplicate results from 3 experiments ± SE.

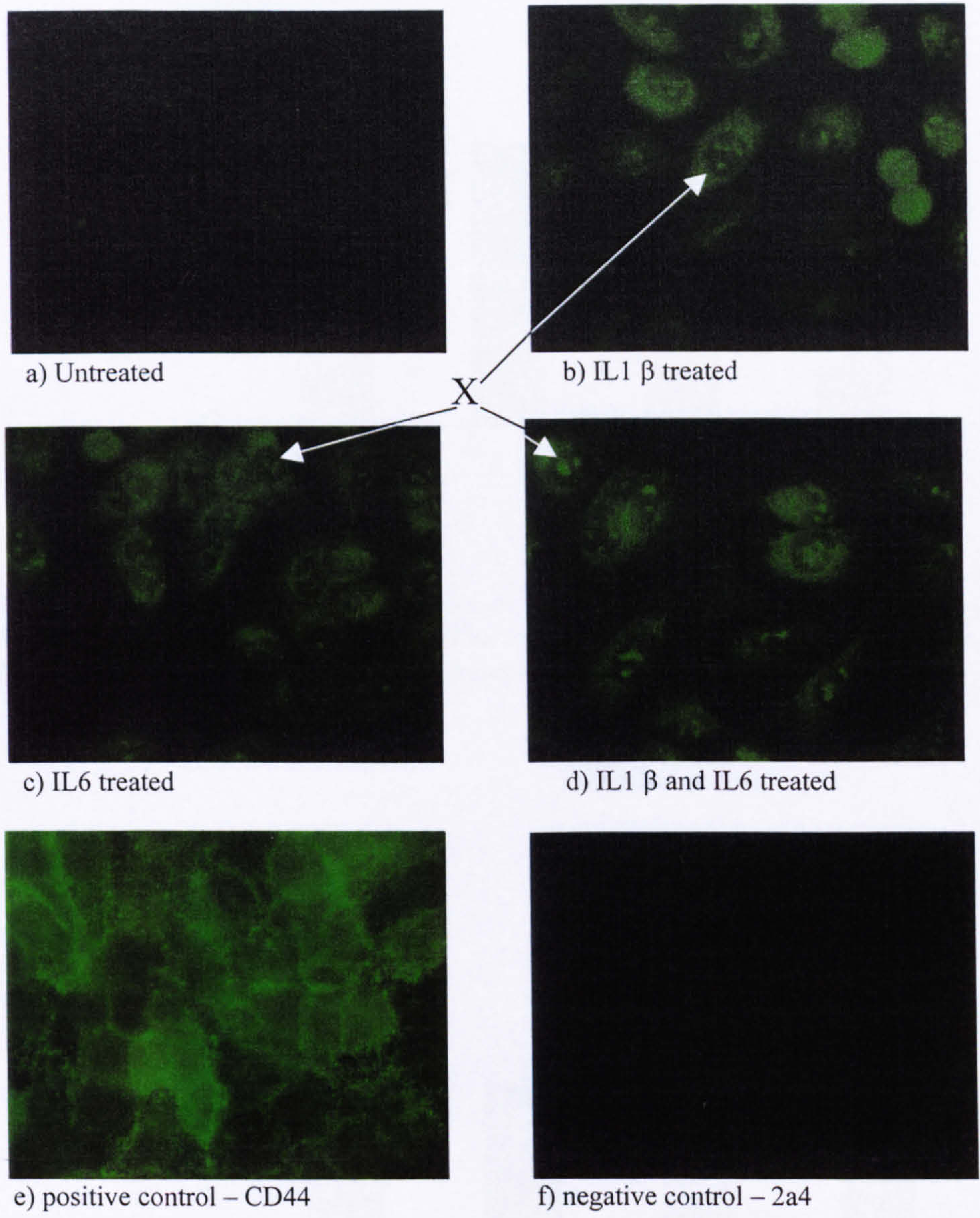


Figure 19: Expression of CRP in A549 cells treated with 10ng/ml IL6 or IL1β for 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation of CRP.

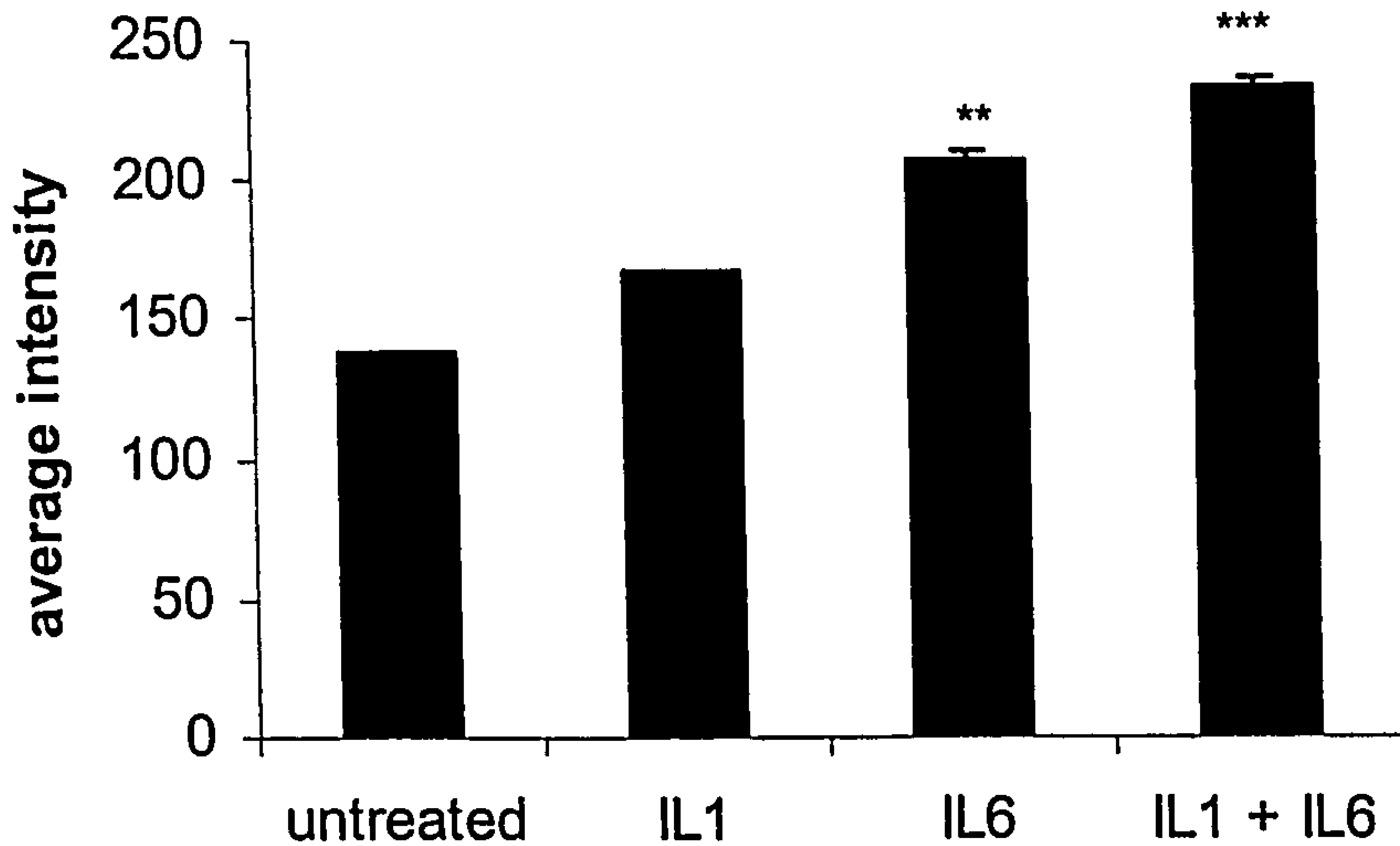


Figure 20: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of treatment with 10ng/ml IL6 or IL1β. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (, ***) denotes significant changes from untreated (control) cells, p< 0.01, p<0.001.**

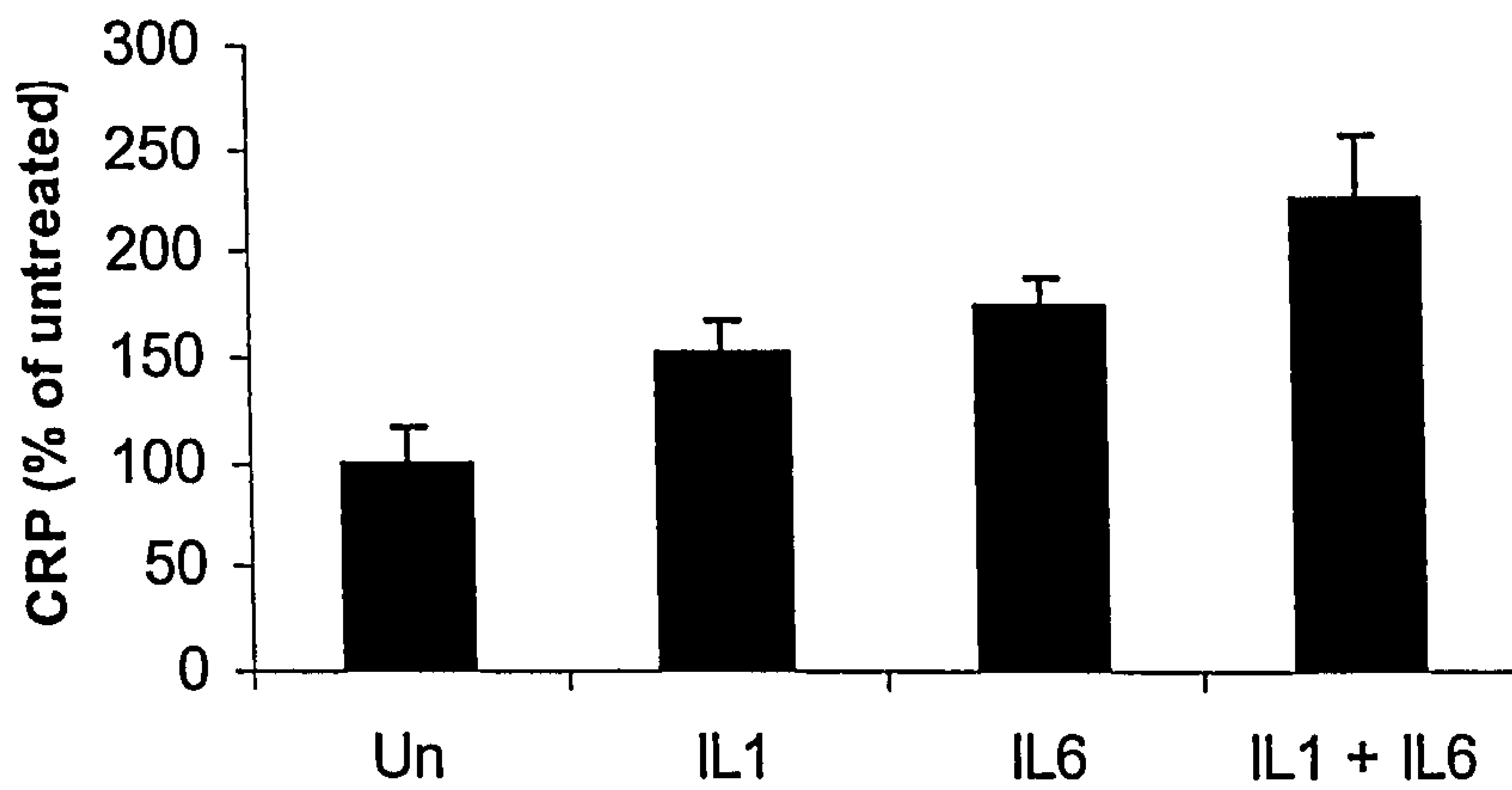
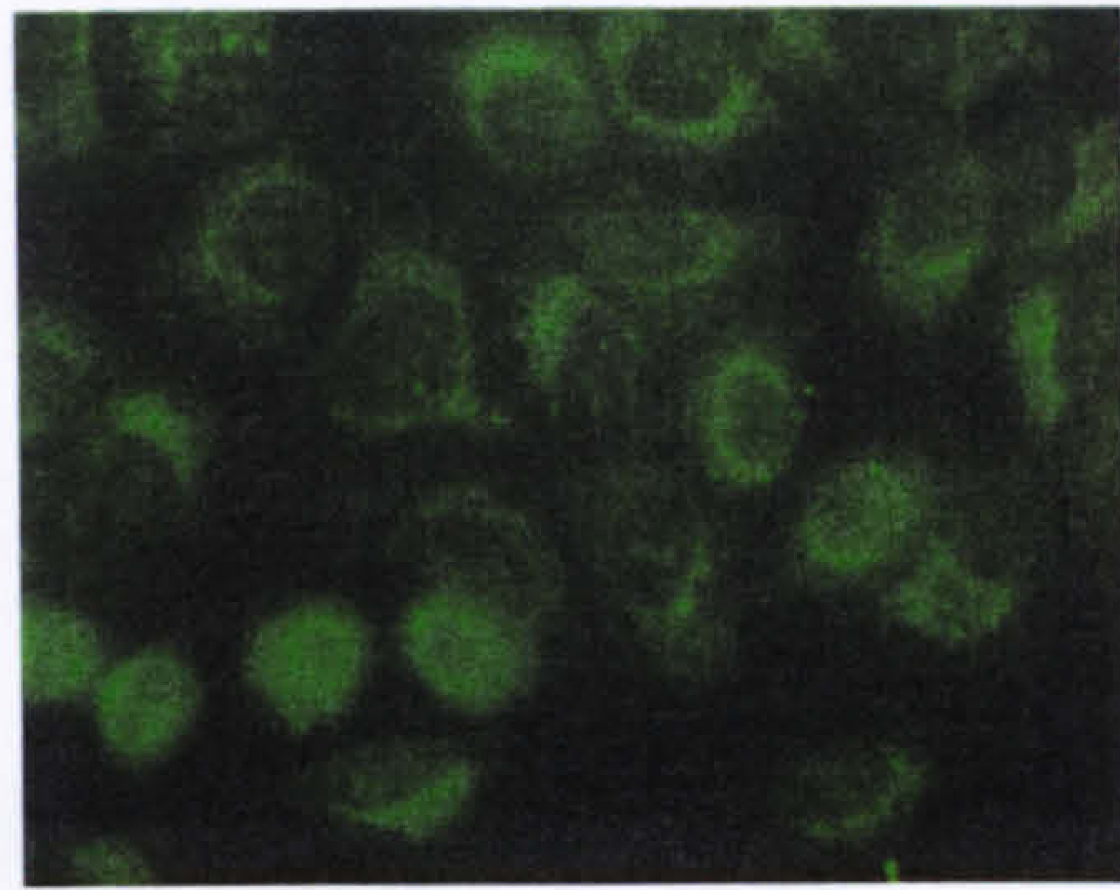


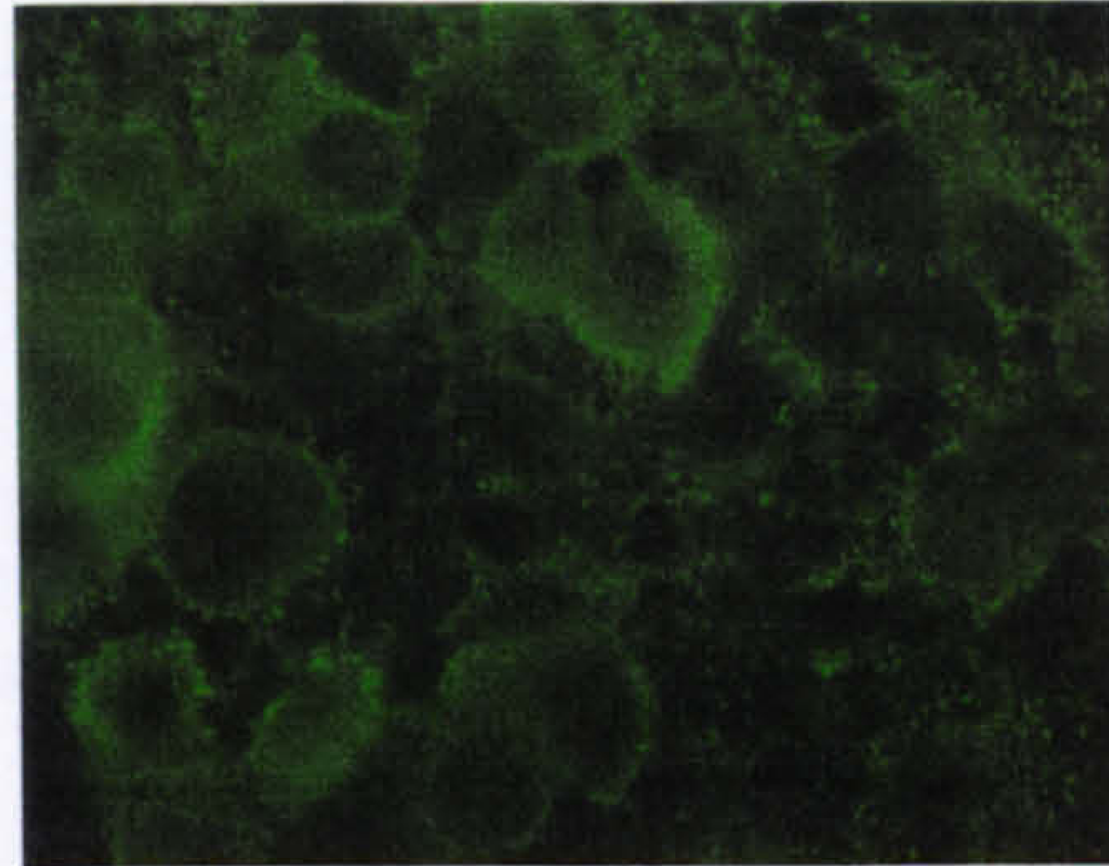
Figure 21: Concentration of CRP in cell supernatant measured by ELISA after 18 hours of treatment with 10ng/ml of IL6 or IL1β treatment. CRP is expressed as a percentage of the concentration present in the untreated cells. The results are the mean of triplicate results from 3 experiments ± SE.

a) Untreated

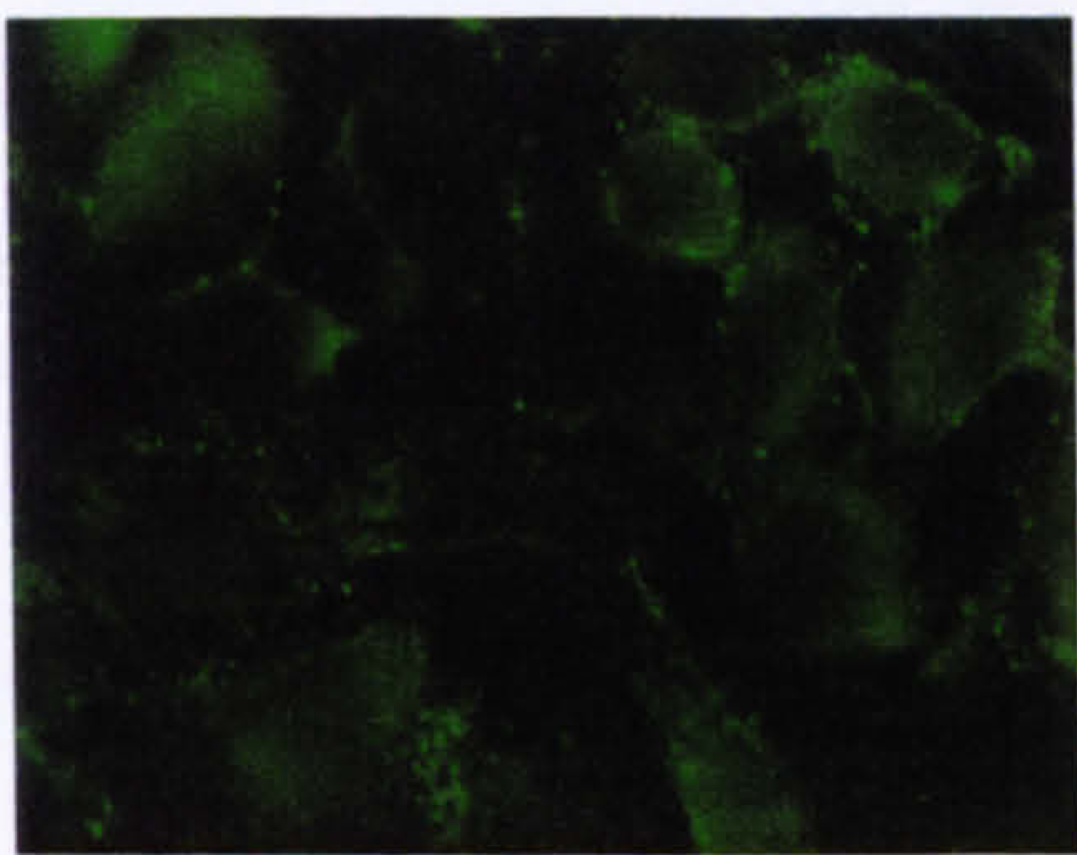


b) IL6 treated

c) IL8 treated



d) IL6 + IL8 treated



e) positive control – CD44

f) negative control – 2a4

Figure 22: Expression of CRP in A549 cells treated with 10ng/ml IL6 or IL8 for 18 hours as shown by indirect immunofluorescence.

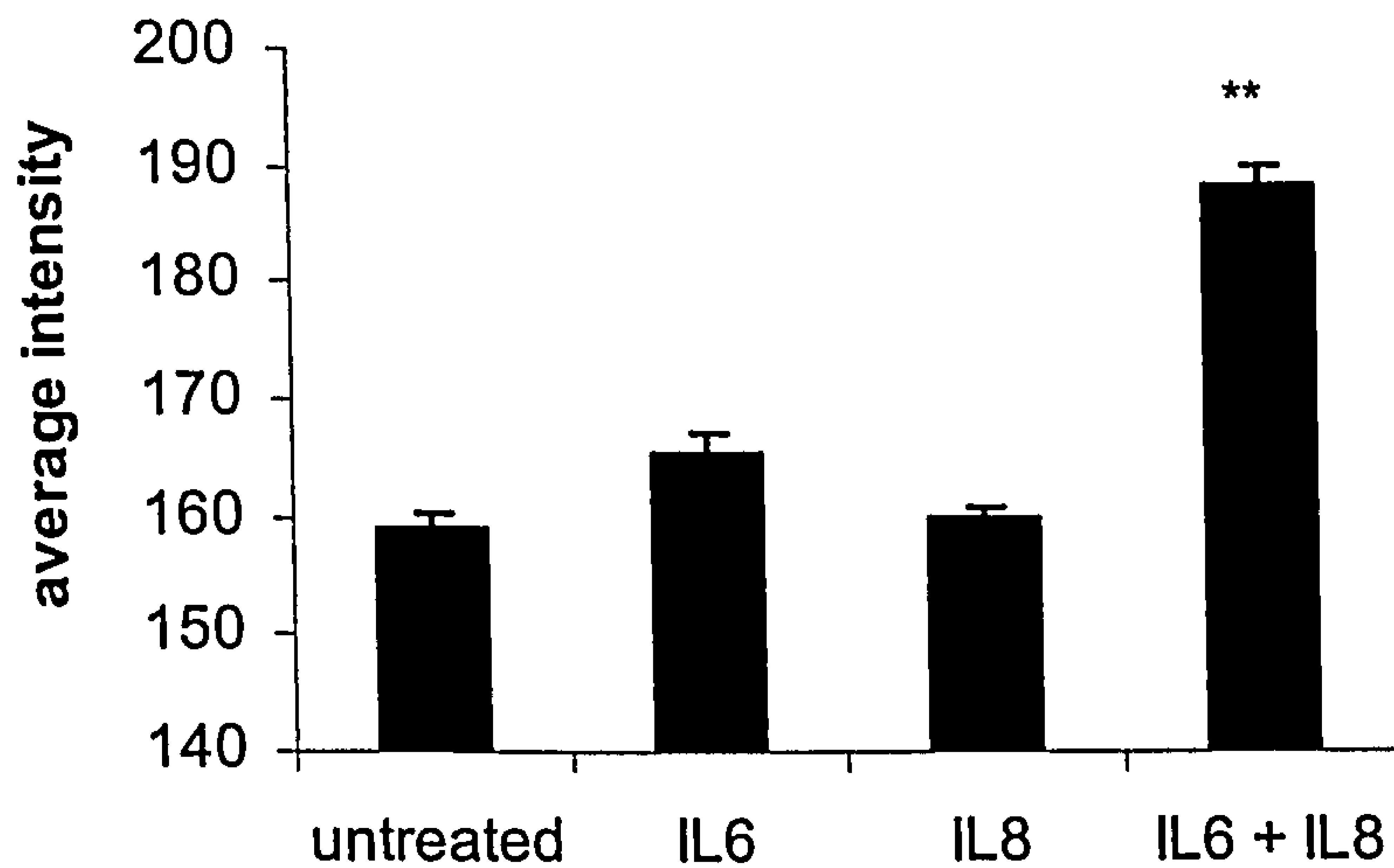


Figure 23: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of treatment with 10ng/ml of IL6 or IL8 treatments. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p < 0.01$.

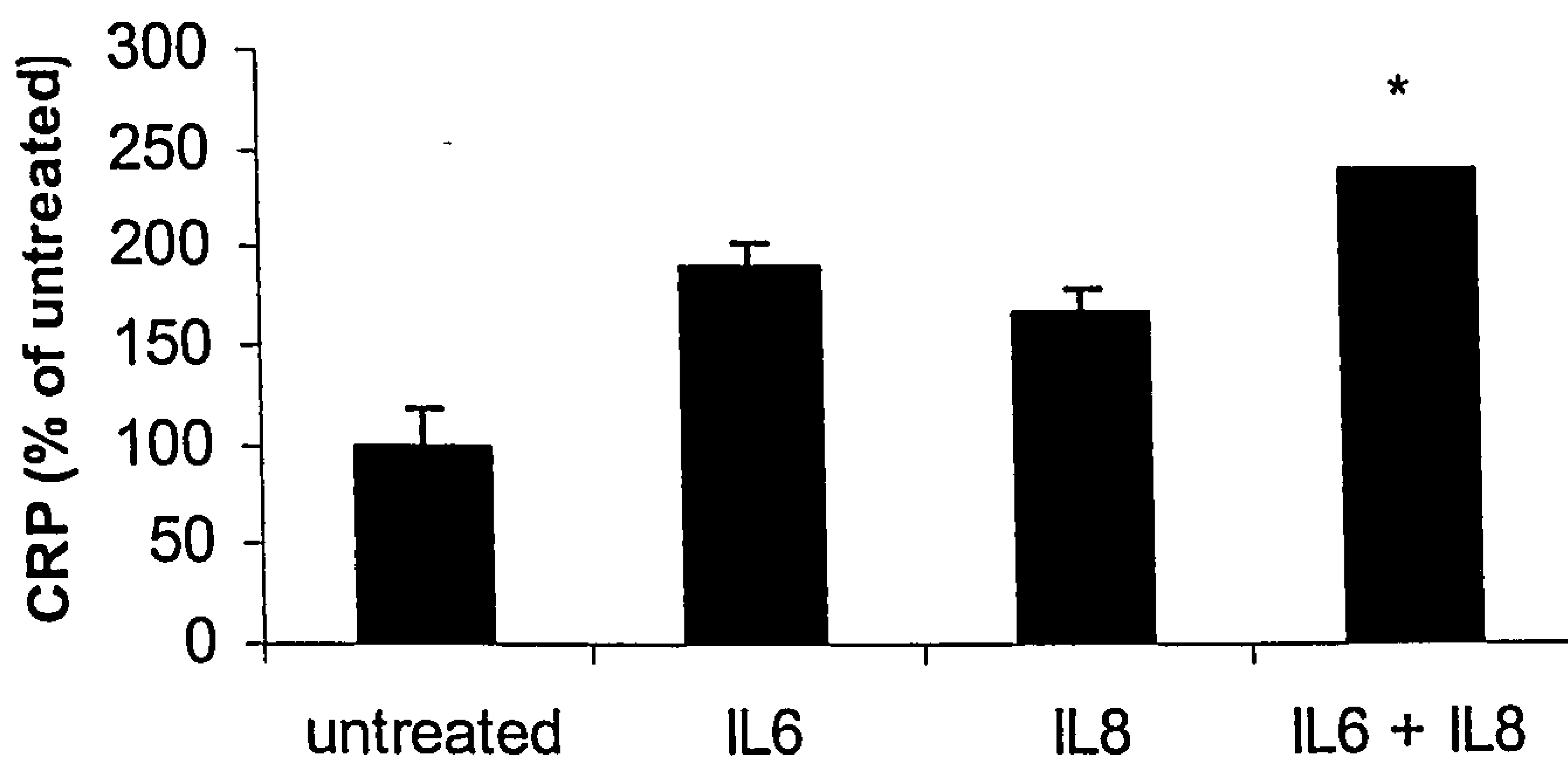


Figure 24: Concentration of CRP in cell supernatant measured by ELISA after 18 hours of treatment with 10ng/ml IL6 or IL1β treatment. CRP is expressed as a percentage of the concentration present in the untreated cells, The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (*) denotes significant changes from untreated (control) cells, $p < 0.05$.

Chapter 4

Effect of air pollution particles, carbon black (CB) and ultrafine carbon black (ufCB), PM10 and the transition metal salt, $FeCl_3$, treatment on inflammatory protein expression in lung epithelial cells.

4.1.0 Effects on CRP expression.

4.1.1 Treatment with CB over 18 hour period.

Cells were treated with 100μg/ml CB for various time points over 18 hour period (0, ½ , 3,6,18 hours). Immunofluorescent staining showed that CRP was expressed in the cells from 3 hours and increased in a time-dependent manner (Fig. 25), with some nuclear localisation at 18 hours. Average intensity of the fluorescent cells for each time point was then calculated using Metamorph software (Fig 26). This shows that there is a significant increase in CRP over the untreated cells with CB treatment at 6 hrs ($p<0.05$) and 18 hours ($p<0.01$). The levels of CRP in the untreated cells do not change greatly in culture over the 18 hour time period and in further experiments were assumed to be the same at the beginning as at the end of the 18 hour incubation.

ELISAs were also carried out on the whole cell lysate and the cell culture supernatant. The cell lysate data (Fig 27) show that CRP increases time-dependently with a three fold increase over the untreated cells after 18 hours. This increase is statistically significant ($p< 0.05$) from 6 hours. The cell supernatant data (Fig 27) show a similar trend to that of the cell lysate with a three fold increase after 18 hours of treatment, however high variability between replicates gives high standard deviations resulting in no statistical significance from untreated cells.

4.1.2. Treatment with ufCB over 18 hour time period.

Cells were treated with 100μg/ml ufCB for the same time points as the CB treatments. The effect of CB particle size (fine or ultrafine) was investigated to find if the smaller particles had an increased or decreased effect of CRP expression. Immunofluorescent staining was carried out to see the localisation of CRP within the cells (Fig 28). Like CB, ufCB induced a time-dependent increase in CRP. However, the localisation of CRP within the cell is different between the two treatments. The ufCB treatment produced a pattern which may indicate polarisation, since the CRP is located at either end of the cell, particularly after 18 hours of treatment. Average intensities of the cells were then calculated (Fig 29). Once again there is a statistical increase in CRP at 6 and 18 hours (both $p<0.01$) of treatment. This shows a greater statistical significance

at the 6 hour time point with ufCB than the CB treatment (graphs of comparison between CB and ufCB in appendix I).

ELISA data indicated that there was an increase of CRP in both the whole cell lysate and the cell culture medium. The cell lysate (Fig 30) showed a statistically significant increase from 6 hours ($p<0.05$) which rose to 18 hours ($p<0.001$) with a 5- fold increase over the untreated cells. The cell supernatant (Fig 30) shows an increase from 3 hours of treatment but once again this is not statistically significant compared to untreated cells (graphs of comparison between CB and ufCB in appendix I).

4.1.3. Treatment with PM10 over 18 hour time period.

Cells were treated for up to 18 hours with 80µg/ml PM10 (collected on TEOM filters from a roadside sampling site in Wolverhampton, UK). Immunofluorescent staining for CRP was carried out and showed that, like the surrogate air pollution particles, CRP could be produced in a time dependent manner (Fig 31). Between 6 and 18 hours there appears to a slight reduction in the levels of CRP when looking at the pictures although this could be the result of differing staining patterns within the cells. At the 18 hour time point there appears to be some aggregation of CRP in the cell cytoplasm whereas at the 6 hour time point there appears to be a smoother appearance with some localisation around the nuclear membrane. Average intensities of the cells reveal that there is an increase in the levels of CRP during the first 3 hours of treatment and there after the levels remains constant until 18 hours (Fig 32). This is likely to be the result of differing staining patterns and the relative insensitivity of the software to differentiate between the different patterns. The levels of CRP in the cells was statistically significant at the 3, 6 and 18 hour time points ($p<0.01$).

ELISAs were carried out on the cell lysates and supernatants. The cell lysate showed that there was an increase in the levels of CRP in a time dependent manner (Fig 33). The levels of CRP are in agreement with the immunofluorescence results and the increase is statistically significant at the 18 hour time point ($p<0.01$). The cell supernatant was also analysed to look for secreted CRP (Fig 33). The appeared to be secretion of CRP from the cells; however, there were high variations between the different days in which the ELISA was carried out but it does appear that there is

indeed secretion at 18 hours although the results from the other time points are a little inconclusive.

4.1.4. Treatment with $FeCl_3$ over 18 hour time period.

Cells were treated with the transition metal salt, iron (III) chloride ($FeCl_3$), for up to 18 hours at a concentration of 100μM. Immunofluorescence staining was carried out and this showed that there was no effect on CRP expression in A549 cells. Intensity analysis was carried out and there was no change with treatment also there was no detectable CRP in either the cell lysate or cell supernatant, therefore data is not shown.

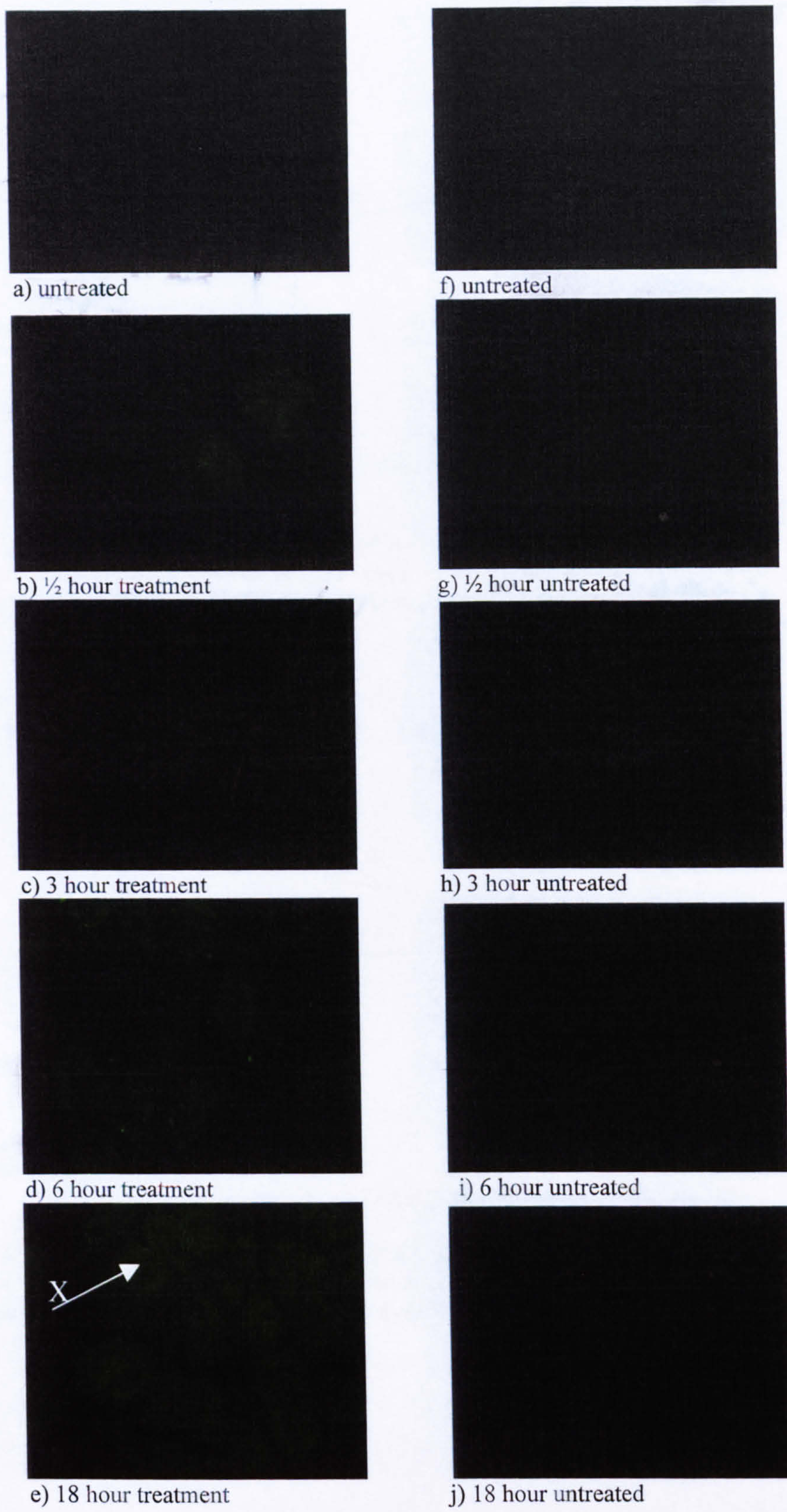


Figure 25: Expression of C-reactive protein (CRP) in A549 cells treated with 100μg/ml CB, for various time points up to 18 hours shown by indirect immunofluorescence. X indicates nuclear localisation. For controls see Appendix I.

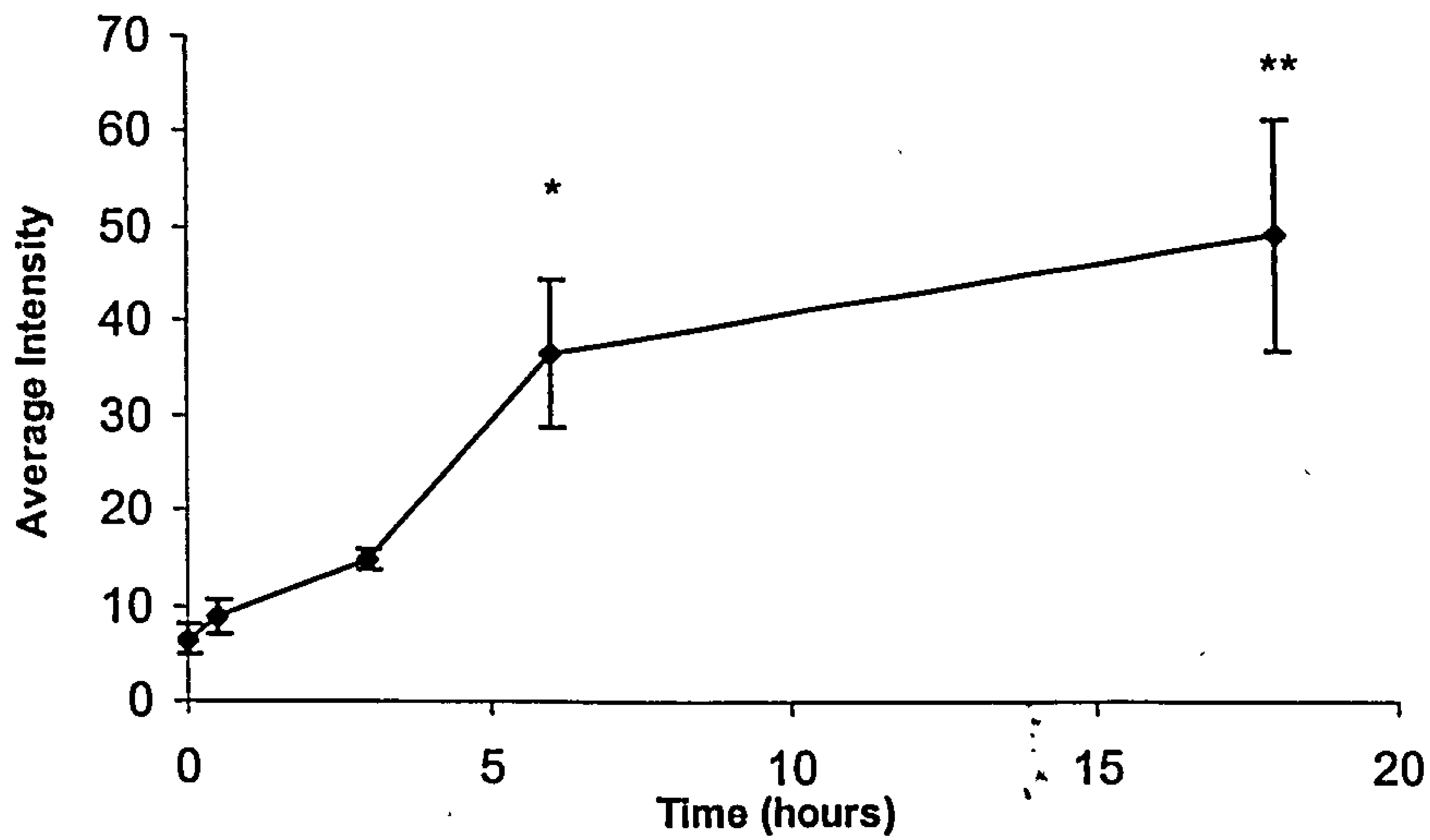


Figure 26: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of 100 µg/ml CB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (*, **) denotes significant changes from untreated (control) cells, p<0.05, p< 0.01.

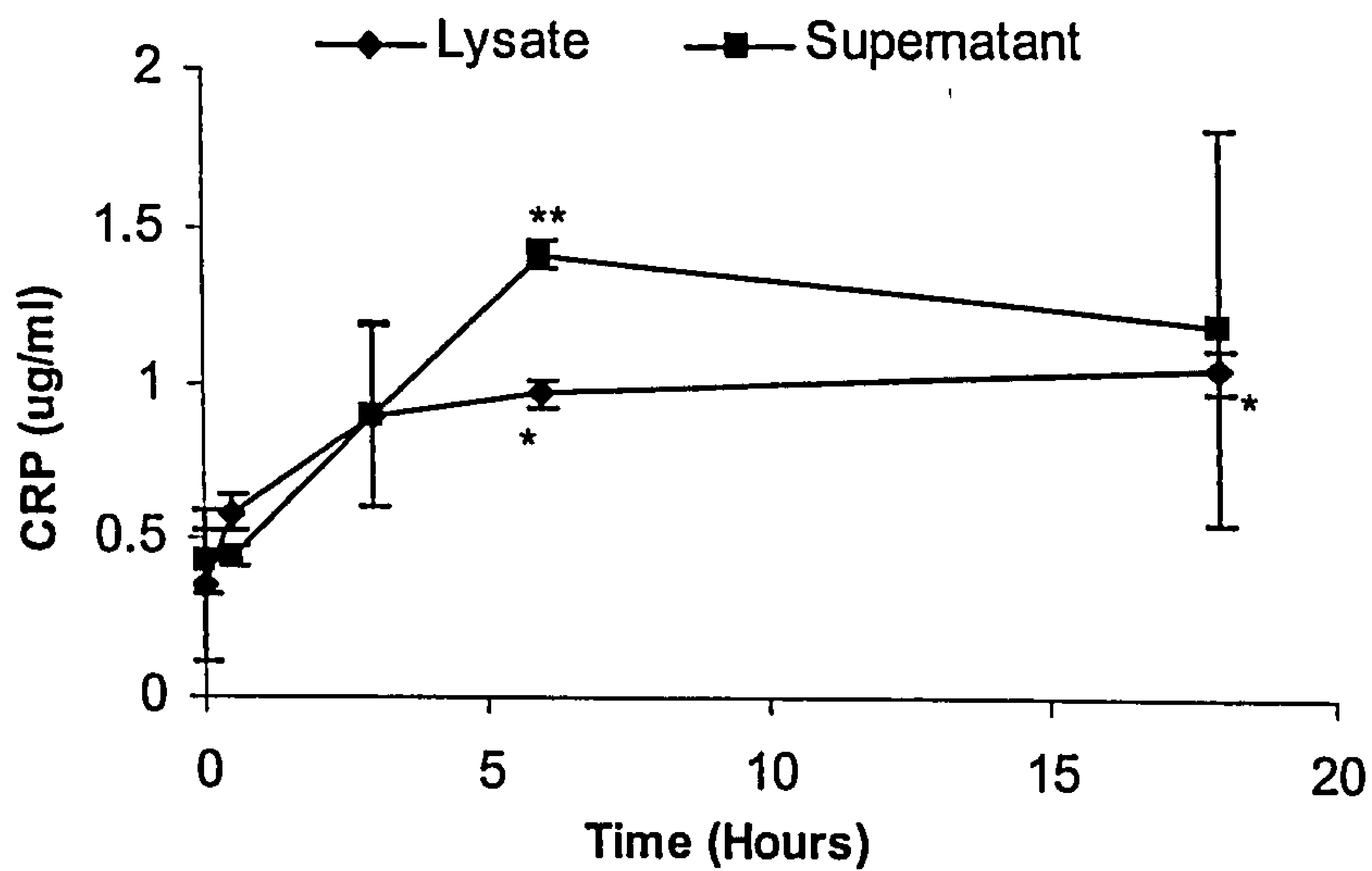


Figure 27: Concentration of CRP in cell lysate and supernatant measured by ELISA after 18 hours of treatment with 100µg/ml CB. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*, **) denotes significant changes from untreated (control) cells, p< 0.05, p< 0.01.

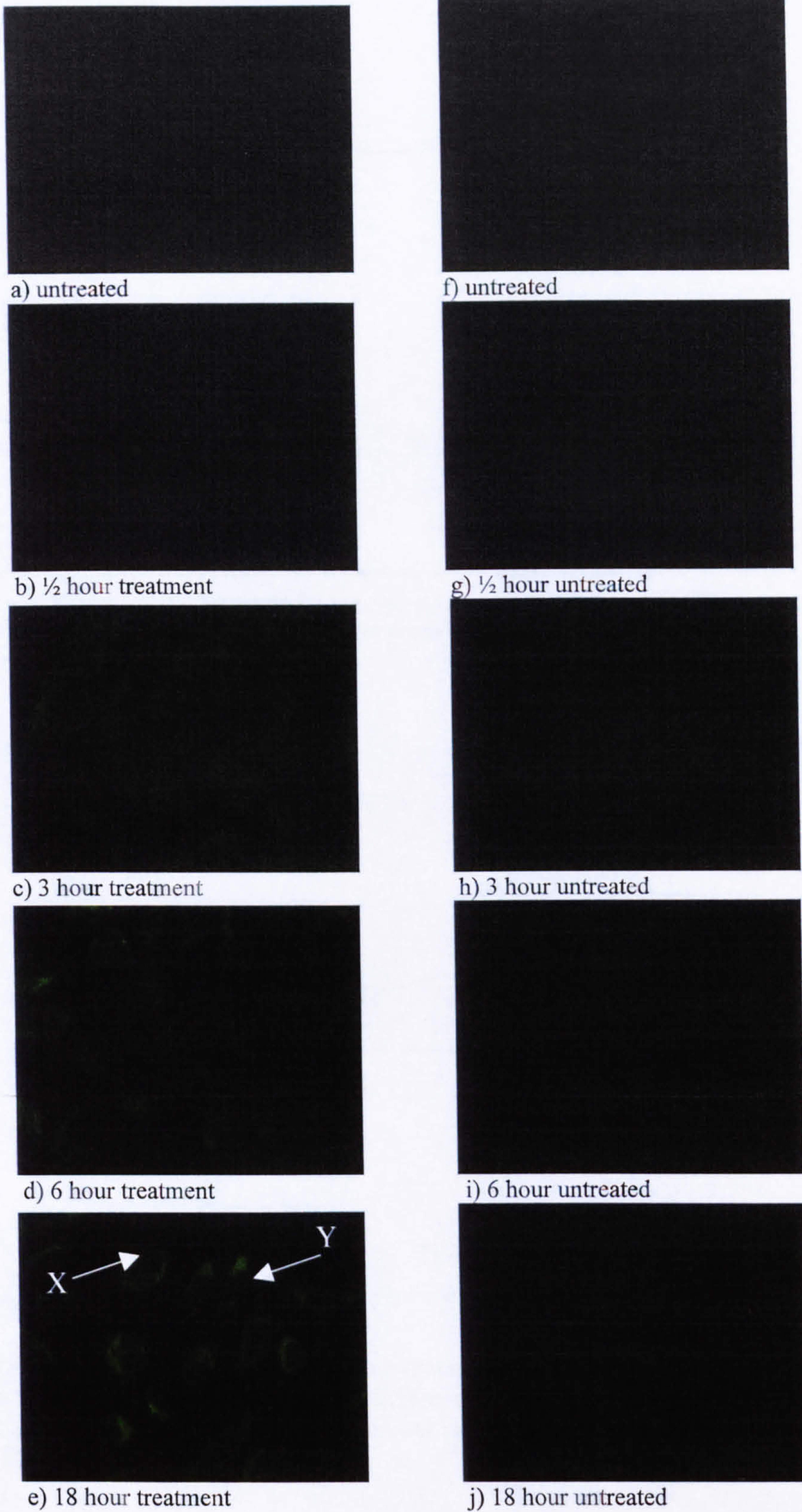


Figure 28: Expression of C-reactive protein (CRP) in A549 cells treated with 100 µg/ml ufCB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation and Y indicates polarisation of staining. For controls see Appendix I.

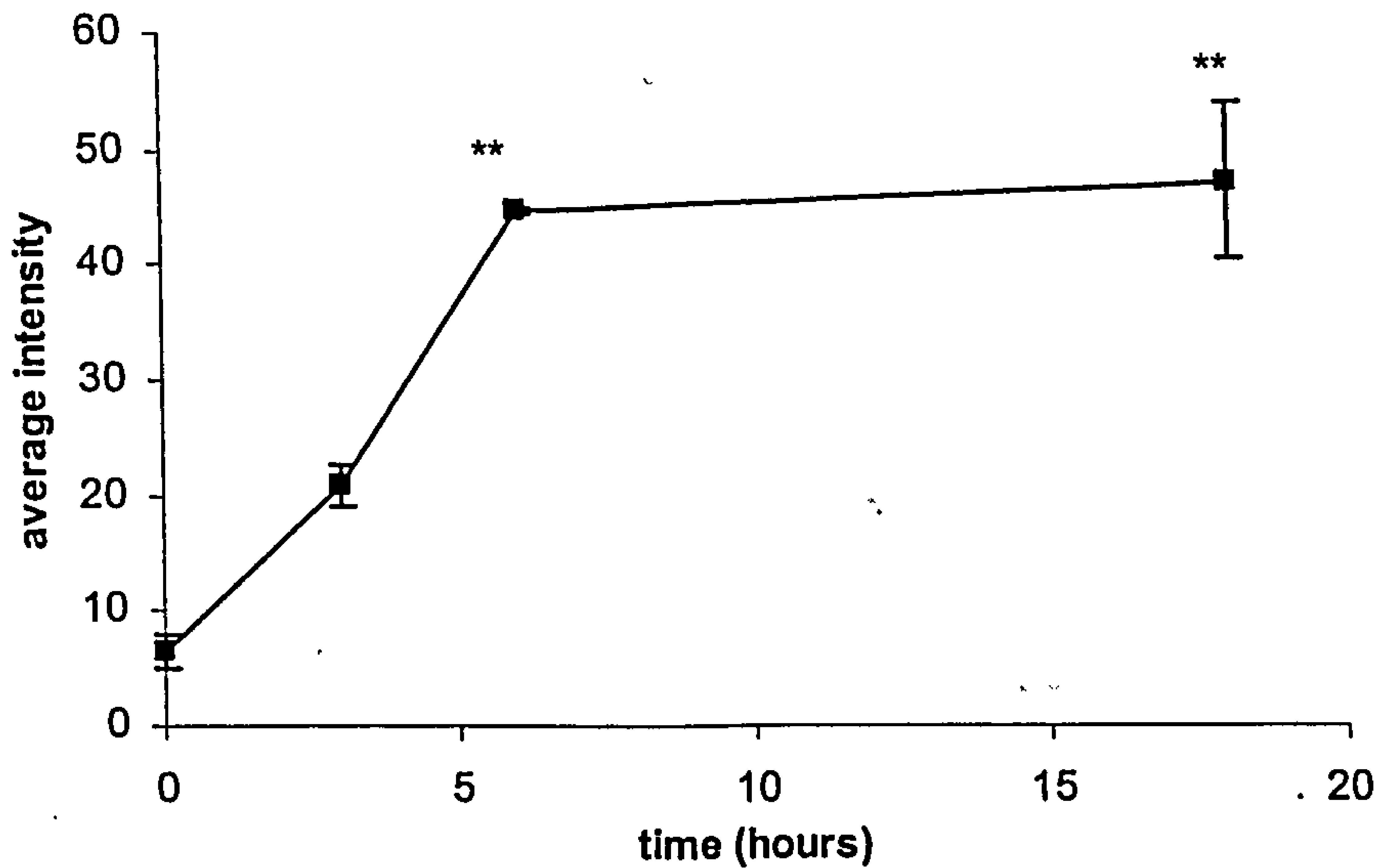


Figure 29: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of 100 μg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p < 0.01$.

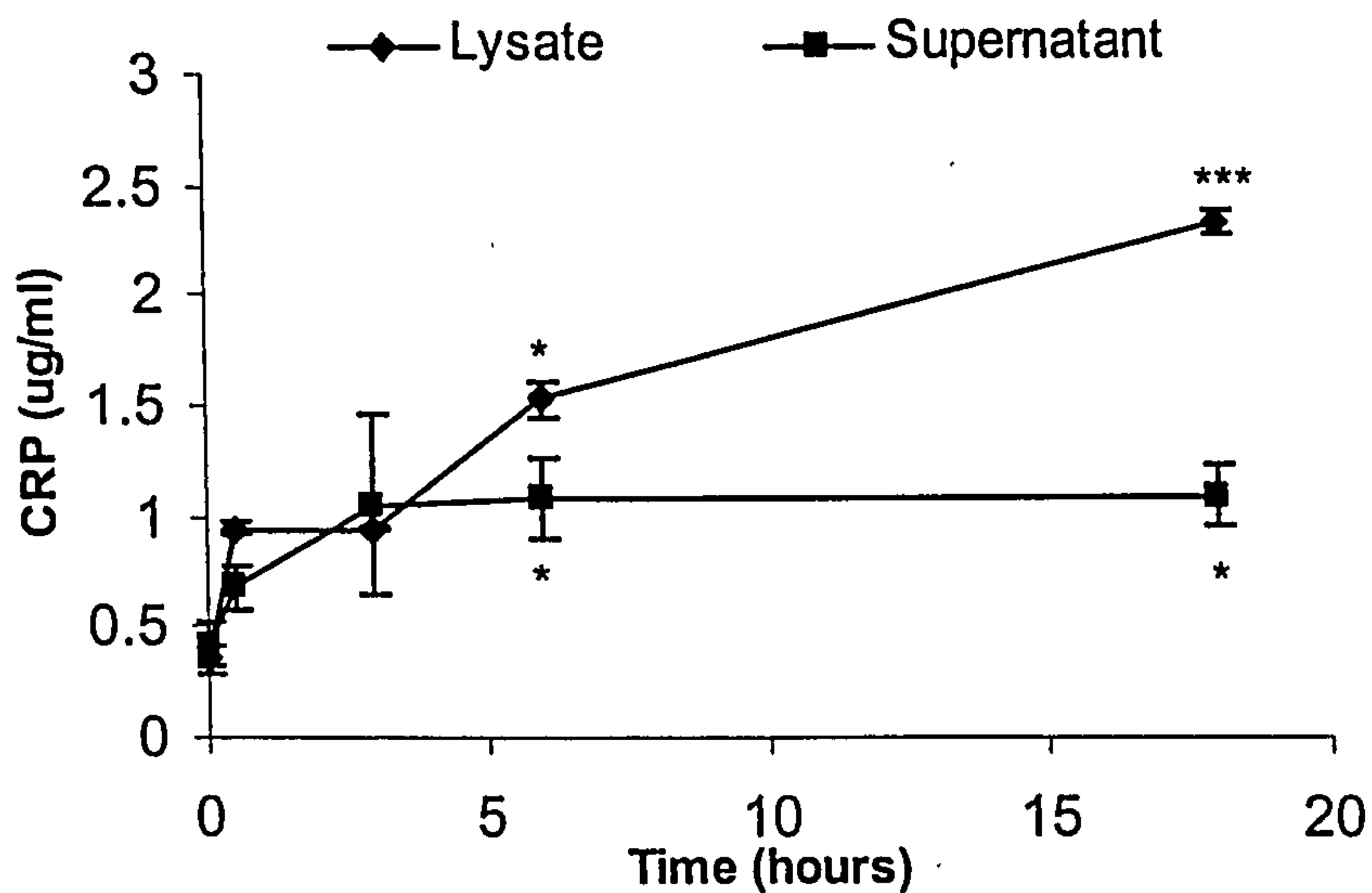


Figure 30: Concentration of CRP in cell lysate and supernatant measured by ELISA after 18 hours of treatment with 100μg/ml ufCB. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (*, *) denotes significant changes from untreated (control) cells, $p < 0.05$, $p < 0.001$.**

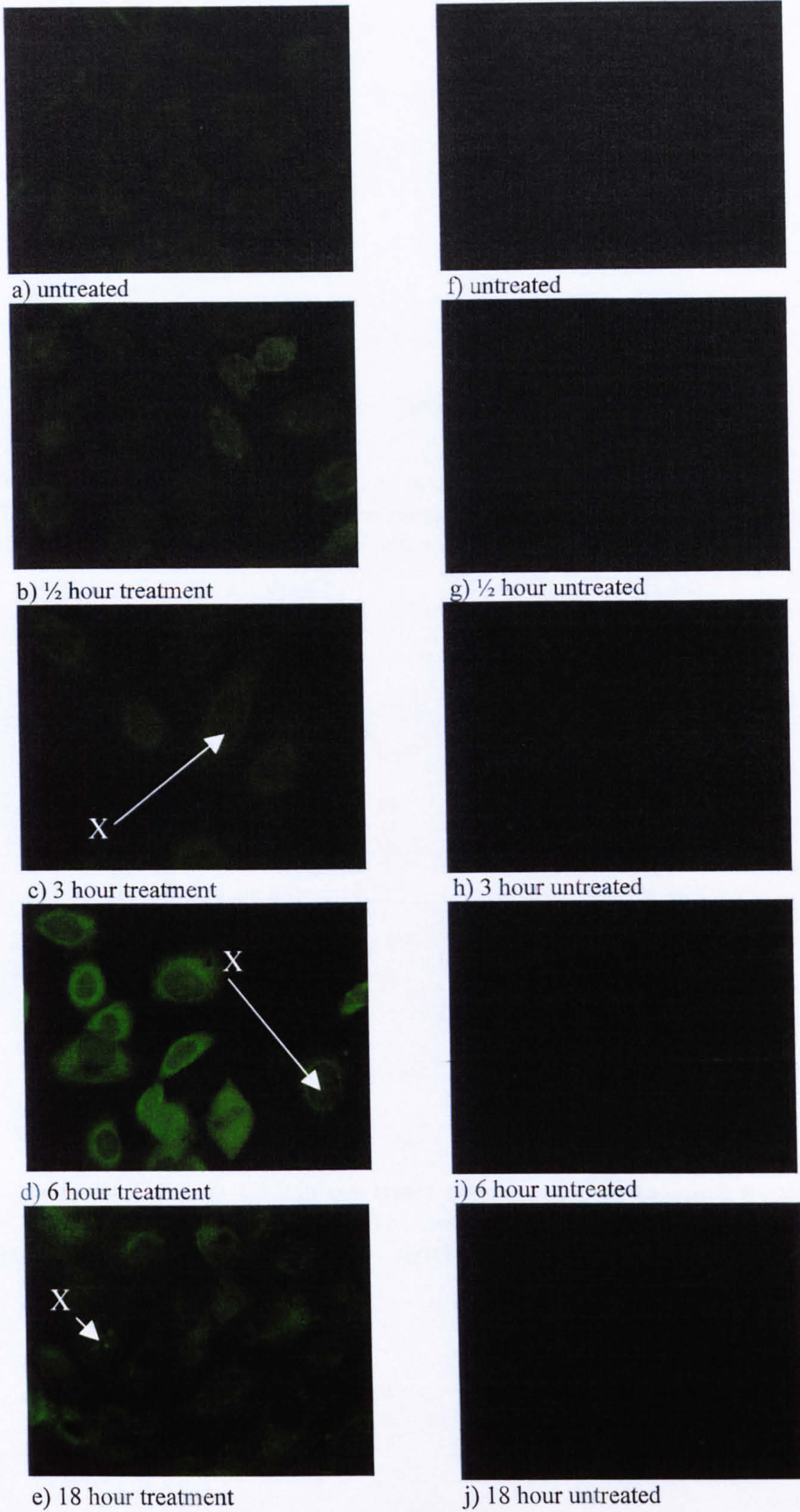


Figure 31: Expression of C-reactive protein (CRP) in A549 cells treated with 80μg/ml PM10, for various time points up to 18 hours, as shown by indirect immunofluorescence. X indicates nuclear staining of CRP. For controls see Appendix I.

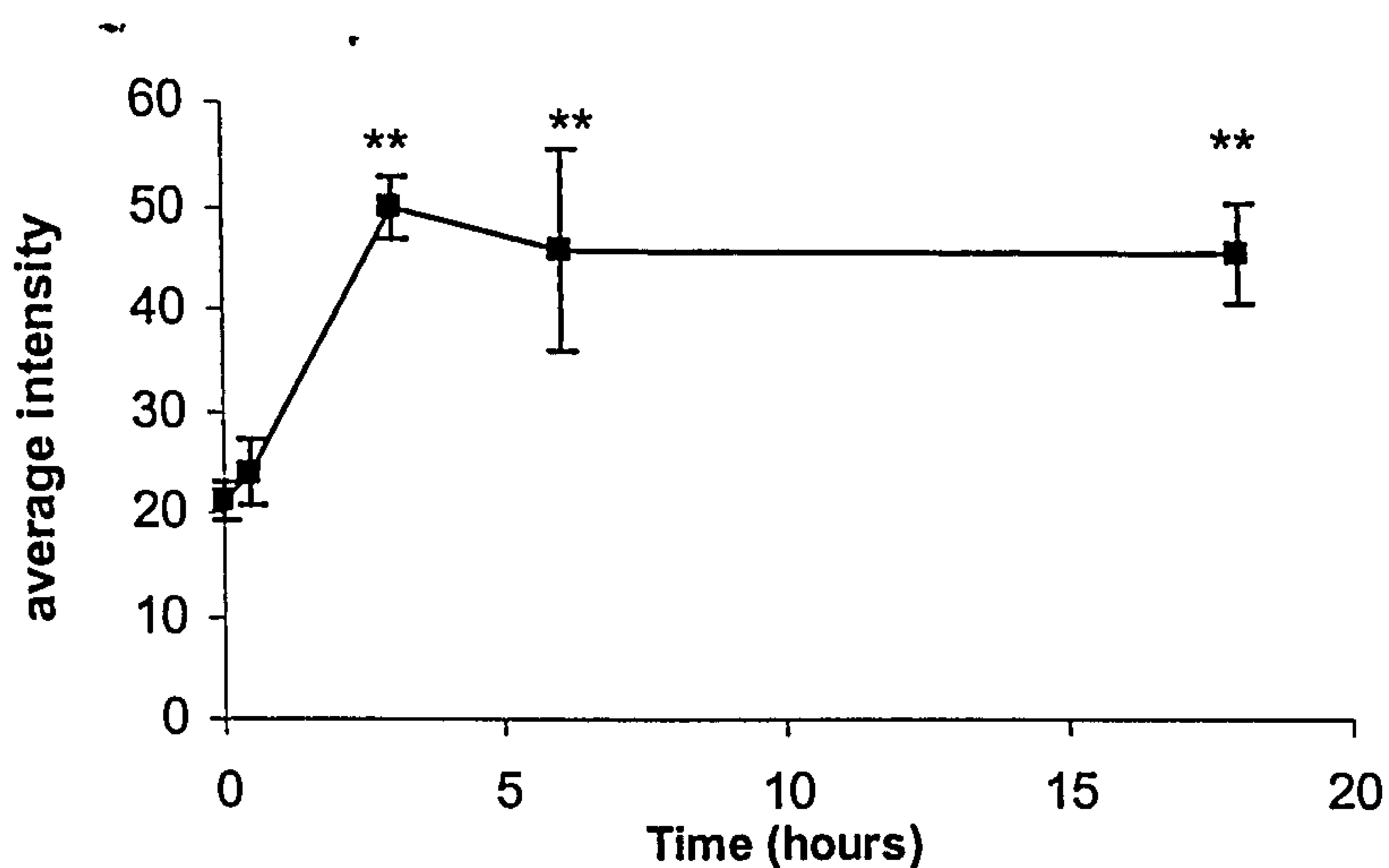


Figure 32: Immunofluorescent intensities of A459 cells stained for CRP after 18 hours of 80 μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p < 0.01$.

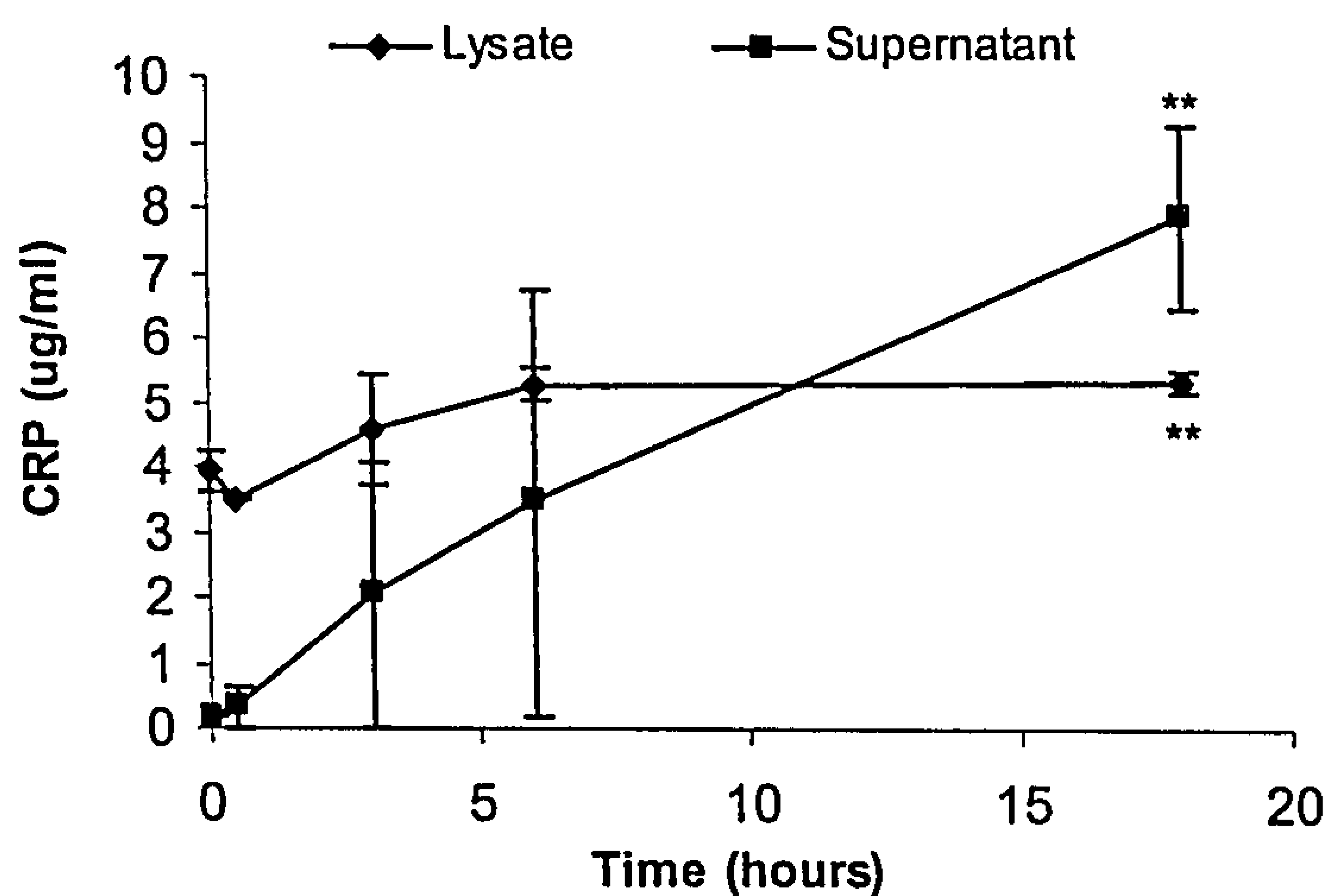


Figure 33: Concentration of CRP in cell lysate and supernatant measured by ELISA after 18 hours of treatment with 80μg/ml PM10. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p<0.01$.

4.2. Effect of CB and ufCB treatment on Fibrinogen expression.

4.2.1 Treatment with CB over 18 hour period.

Immunofluorescent staining after treatment with CB showed a time-dependent increase in fibrinogen similar to that of CRP (Fig 34). After 3 hours of treatment the levels of fibrinogen appear to be increased compared to the untreated cells. Fibrinogen was found localised in the cell cytoplasm and distributed evenly throughout the cell. Intensity analysis (Fig 35) reveals that there is almost a linear relationship between time of treatment and amount of fibrinogen within the cells, with statistical significance at 18 hours ($p<0.01$).

ELISAs carried out on the whole cell lysate and cell culture medium show similar results to CRP. In the whole cell lysate (Fig 36) the levels of fibrinogen increase with time showing statistical significance at 18 hours ($p< 0.05$). The analysis of the cell culture medium (Fig 36) indicates that there is also secretion of fibrinogen from the cells. There is an increase with time, but at the 6 hour time point there appears to be a decrease in the concentration of fibrinogen increasing to 18 hours. The decrease in fibrinogen levels at 6 hours is unexpected and is likely to be the result of experimental errors.

4.2.2. Treatment with ufCB over 18 hour time period.

The effect of treatment with ufCB on fibrinogen expression was then investigated. Cells were treated as described earlier for CRP. Immunofluorescent staining (Fig 37) shows that there is an increase in expression of fibrinogen with time. The levels of fibrinogen start to increase from 3 hours but are greatly increased from 6 hours. The staining at the 6 and 18 hour time points show distinct polarisation of the fibrinogen in the cells, which was not present in the CB treated cells. Intensity analysis was carried out (Fig 38) and this showed that there was little change in the intensity in the first three hours. After 6 hours of treatment there is statistical significance compared to untreated cells ($p< 0.01$) rising to 18 hours ($p<0.001$).

ELISAs were carried out on the whole cell lysate and the cell culture medium. The whole cell lysates (Fig 39) showed an increase with time and a statistical significance

at 18 hours after treatment. The cell culture medium (Fig 39) showed that there is some secretion of fibrinogen although it never increases enough to be statistically significant. This could be due to adsorption of fibrinogen on to the particle surface which would result in an underestimation of the amount of secreted fibrinogen (see appendix II).

4.2.3. Treatment with PM10 over 18 hour time period.

Treatment with 80mg/ml PM10 from a roadside TEOM sampler in Wolverhampton, UK, was carried out over 18 hours. Immunofluorescent staining was carried out at various time points and showed that fibrinogen was produced in a time dependent manner (Fig 40). Fibrinogen was found to be present in the cytoplasm of the cells with fairly high density of fibrinogen localised around the cell nucleus. Intensity analysis showed that there was an increase in fibrinogen but this was not statistically significant (Fig 41).

ELISAs were carried out on the cell lysates and cell supernatants of the PM10 treated cells. The cell lysates (Fig 42) showed that there was an increase in the levels of fibrinogen with time of PM10 treatment. The cell supernatants showed that there was some secretion of fibrinogen from the cells (Fig 42). Although the levels of fibrinogen are only increased above the control cell level at the 18 hour time point and even then there are high variations.

4.2.4. Treatment with $FeCl_3$ over 18 hour time period.

Cells were treated with 100μM of the transition metal salt $FeCl_3$ for various time points up to 18 hours. Immunofluorescent staining was carried out for fibrinogen at each time point and this revealed that fibrinogen was stimulated in a time dependent manner (Fig 43). At all the time points fibrinogen is found in the cytoplasm of the cells and at the latter time points there is fibrinogen expressed at high levels in a higher proportion of the cells. Intensity analysis of the cells showed that there is an increase in the levels of fibrinogen with $FeCl_3$ treatment over the time course and that at 18 hours this increase is statistically significant ($p<0.01$)(Fig 44).

ELISAs were carried out in the cell lysate revealed that fibrinogen expression was increased over the time course; however, the errors between treatments were high (Fig 45). The high deviation between the samples means that it is difficult to tell if the values actually represent what is happening within the cells and the errors are too great to achieve statistical significance. Analysis of the cell supernatant indicates that there may be an increase in fibrinogen after 18 hours (Fig 45) however at this time point there are is a high degree of error between the samples resulting in no statistical significance. Both the cell lysate and supernatants were repeated 5 times and this did not affect the results.

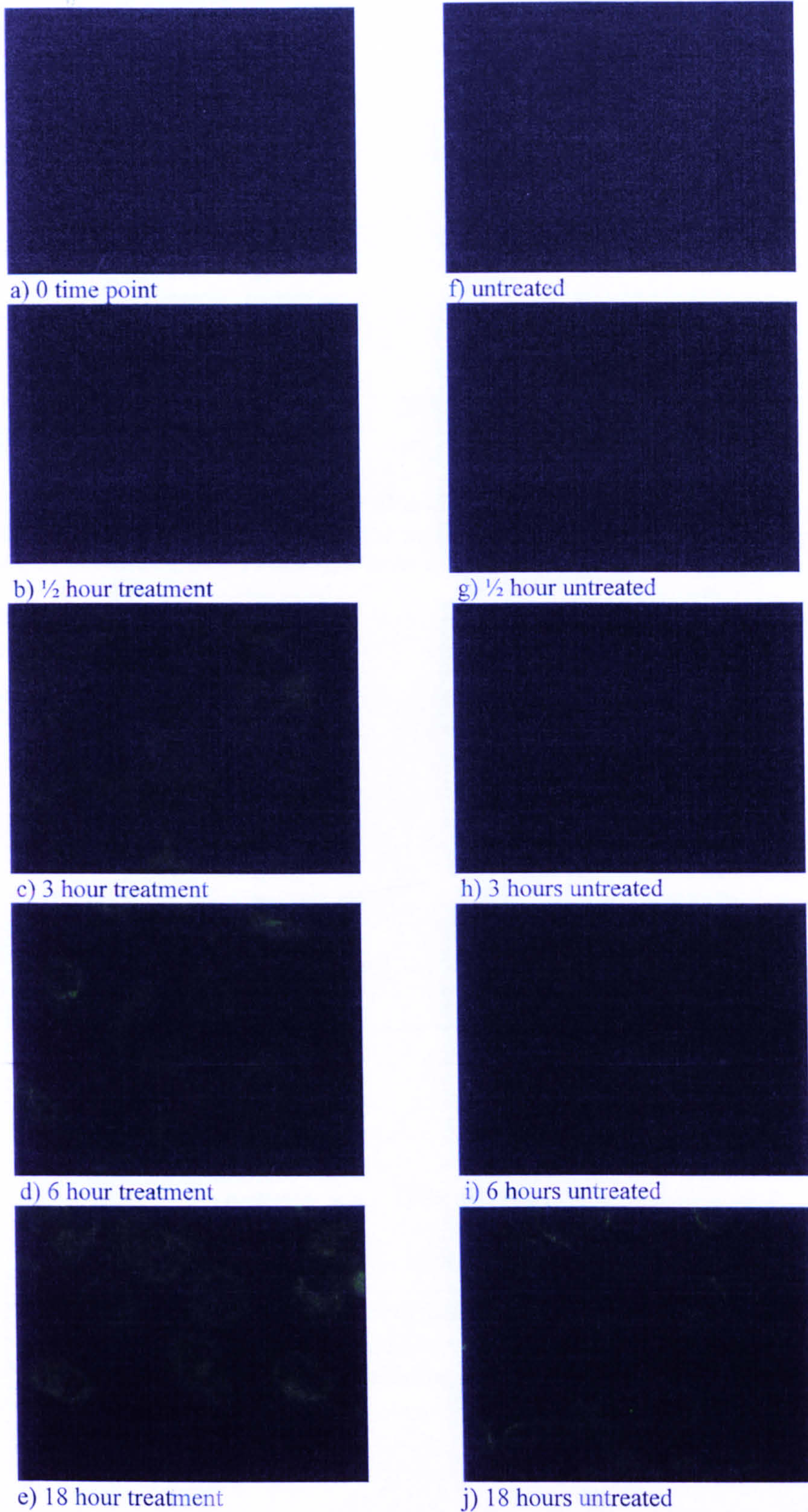


Figure 34: Expression of fibrinogen in A549 cells treated with 100μg/ml CB, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

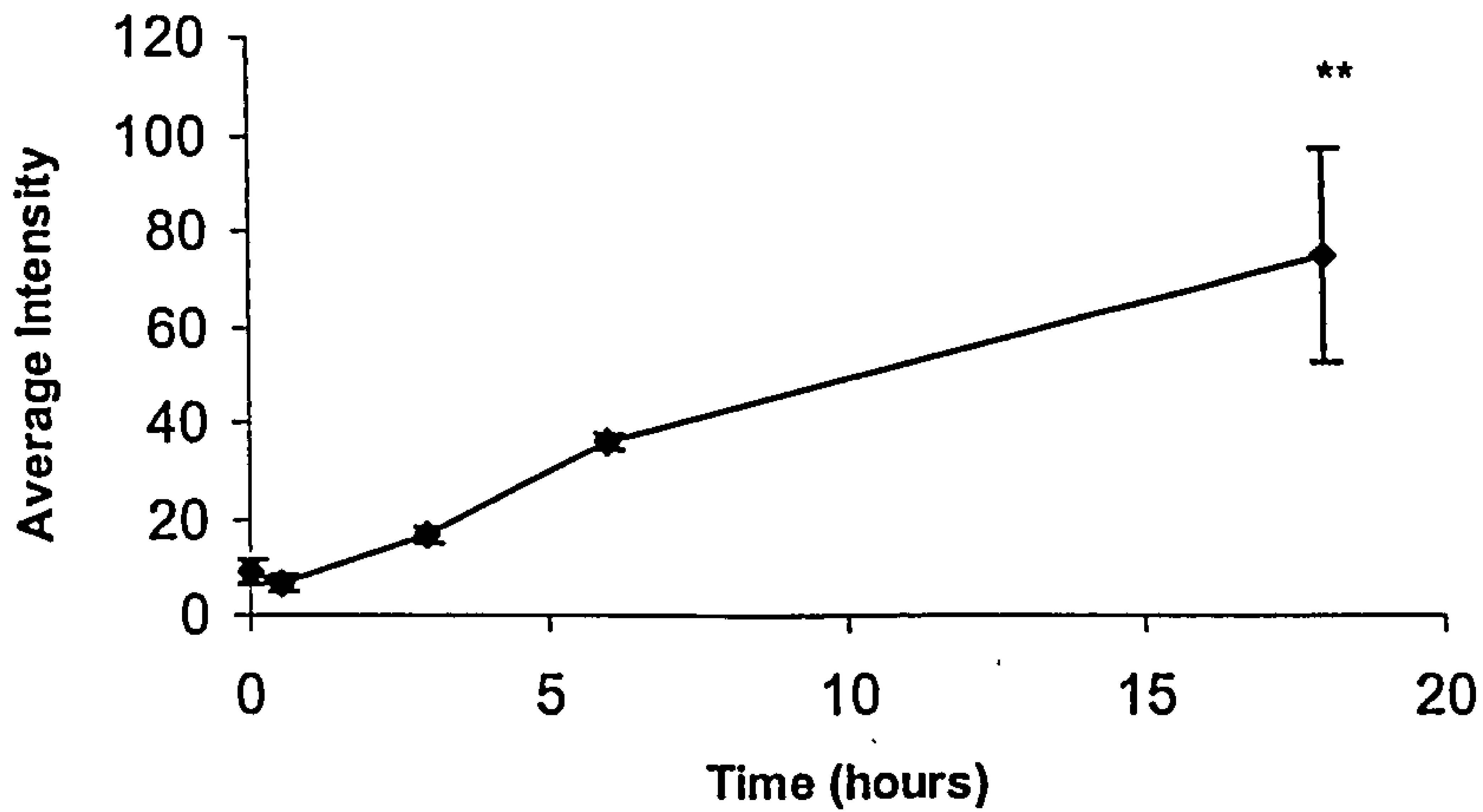


Figure 35: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 100 μg/ml CB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p < 0.01$.

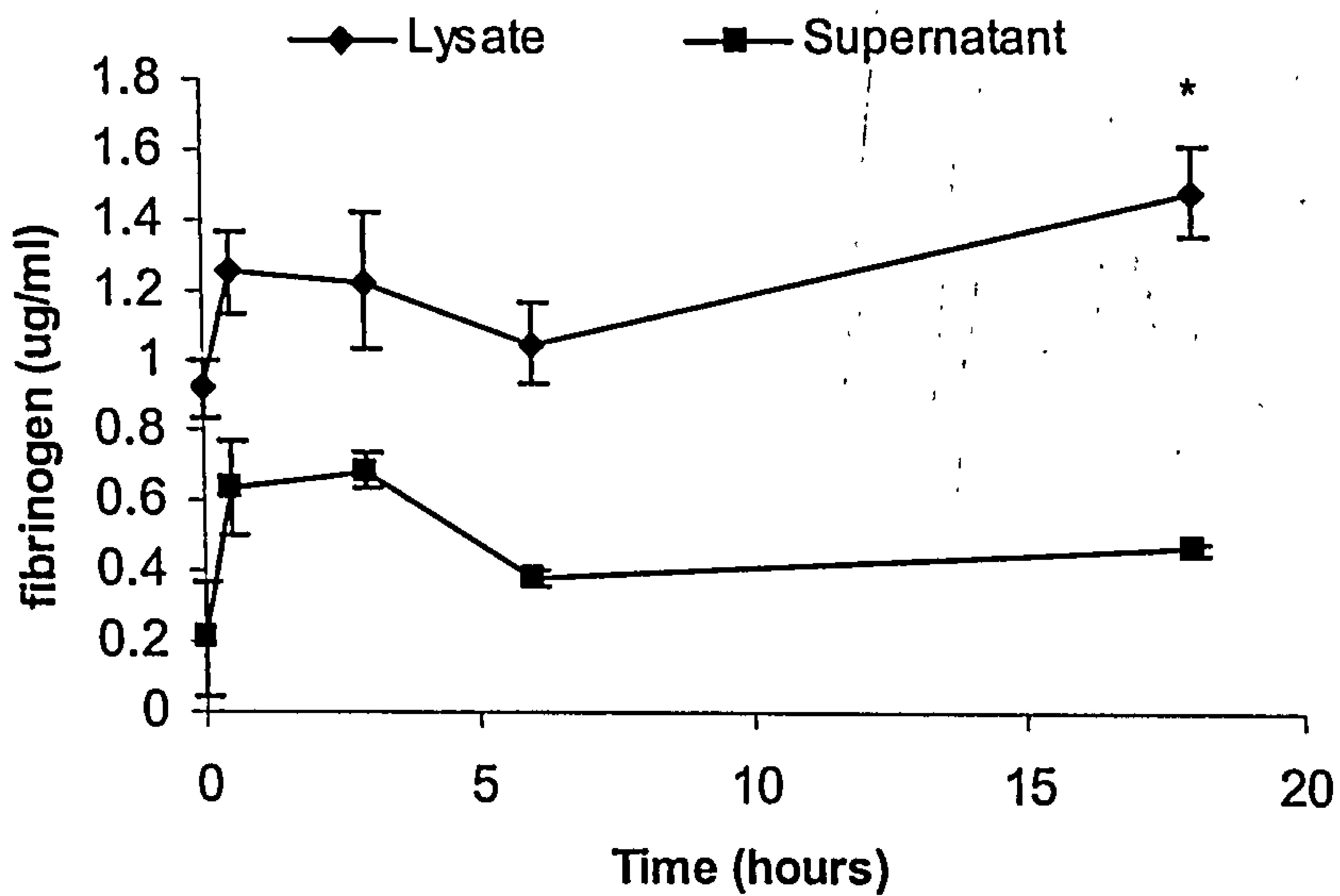


Figure 36: Concentration of fibrinogen in cell lysate and supernatant measured by ELISA after 18 hours of treatment with 100μg/ml CB. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated (control) cells, $p < 0.05$.

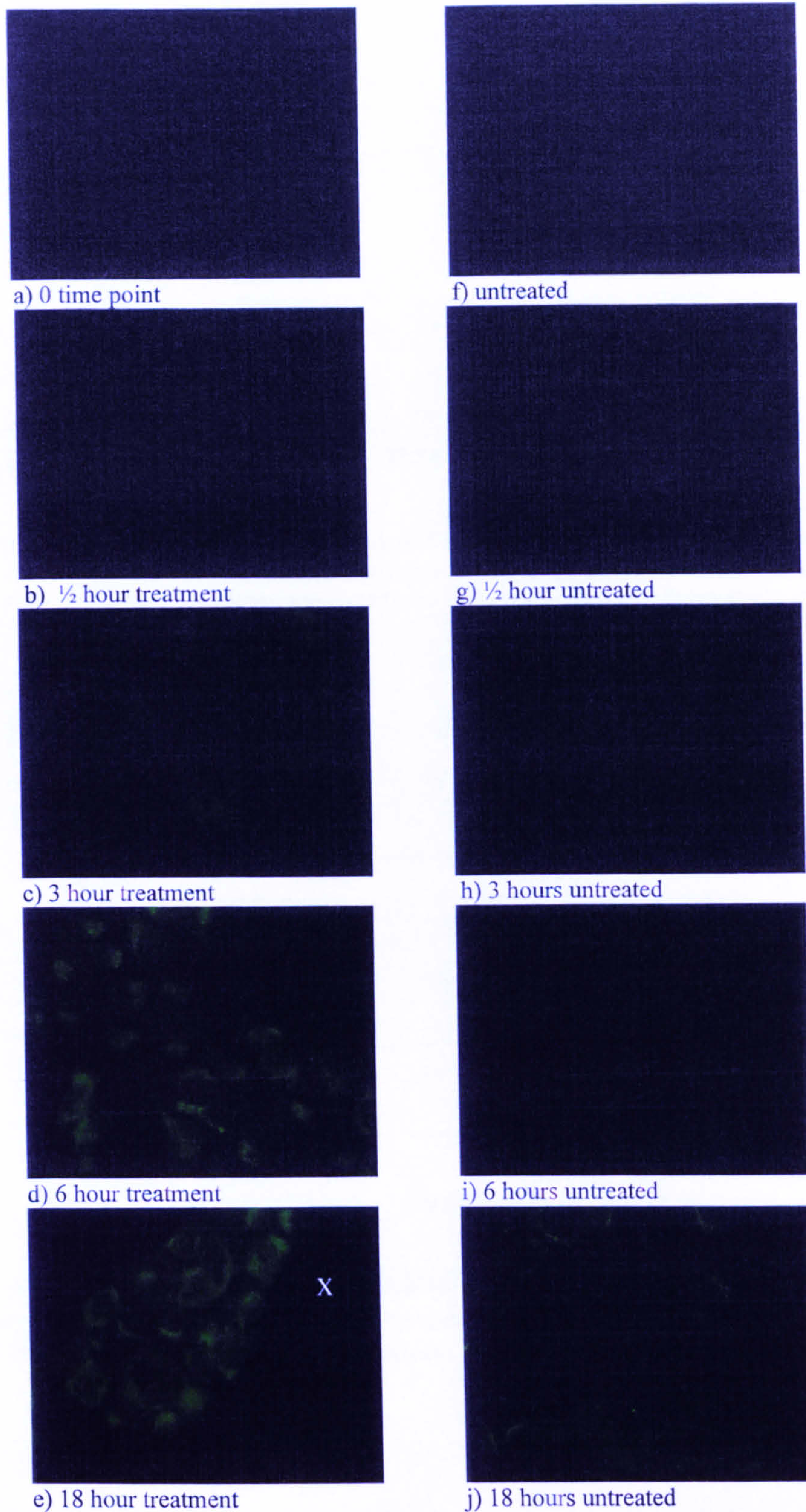


Figure 37: Expression of fibrinogen in A549 cells treated with 100 μg/ml ufCB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates polarisation of fibrinogen. For controls see Appendix I.

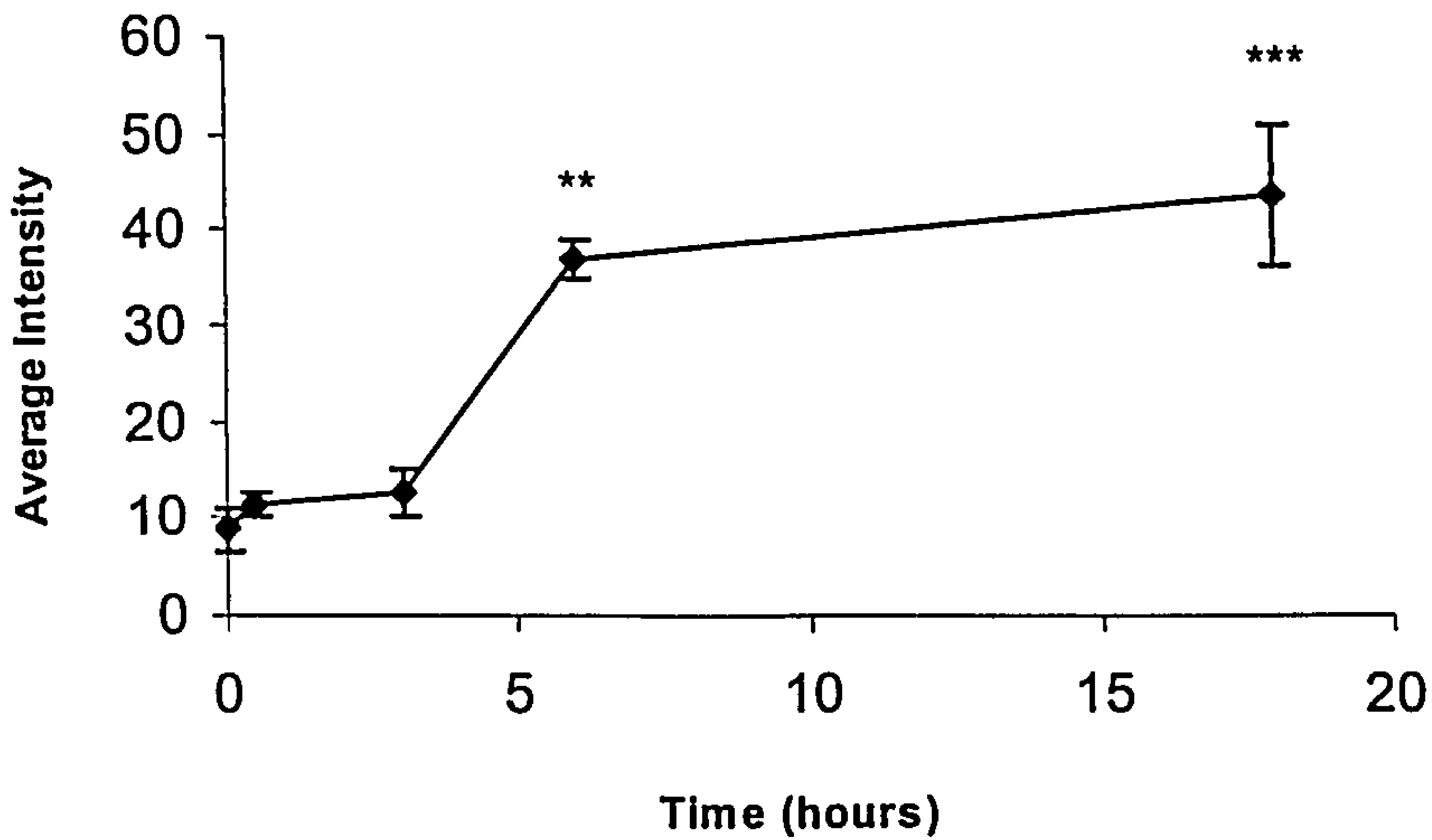


Figure 38: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 100 µg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (, ***) denotes significant changes from untreated (control) cells, $p < 0.01$, $p < 0.001$.**

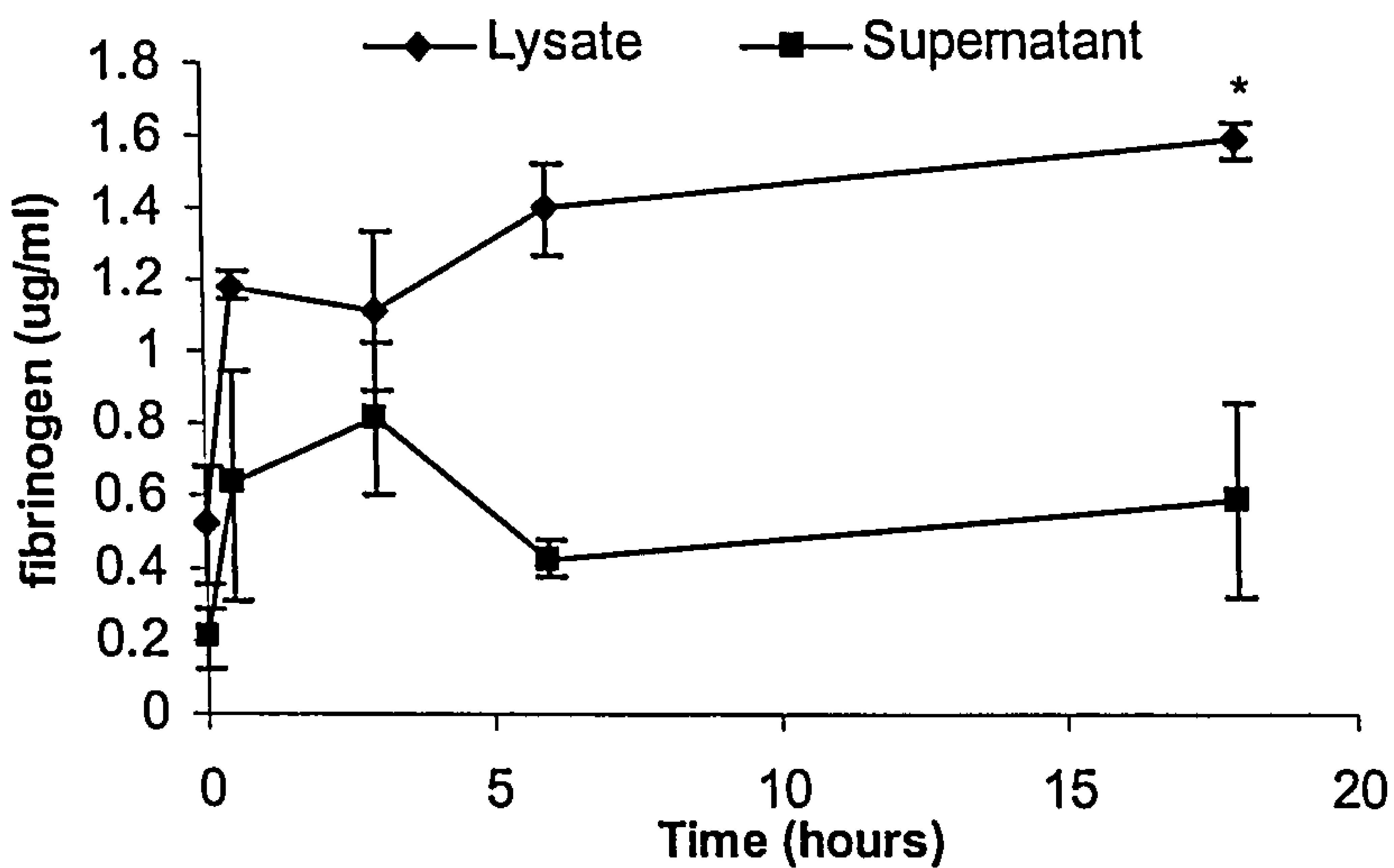
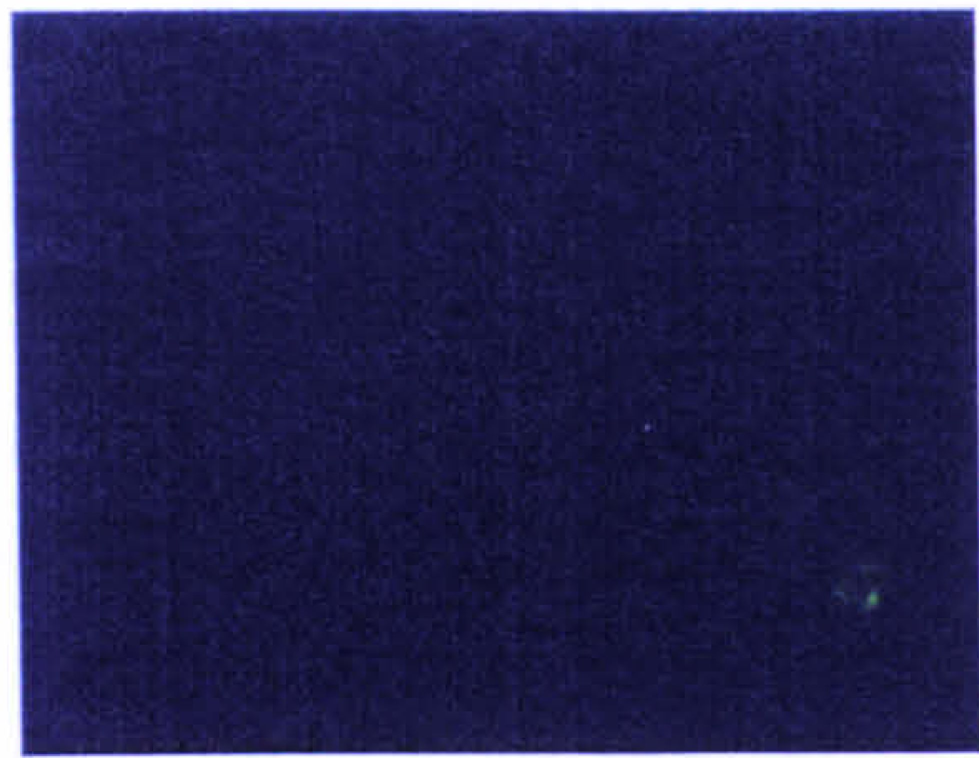
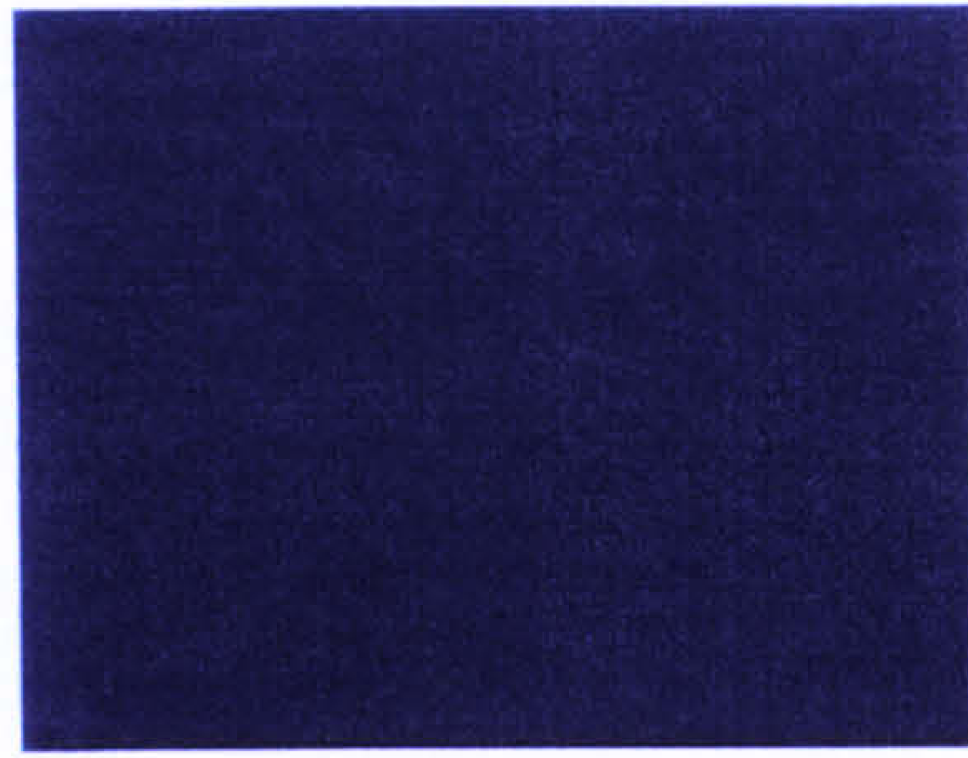
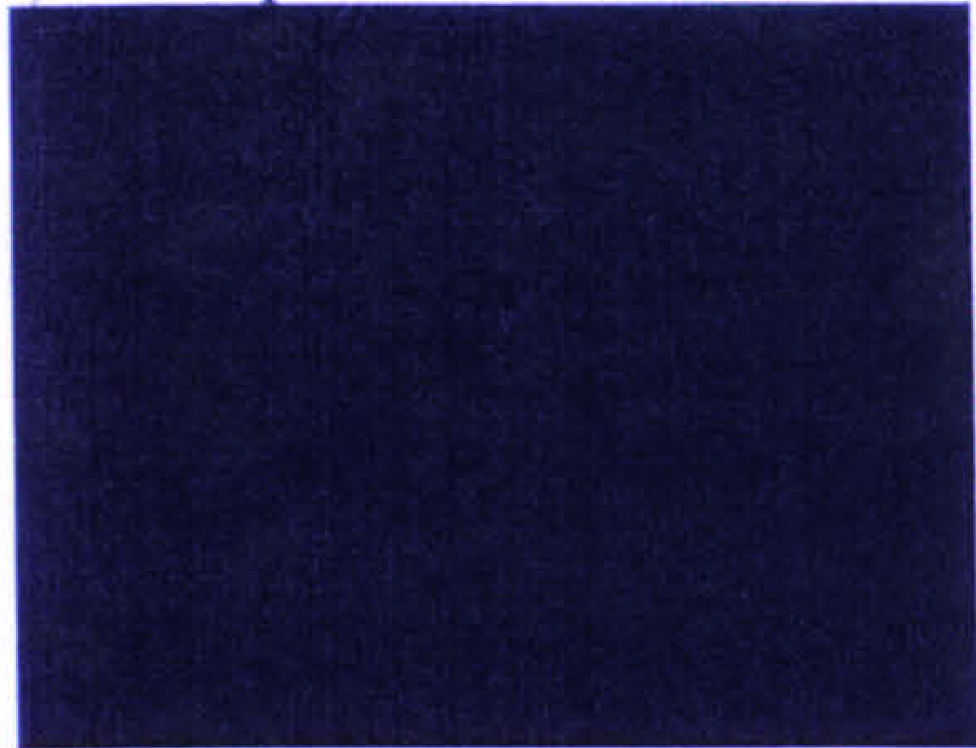
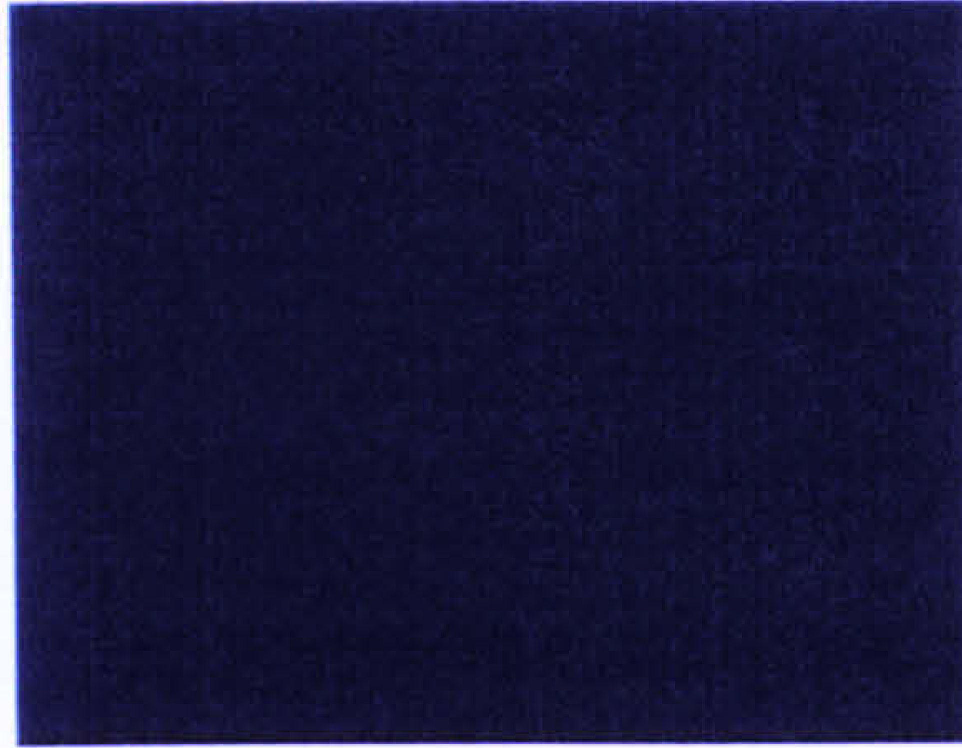


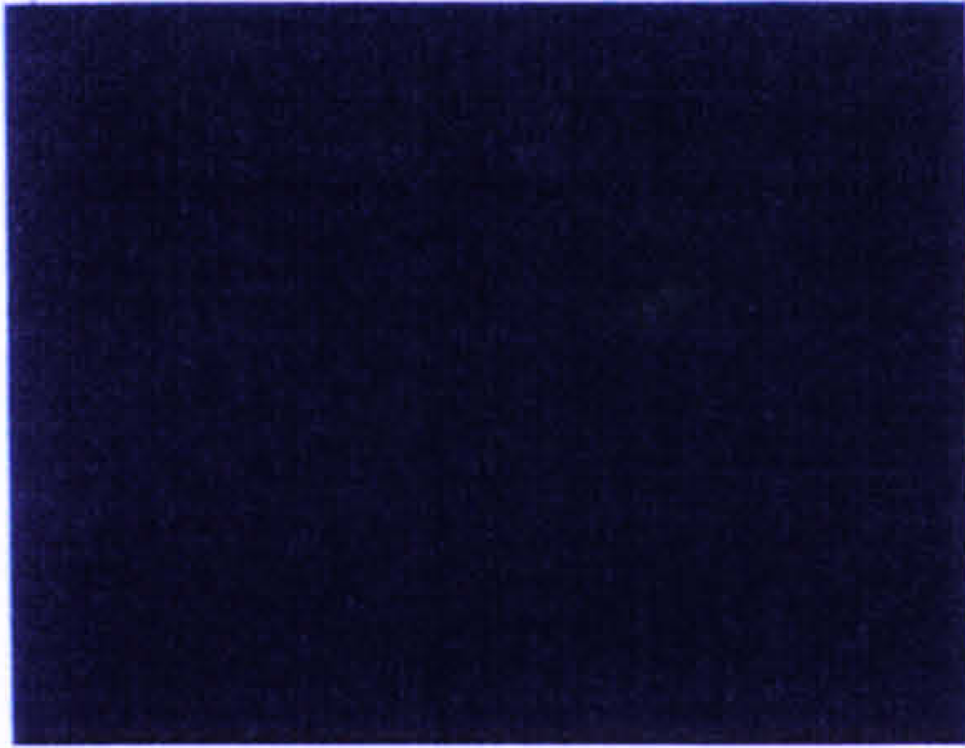
Figure 39: Concentration of fibrinogen in cell lysate and supernatant measured by ELISA after 18 hours of 100 µg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated (control) cells, $p < 0.05$.


a) 0 time point


f) untreated

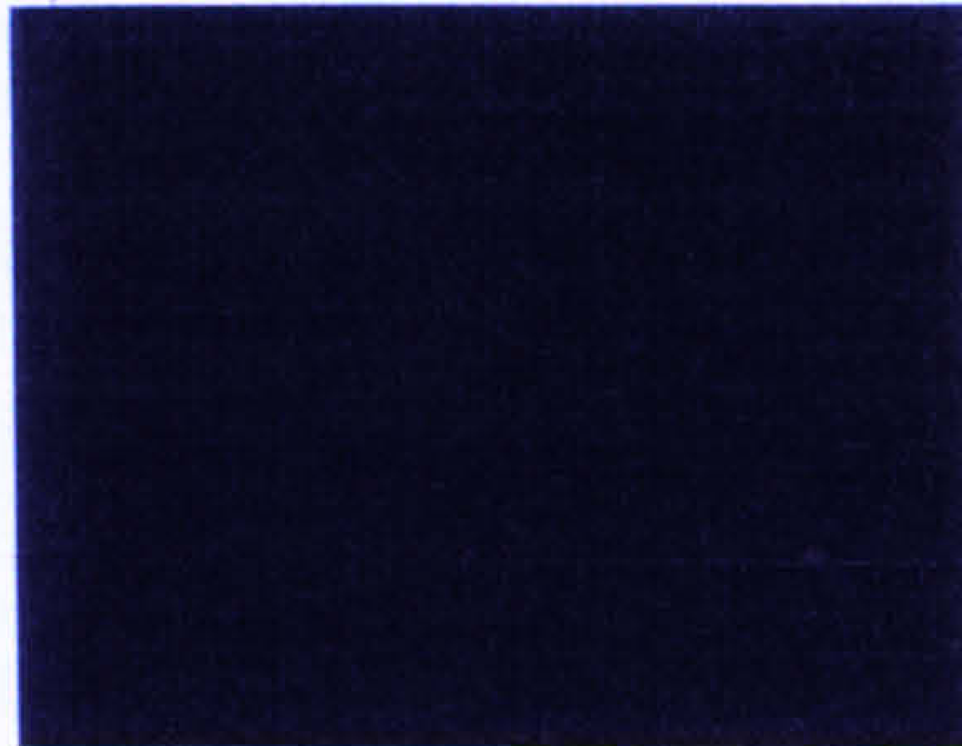

b) ½ hour treatment

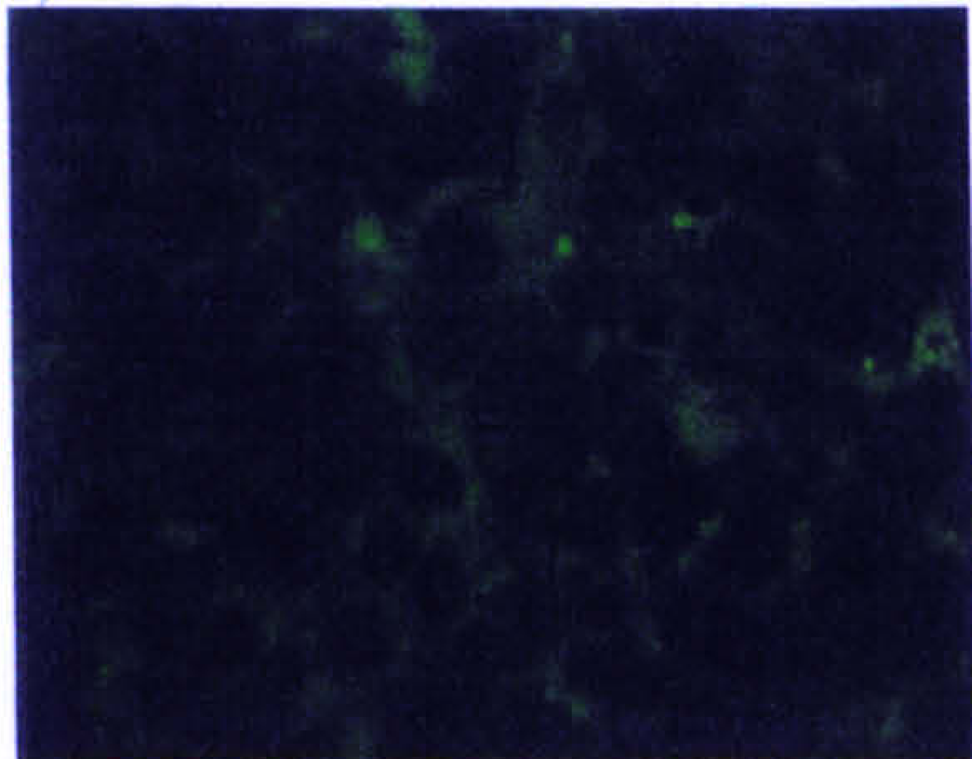

g) ½ hour untreated


c) 3 hour treatment

h) 3 hours untreated

d) 6 hour treatment


i) 6 hours untreated


e) 18 hour treatment

f) 18 hours untreated

Figure 40: Expression of fibrinogen in A549 cells treated with 80µg/ml PM10, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

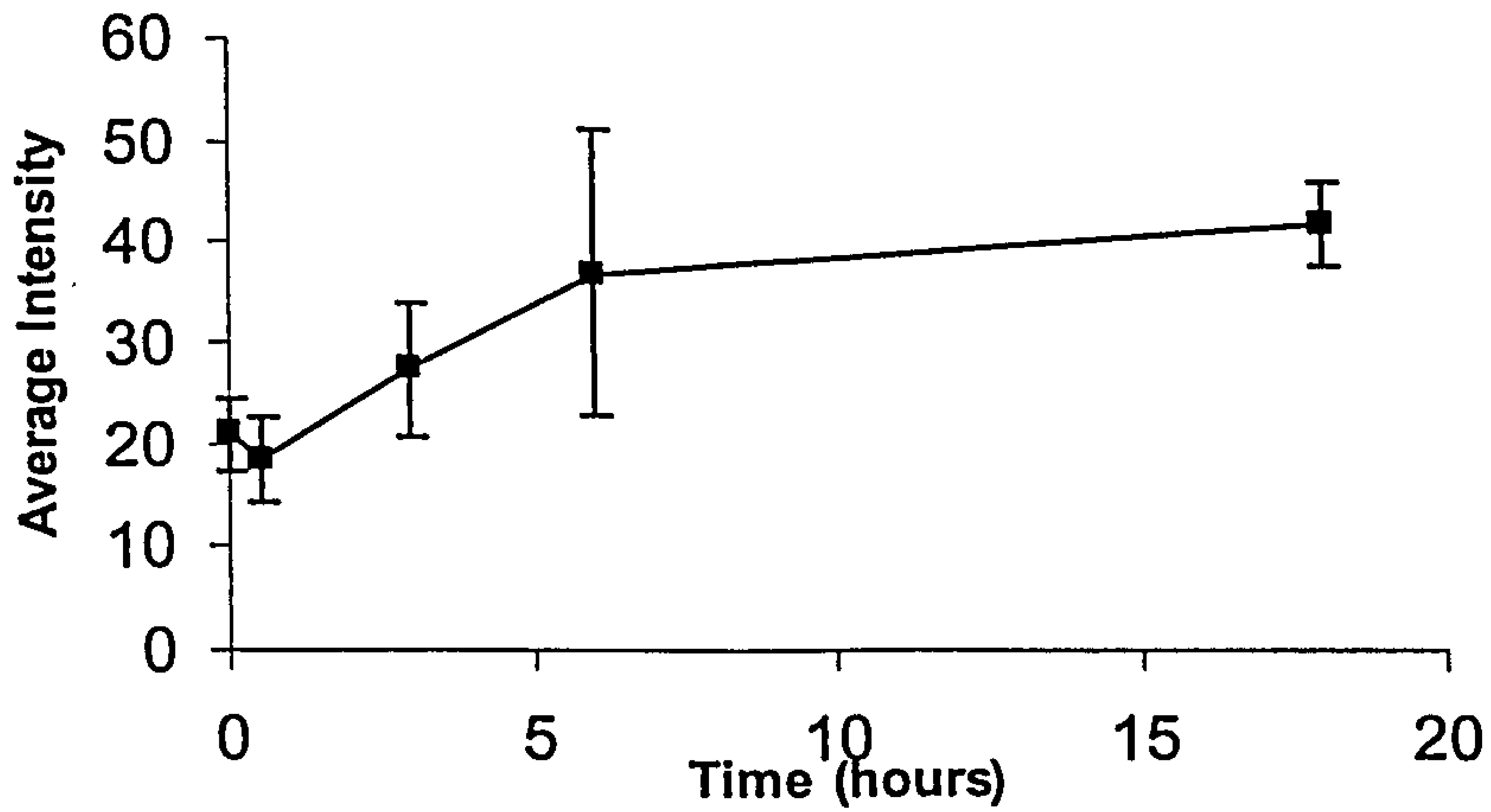


Figure 41: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 80 μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE.

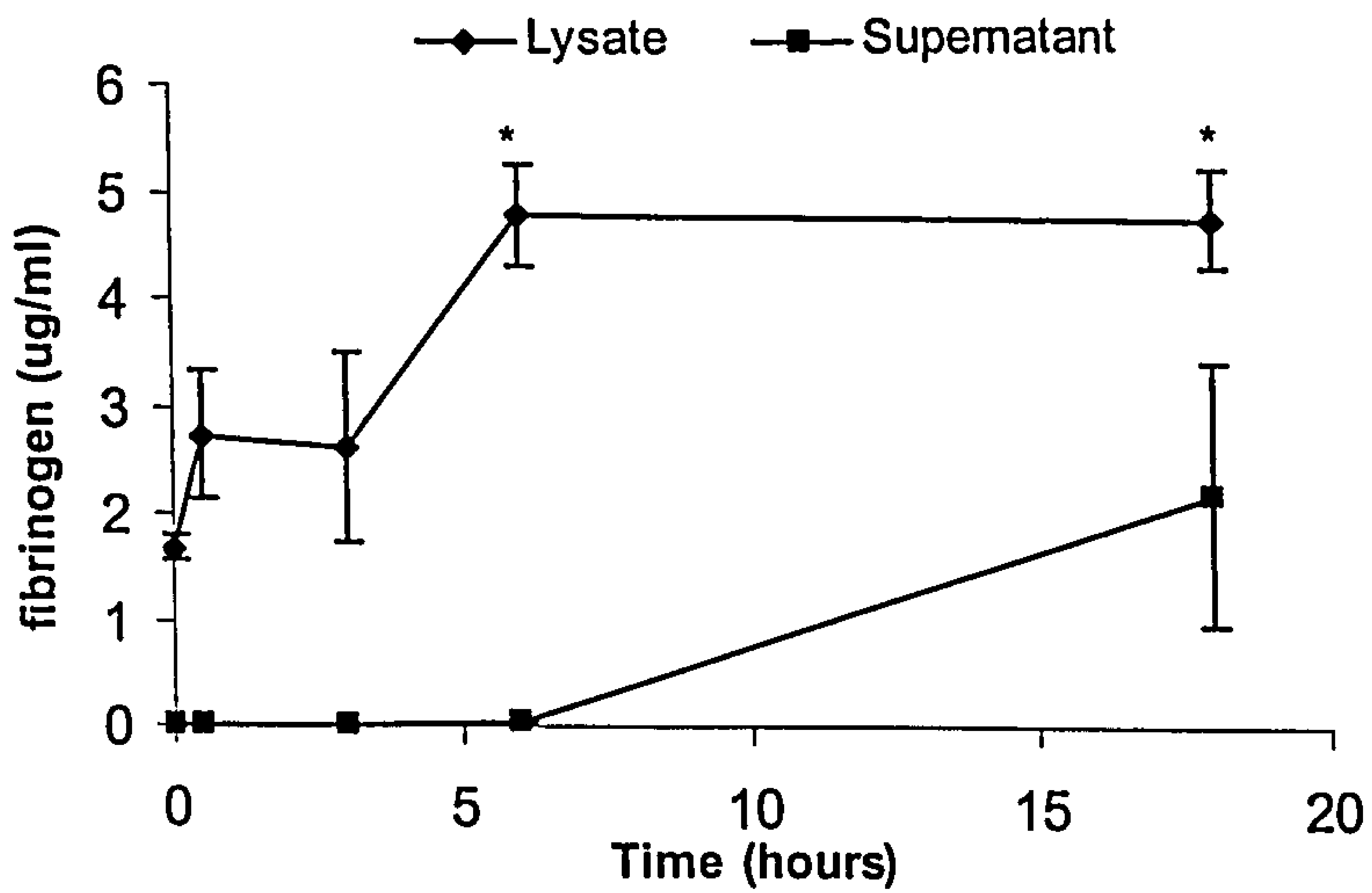
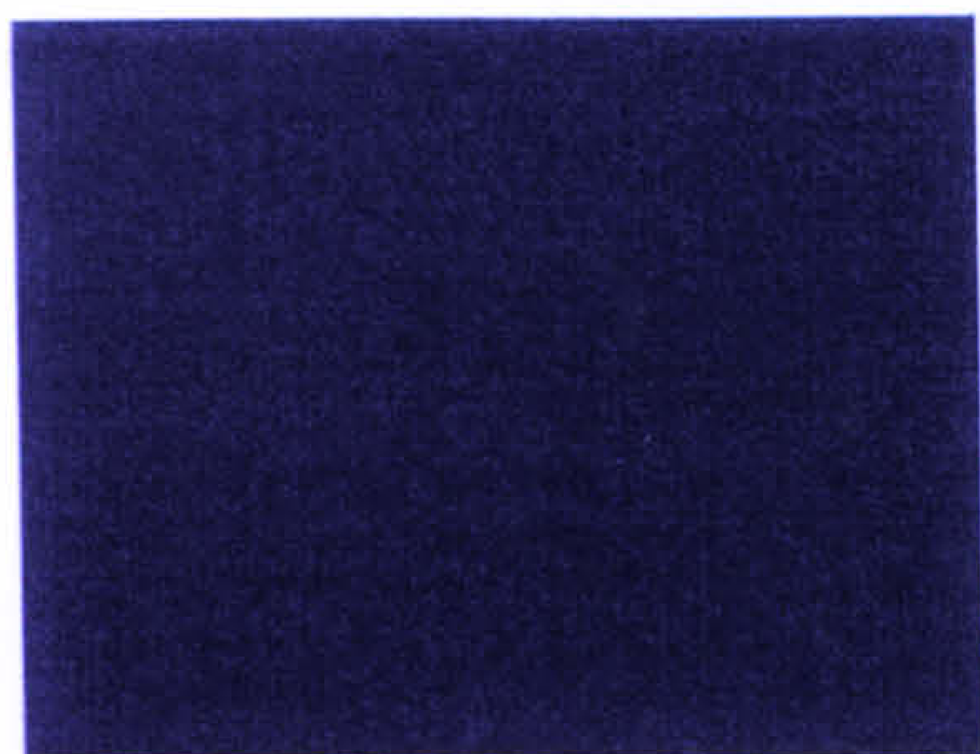
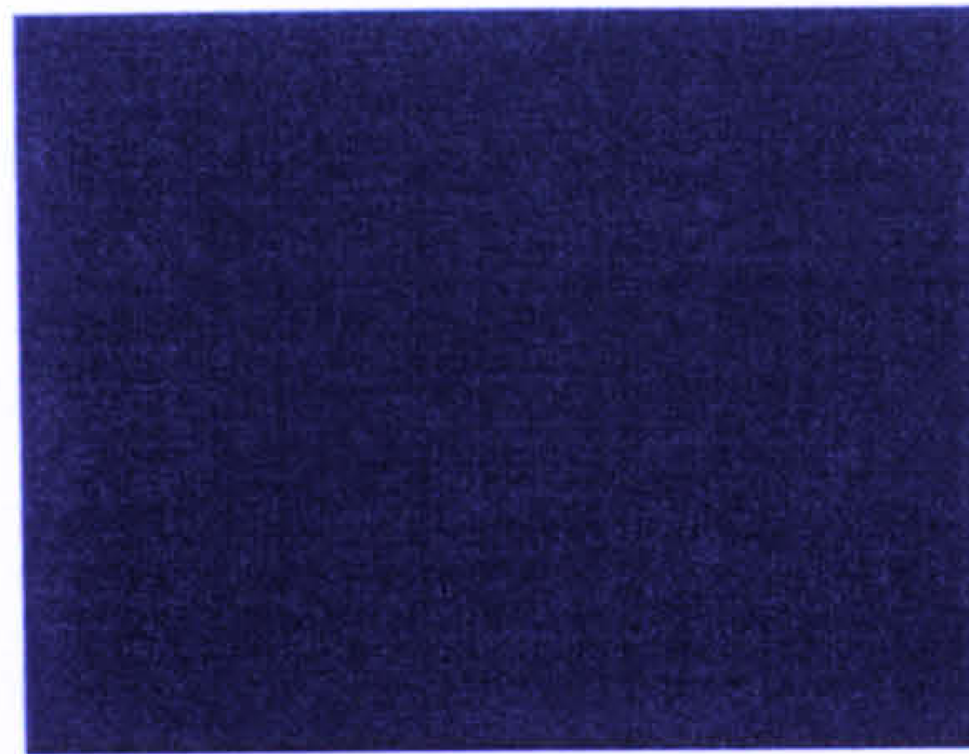
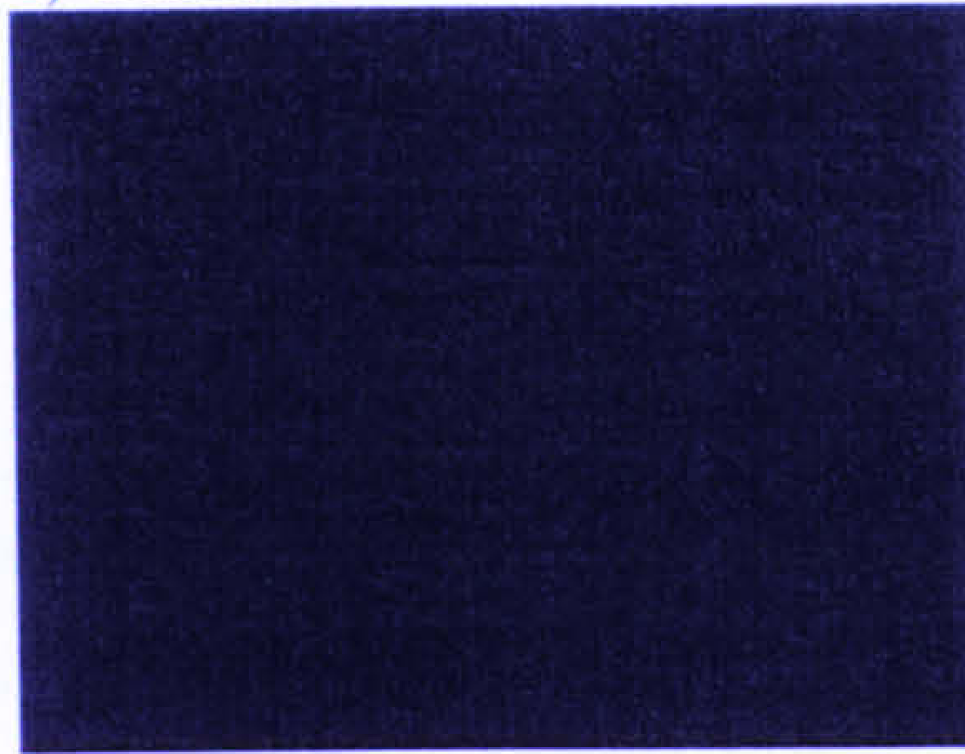


Figure 42: Concentration of fibrinogen in cell lysate and supernatant measured by ELISA after 18 hours of 80μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated (control) cells, p< 0.05.

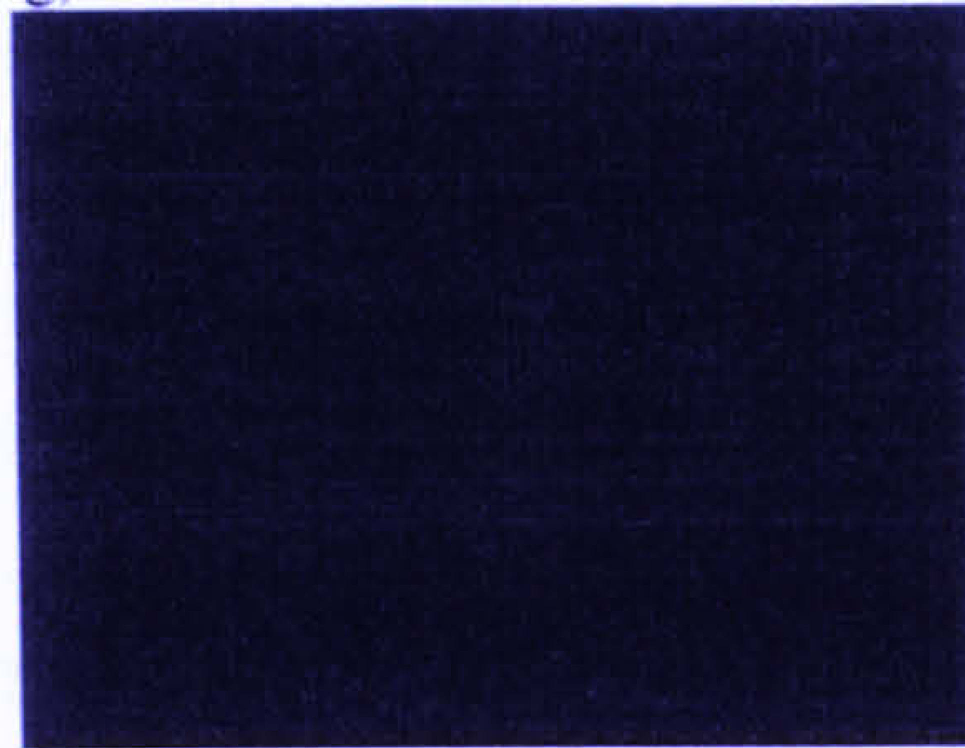

a) 0 time point


f) untreated

b) ½ hour treatment


g) ½ hour untreated

c) 3 hour treatment


h) 3 hours untreated

d) 6 hour treatment

i) 6 hours untreated

e) 18 hour treatment

j) 18 hours untreated

Figure 43: Expression of fibrinogen in A549 cells treated with100mM $FeCl_3$, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

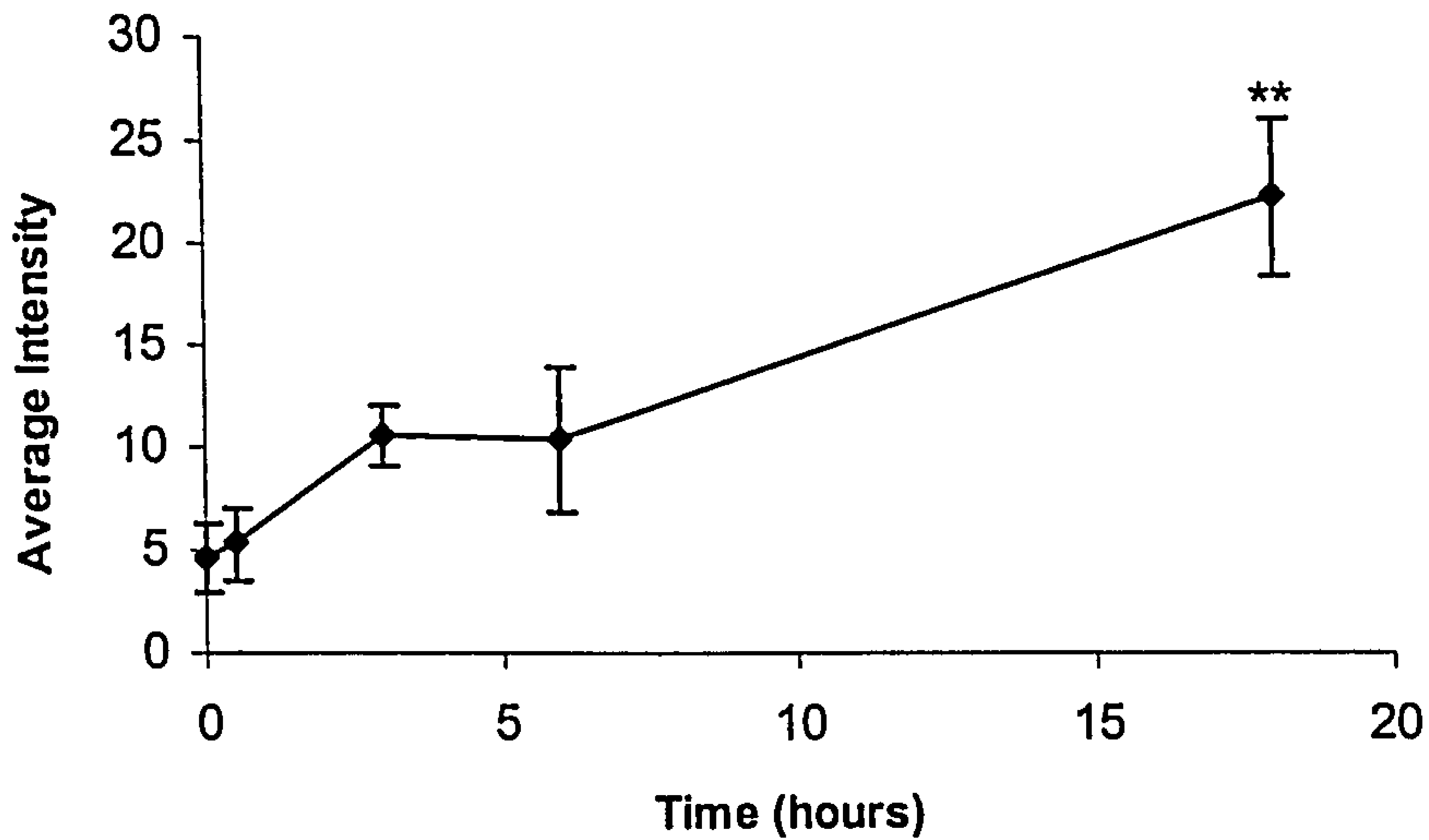


Figure 44: Immunofluorescent intensities of A459 cells stained for fibrinogen after 18 hours of 100μM $FeCl_3$ treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, $p < 0.01$

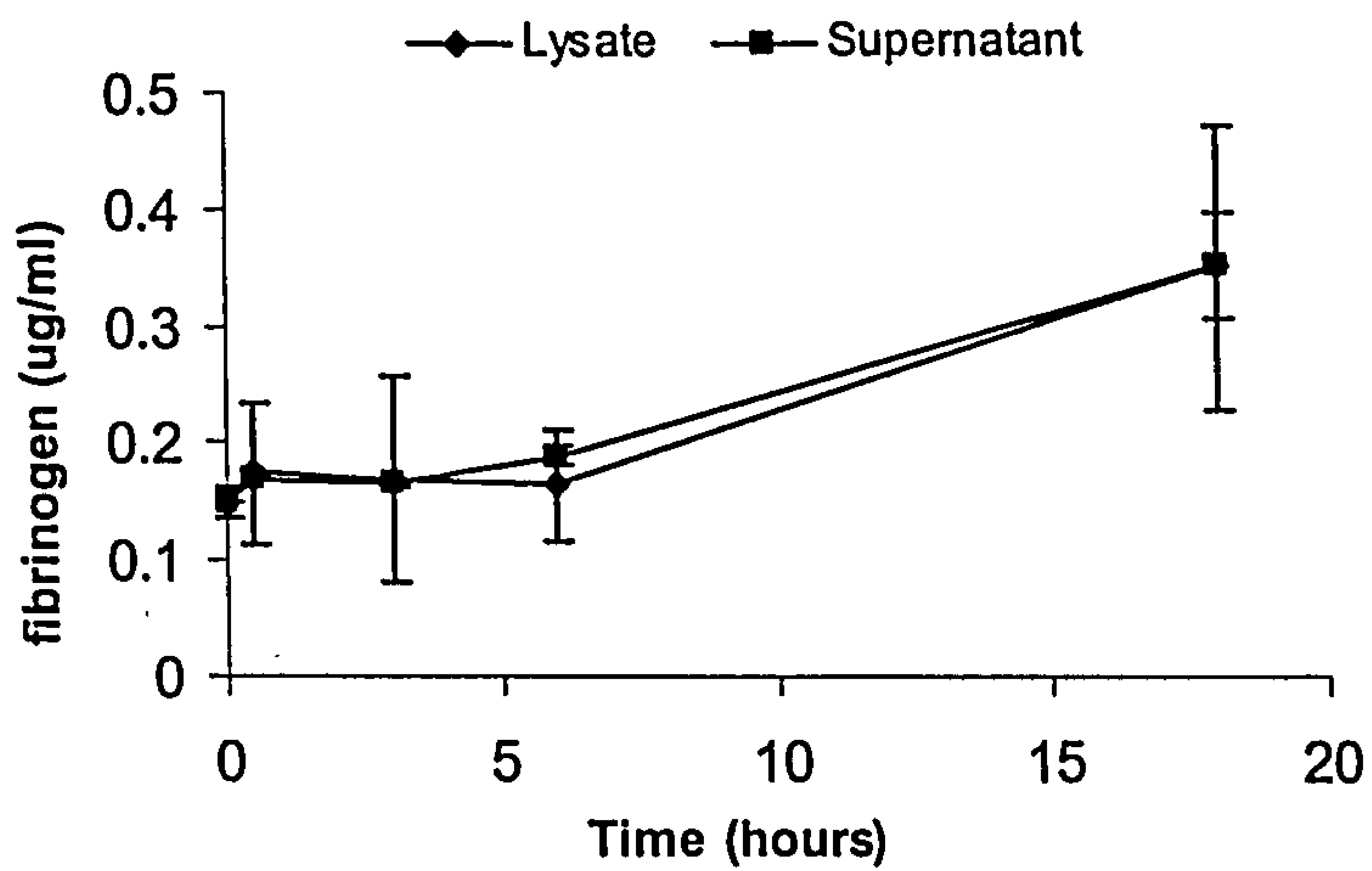


Figure 45: Concentration of fibrinogen in cell lysate and supernatant measured by ELISA after 18 hours of 100mM $FeCl_3$ treatment. The results are the mean of triplicate results from 3 experiments ± SE.

4.3. Effect of CB and ufCB treatment on Hsp70 expression.

4.3.1 Treatment with CB over 18 hour period.

Incubation of cells with CB for up to 18 hours showed that Hsp70 expression could be stimulated in a time dependent manner. Immunofluorescence microscopy (Fig 46) showed that low levels of Hsp70 were present in the cell cytoplasm for the first 3 hours of treatment. After 6 hours of treatment there were high levels of Hsp70 present, much of which was located around the nucleus of the cells with some nuclear localisation present. The nuclear localisation of Hsp70 gives a punctuated staining pattern. At 18 hours of treatment there are very high levels of Hsp70 in the cytoplasm which may be masking nuclear localisation although there appears to be a faint punctuated pattern. Intensity analysis of the immunofluorescence photographs (Fig 47) shows that there is indeed a high increase in intensity of Hsp70 after 3 hours of treatment which is statistically significant from the untreated cells ($p<0.001$ at 6 and 18 hours).

Western blots of the cell lysate and cell supernatant were carried out (Fig 48). ELISAs were not available for this protein and therefore this was the most sensitive technique available. This revealed that in the cell lysate detectable levels of Hsp70 were present from 3 hours in the cell lysate. In the cell supernatants there also appeared to be an increase in Hsp70 after 18 hours of treatment (Fig 49)

4.3.2 Treatment with ufCB over 18 hour period.

Incubation of cells with ufCB for up to 18 hours showed that Hsp70 expression was induced by ufCB after 6 hours, similarly to that with CB treatment. Immunofluorescent microscopy (Fig 50) showed that very low levels of Hsp70 were present during the first 3 hours of treatment, but after 6 hours there were very high levels of Hsp70. At 6 hours of treatment Hsp70 is localised mainly in the nucleus of the cells with some cytoplasmic staining, and at 18 hours there is high levels of staining in both the cytoplasm and the nucleus. Intensity analysis (Fig 51) revealed that there is a statistically significant increase in Hsp70 after 6 hours of treatment ($p<0.001$).

Western blots were carried out on the cell lysate and cell supernatant of the treated cells. This revealed that once again high levels of Hsp70 were present in the cell lysate after 3 hours as indicated by the bands on the blot (Fig 52). Analysis of the cell supernatant showed that there were high increases in the level of Hsp70 after half an hour of treatment (Fig 53).

4.3.3 Treatment with PM10 over 18 hour period.

Cells were treated with 80μg/ml PM10 from Wolverhampton for various time points before immunofluorescent staining for Hsp70. The results (Fig 54) show that Hsp70 was expressed in the cells in a time-dependent manner. At the 6 and 18 hour time points there appears to be Hsp70 present in the nucleus of the cells; however, the staining pattern is continuous and not the punctuated appearance that the CB and ufCB treated cells have. This may be due to a later stage of nuclear localisation than the CB and ufCB particles or possibly PM10 is inducing Hsp70 nuclear localisation by a different mechanism. Average immunofluorescent intensities were calculated (Fig 55) and show that Hsp70 levels are statistically significant after 3 hours ($p<0.05$) and also at 6 and 18 hours ($p<0.001$).

Western blots were carried out on the cells lysates and cell supernatants. Cell lysate Western blots show that Hsp70 is increased with time in particular at the 18 hour time point (Fig 56). The cell supernatants show that there is some secretion of Hsp70 from the untreated cells with a slight increase with time of treatment (Fig 57), high levels in the untreated cells may be due to some stressing of the cells prior to treatment.

4.3.4 Treatment with $FeCl_3$ over 18 hour period.

Cells were treated with 100μM $FeCl_3$ over the 18 hour time period. Immunofluorescence staining revealed that there was some Hsp70 staining in the untreated cells and that this was increased during the first three hours of treatment (Fig 58). The average immunofluorescent intensities of the cells revealed that the level of Hsp70 in the cells peaks at 3 hours with statistical significance ($p<0.01$), before decreasing (Fig 59). This may be the result of Hsp70 preventing any cellular damage and reducing transcription events. However, a mild stress response may have

occurred when the cells were in contact with the iron solution and this could have been quickly resolved.

Western blots were carried out on the cell lysates and cell supernatants of the cells. The cell lysate Western blots revealed that Hsp70 was present at high levels in the cells at all time points and that little increase was seen over the already high level in the untreated cells (Fig 60). Once again it would appear that the cells are stressed before the treatment. This may be due to stressing of the cells during the lysis procedure. Western blots carried out for the cell supernatants reveals that small amounts of Hsp70 were being secreted from the cells before and after treatment (Fig 61). There was little increase in the levels of Hsp70 being secreted before or during treatment. This strengthens the evidence that the cells may have been stressed during the lysis procedure since the cell supernatant was removed prior to lysis and little secreted Hsp70 was present indicating the stress occurred after the supernatant was removed.

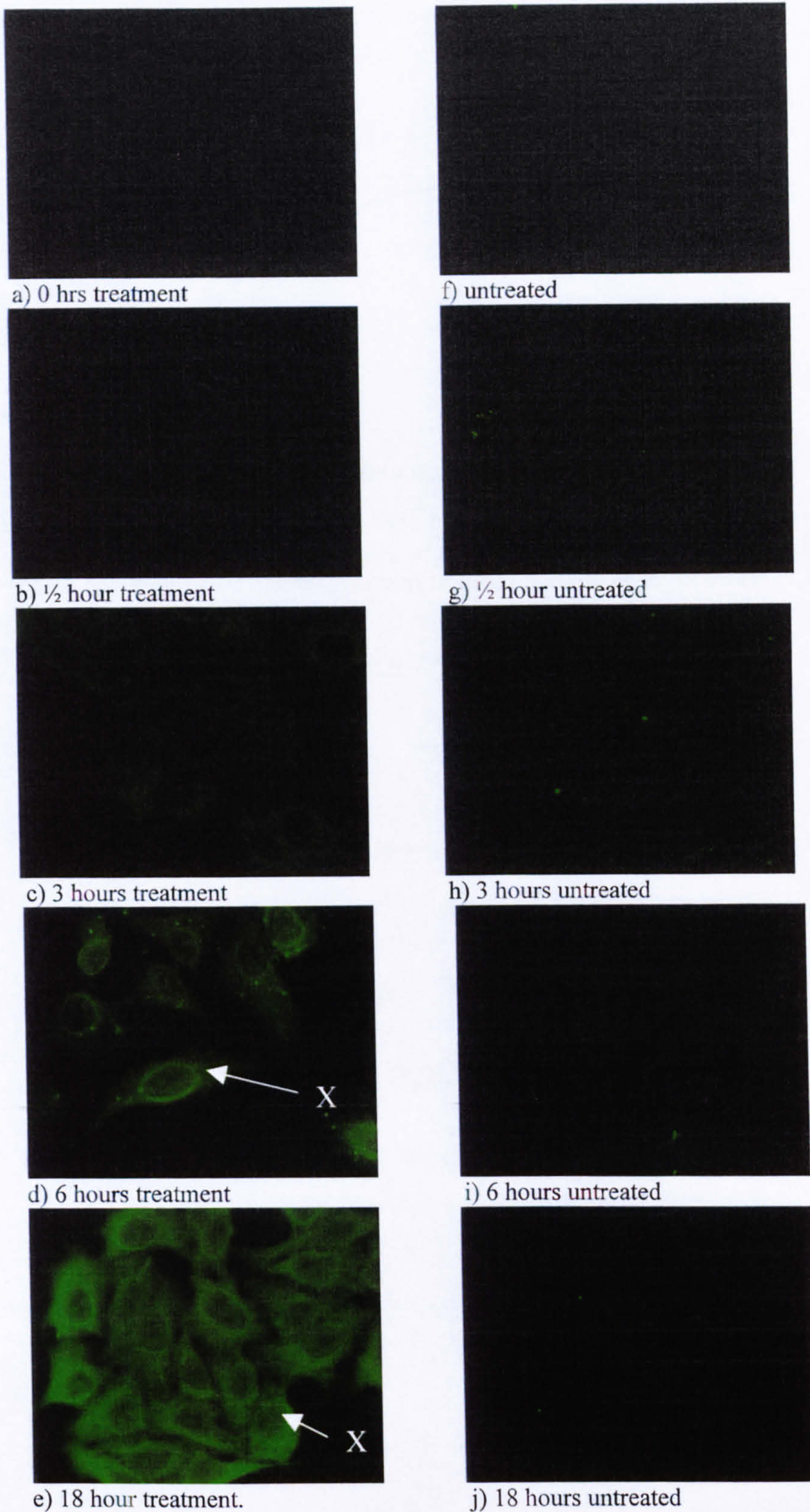


Figure 46: Expression of Hsp70 in A549 cells treated with 100µg/ml CB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear and peri-nuclear localisation. For controls see Appendix I.

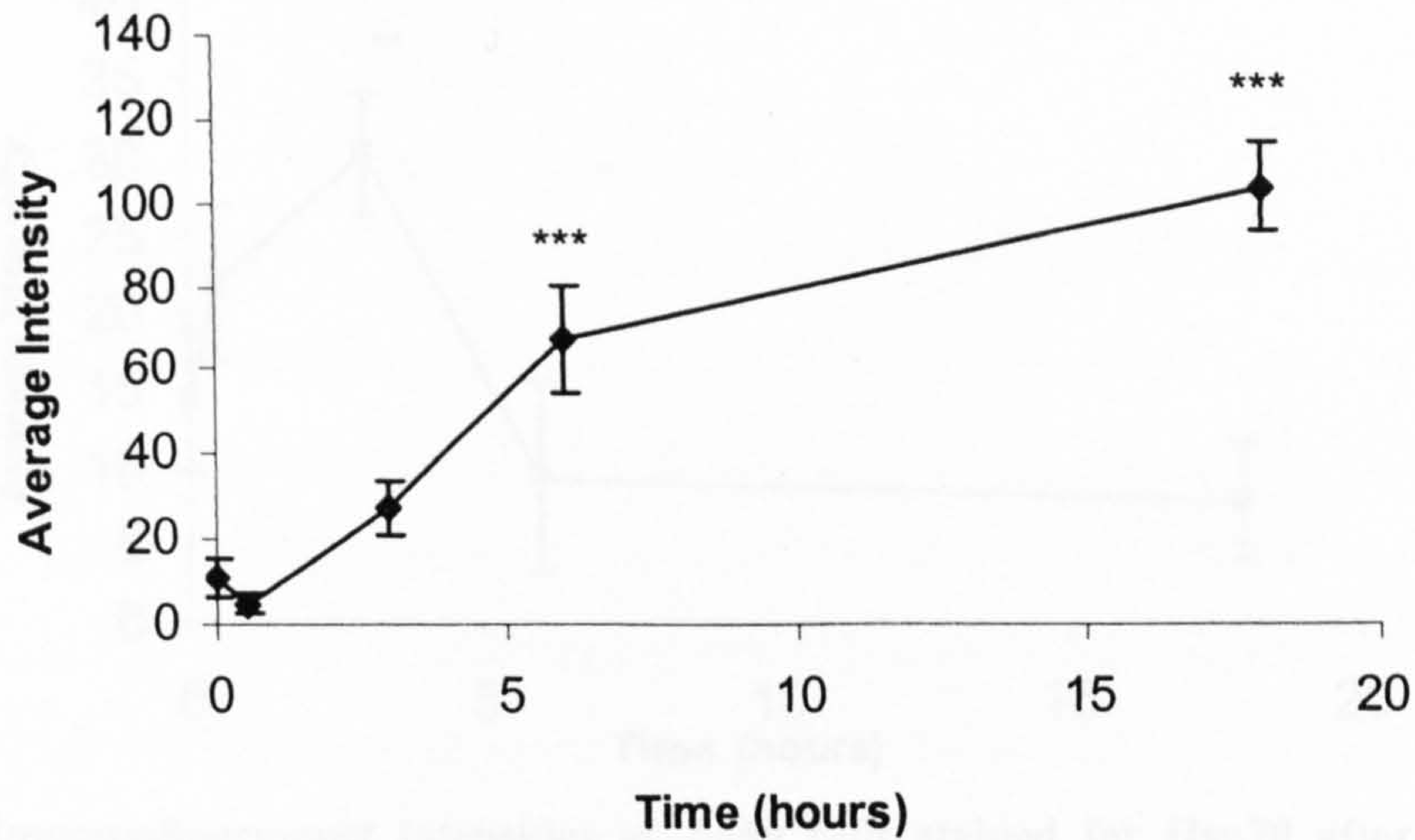


Figure 47: Immunofluorescent intensities of A459 cells stained for Hsp70 after 18 hours of 100 μg/ml CB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denotes significant changes from untreated (control) cells, p< 0.001

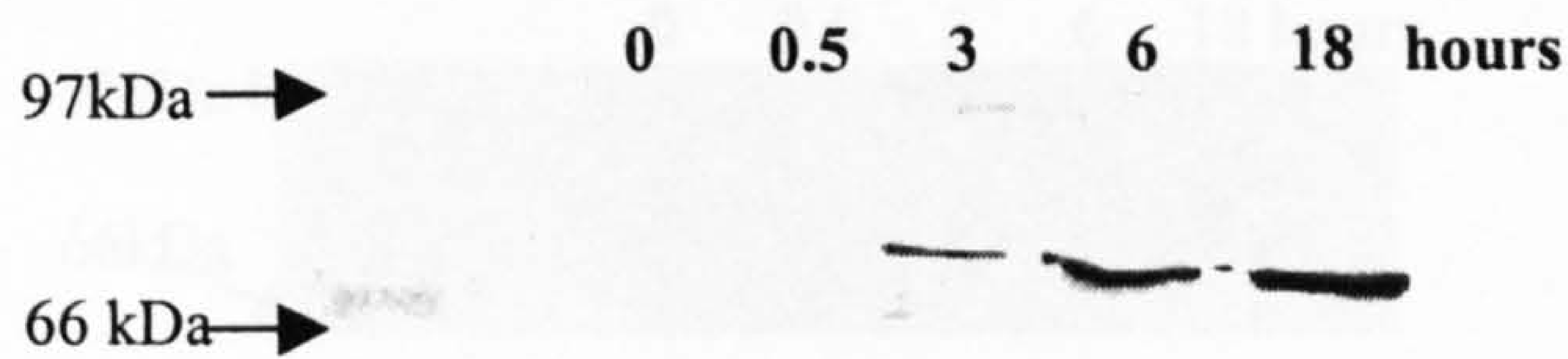


Figure 48: Level of Hsp70 expression in cell lysate measured by Western blot after 18 hours of 100μg/ml CB treatment.

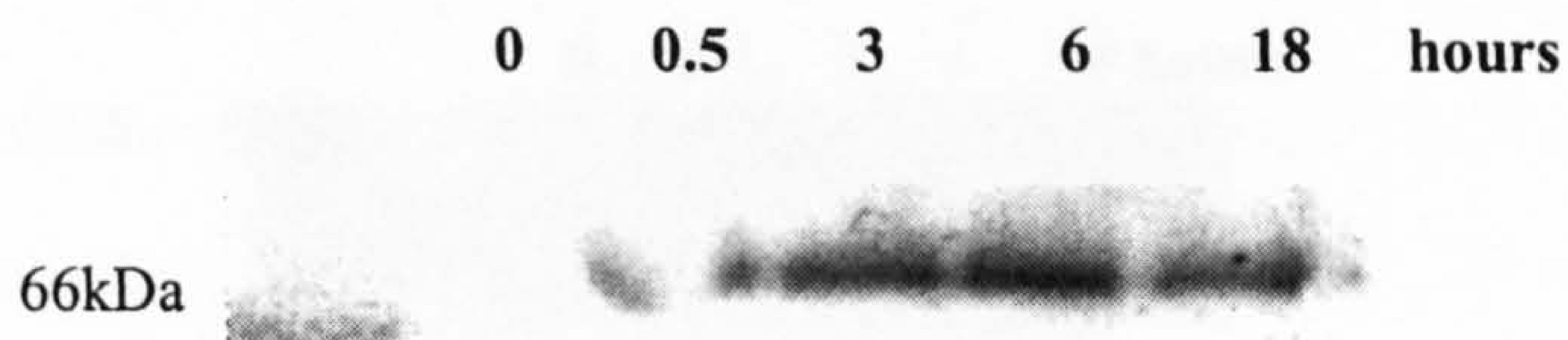


Figure 49: Level of Hsp70 expression in cell supernatant measured by Western blot after 18 hours of 100μg/ml CB treatment.

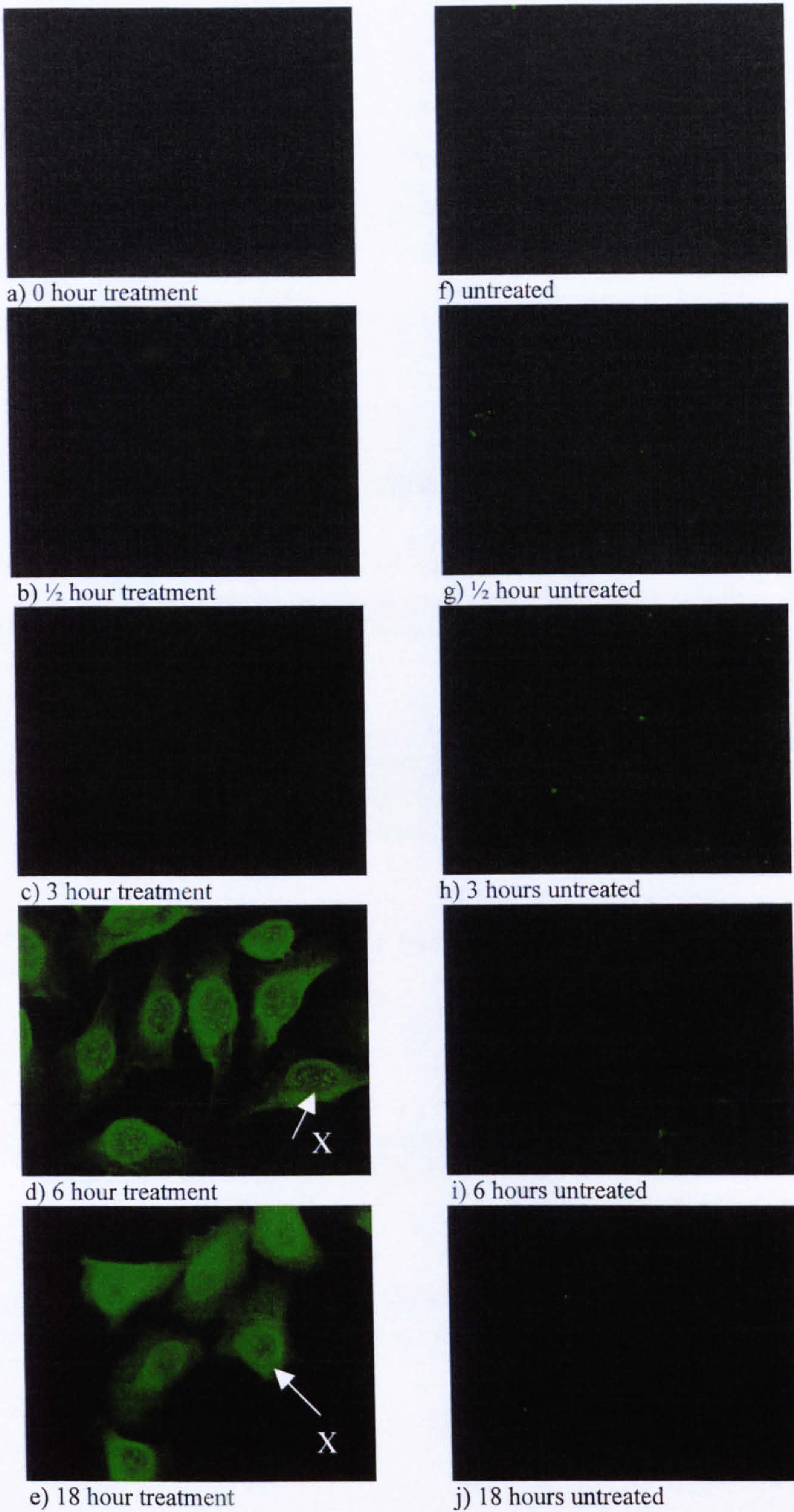


Figure 50: Expression of Hsp70 in A549 cells treated with 100μg/ml ufCB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation. For controls see Appendix I.

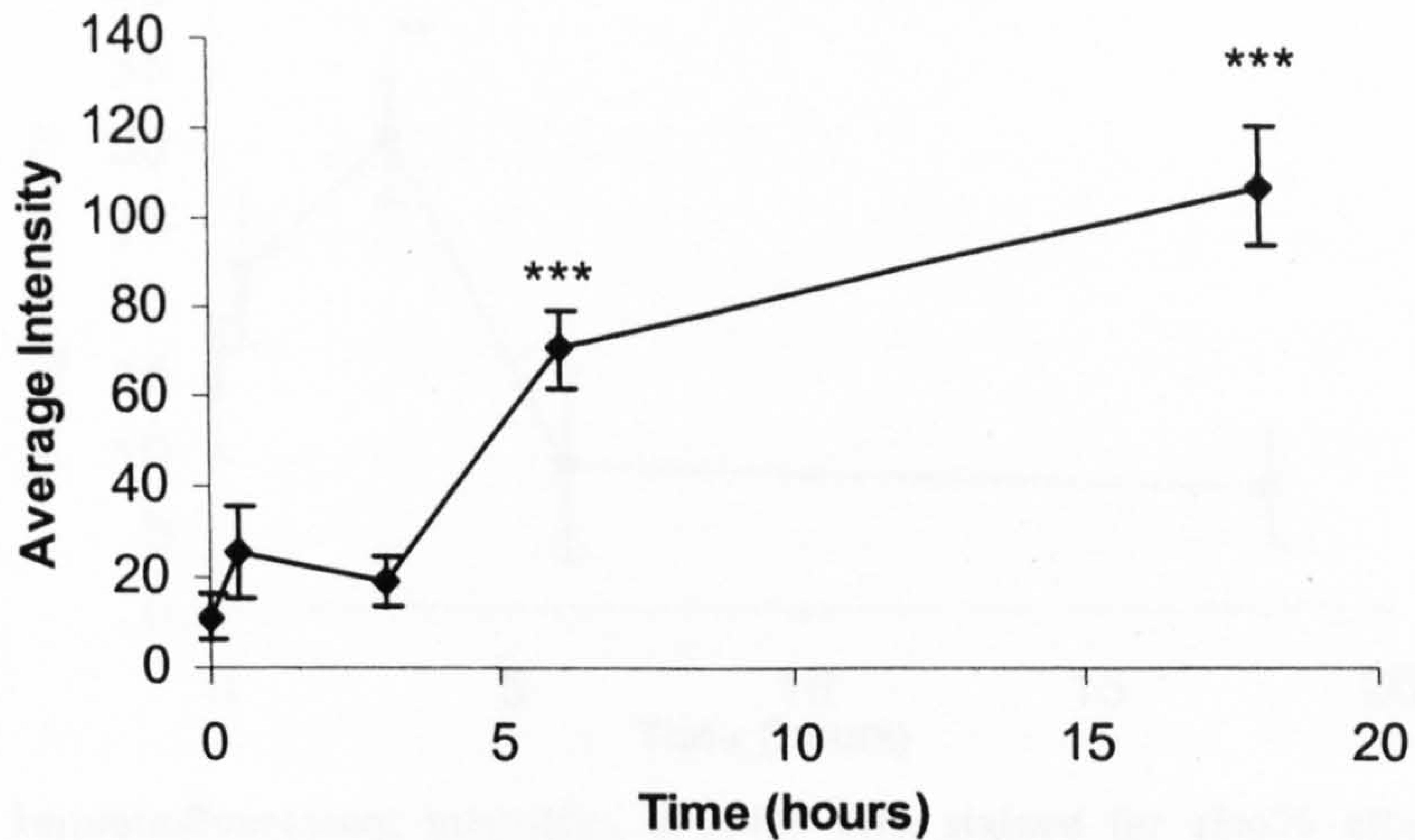


Figure 51: Immunofluorescent intensities of A459 cells stained for Hsp70 after 18 hours of 100µg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denotes significant changes from untreated (control) cells, p< 0.001

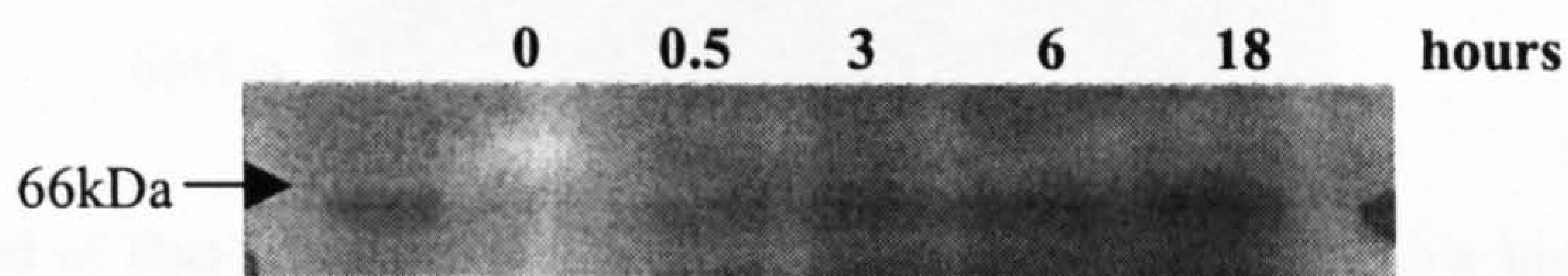


Figure 52: Level of Hsp70 expression in cell lysate measured by Western blot after 18 hours of 100µg/ml ufCB treatment.

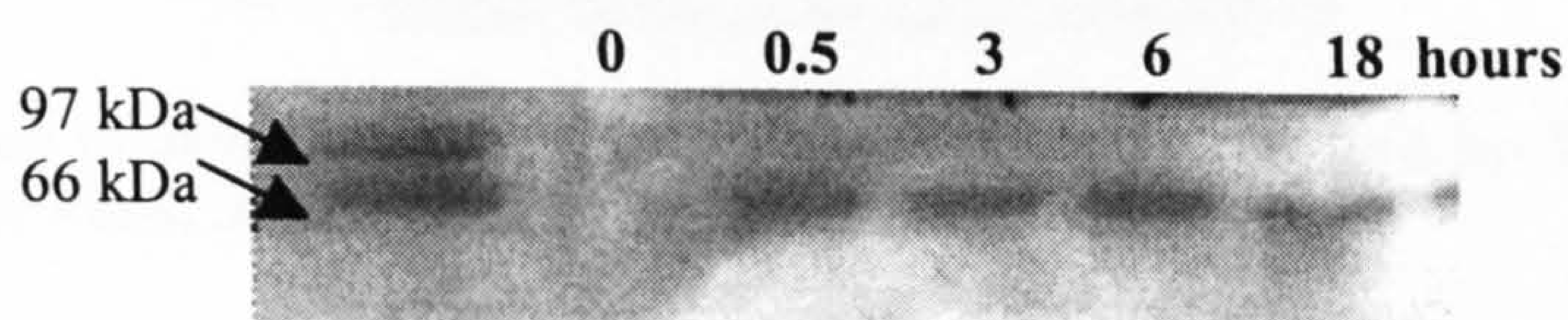


Figure 53: Level of Hsp70 expression in cell supernatant measured by Western blot after 18 hours of 100µg/ml ufCB treatment.

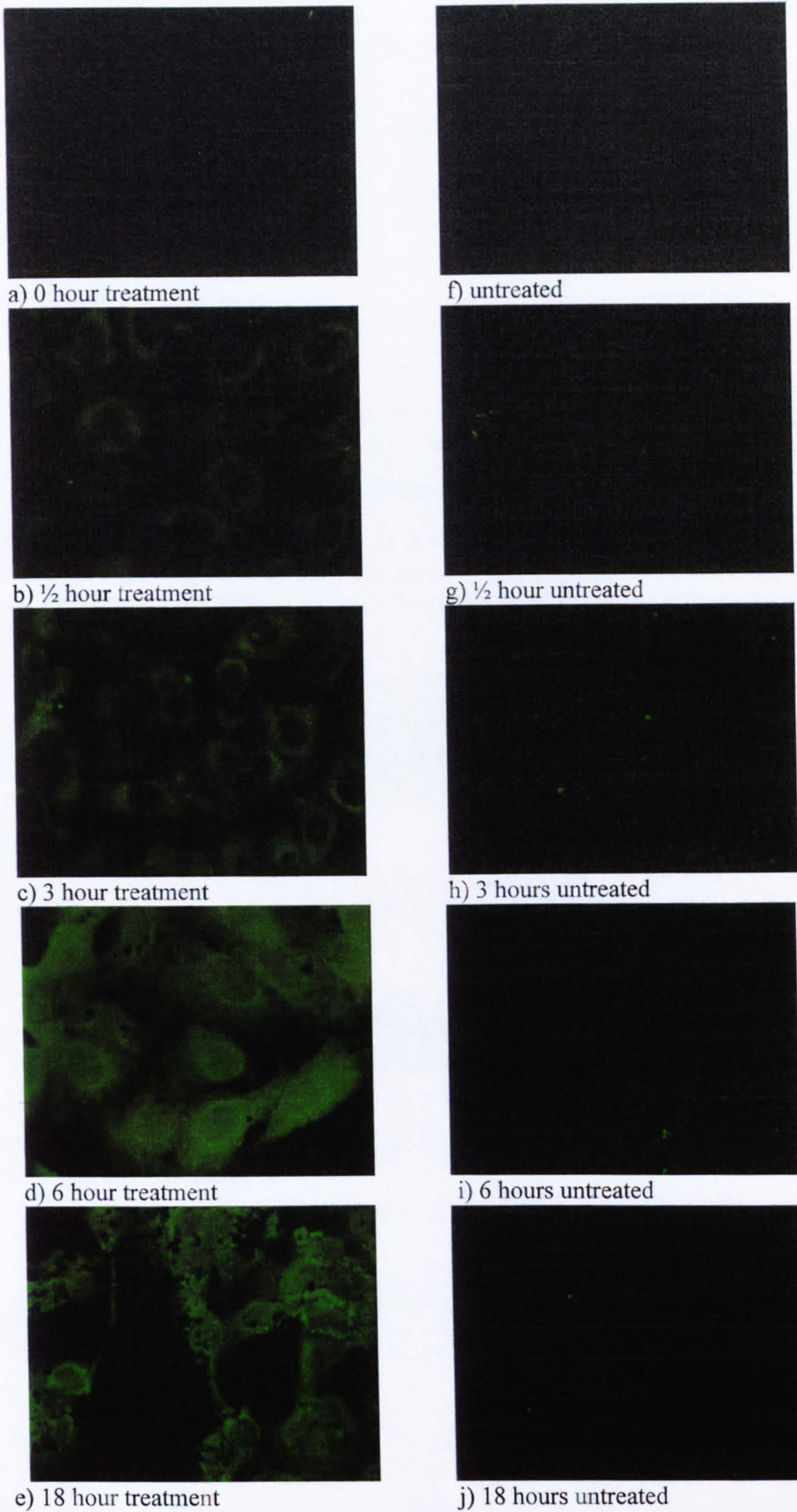


Figure 54: Expression of Hsp70 in A549 cells treated with 80μg/ml PM10, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

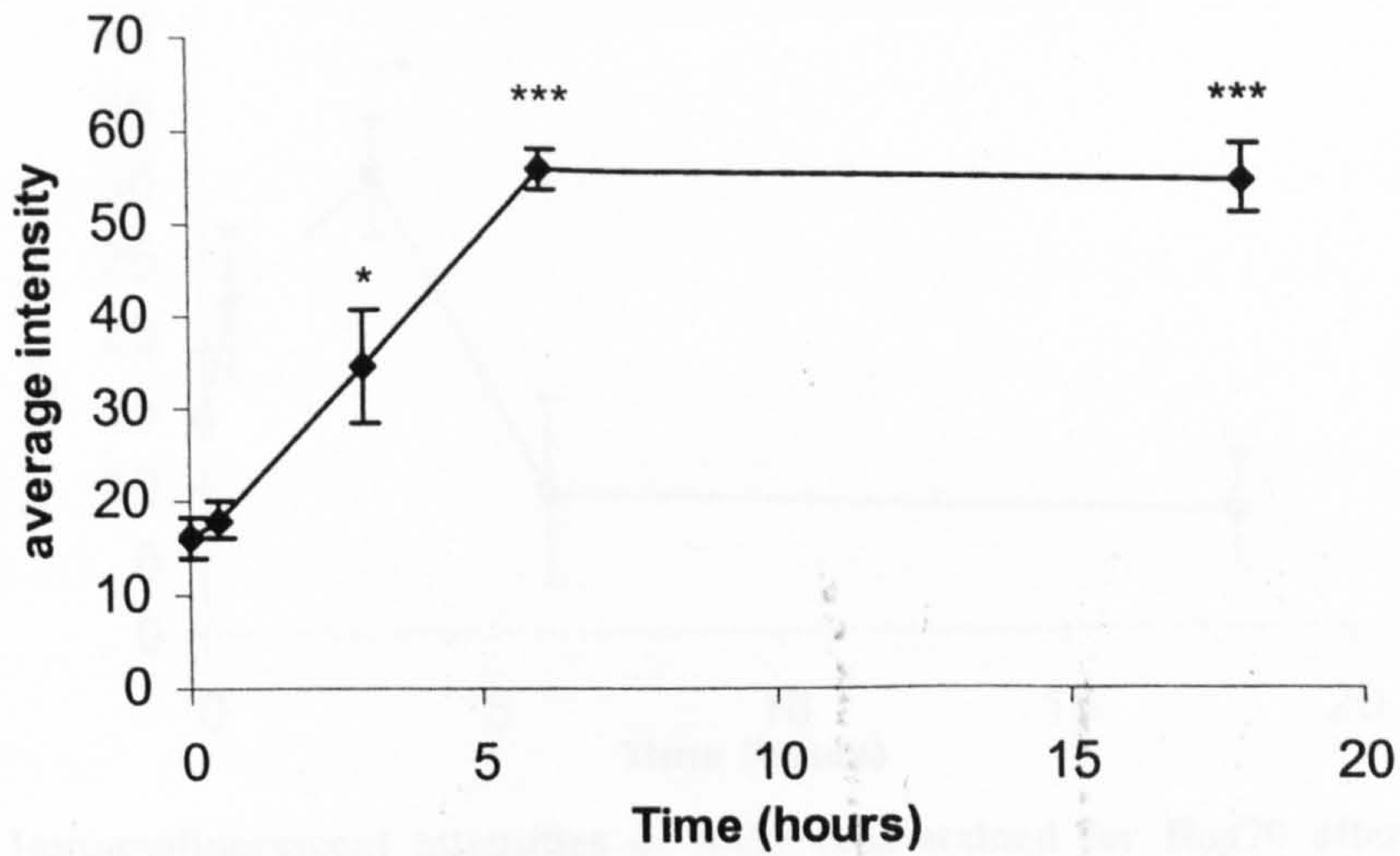


Figure 55: Immunofluorescent intensities of A459 cells stained for Hsp70 after 18 hours of 80μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (*, *) denotes significant changes from untreated (control) cells, p<0.05, p< 0.001.**

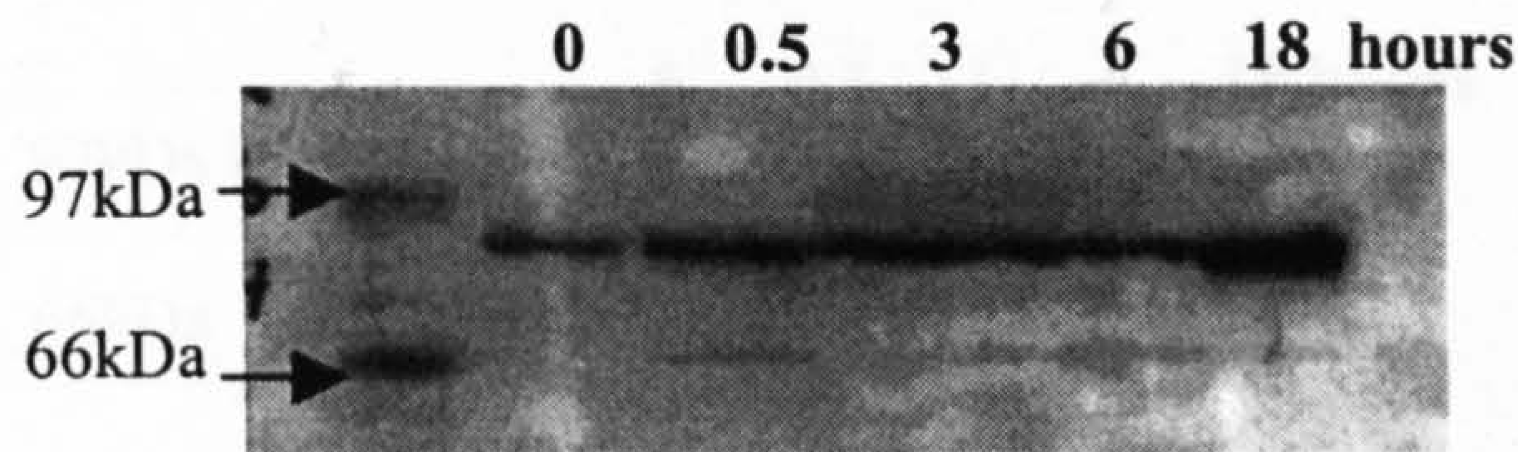


Figure 56: Level of Hsp70 expression in cell lysate measured by Western blot after 18 hours of 80μg/ml PM10 treatment.

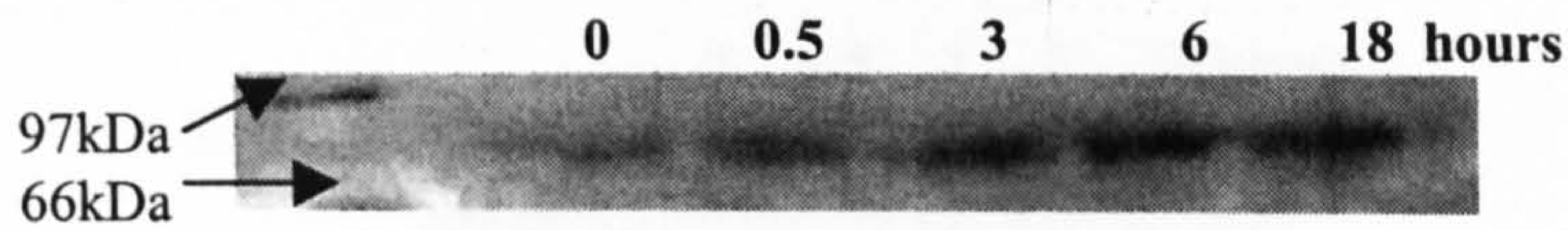


Figure 57: Level of Hsp70 expression in cell supernatant measured by Western blot after 18 hours of 80μg/ml PM10 treatment

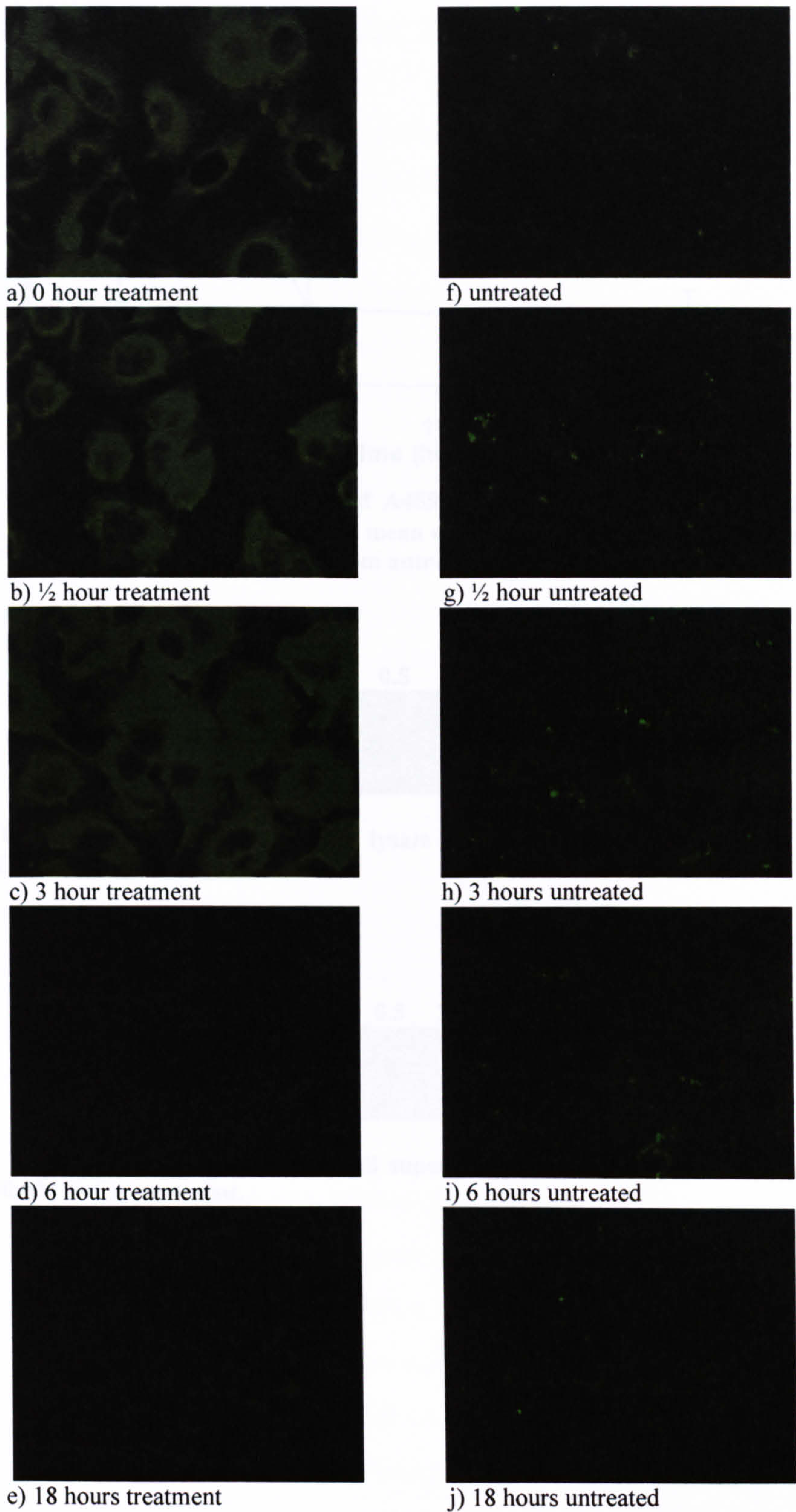


Figure 58: Expression of Hsp70 in A549 cells treated with 100mM $FeCl_3$, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

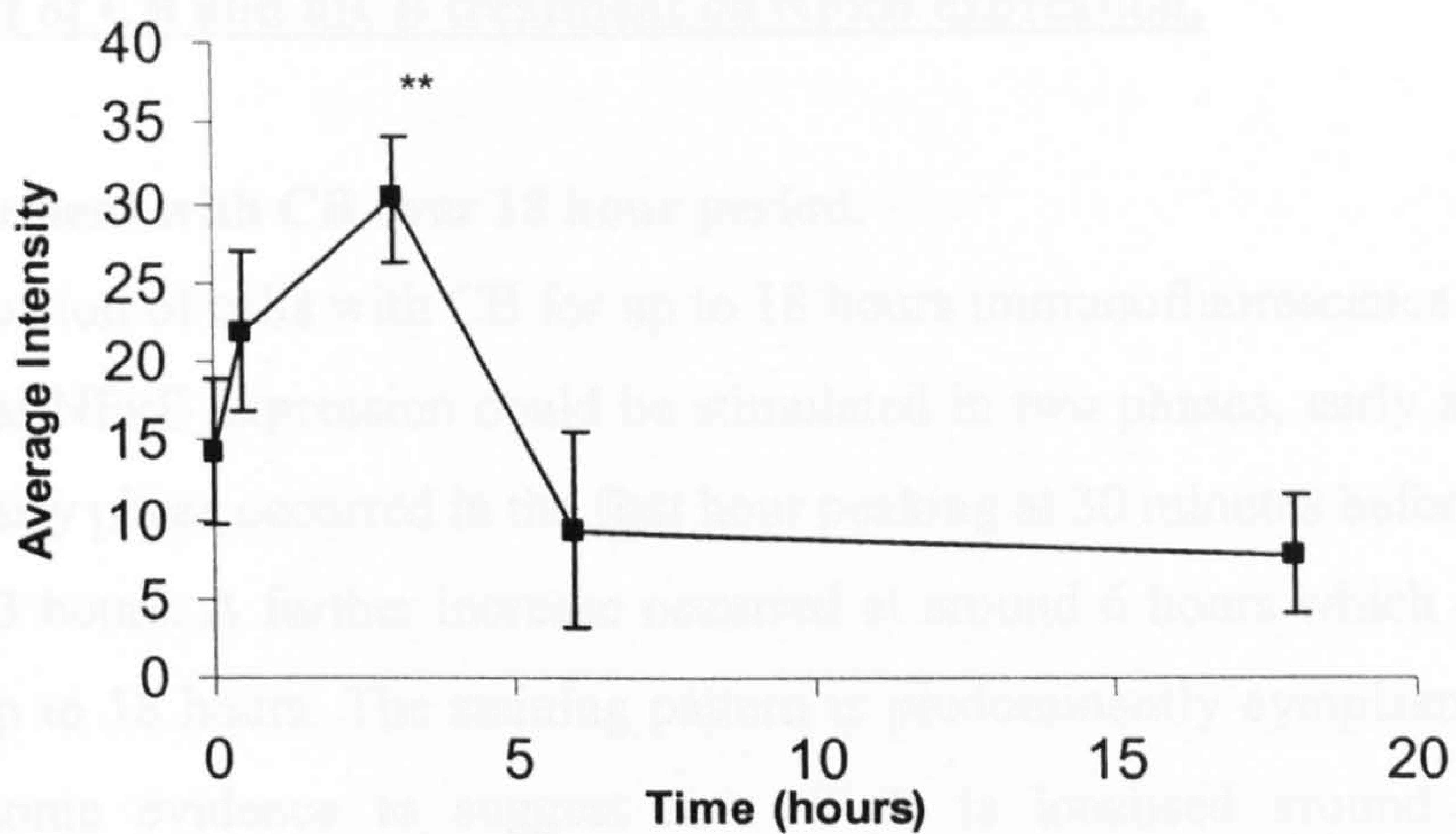


Figure 59: Immunofluorescent intensities of A459 cells stained for Hsp70 after 18 hours of 100mM $FeCl_3$ treatment. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (**) denotes significant changes from untreated (control) cells, p< 0.01.

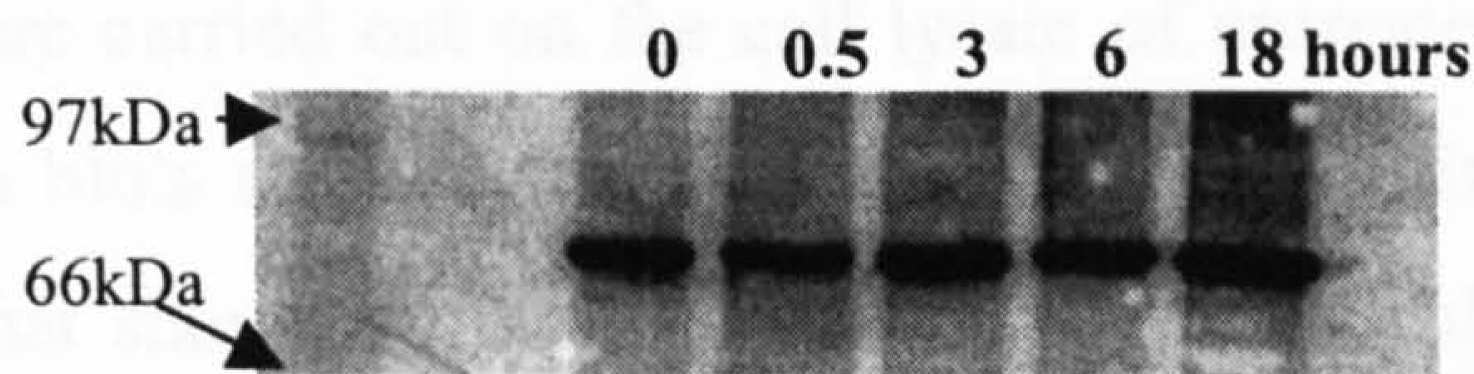


Figure 60: Level of Hsp70 expression in cell lysate measured by Western blot after 18 hours of 100mM $FeCl_3$ treatment.

Figure 61: Level of Hsp70 expression in cell supernatant measured by Western blot after 18 hours of 100mM $FeCl_3$ treatment.

4.4.0 Effect of CB and ufCB treatment on NFκB expression.

4.4.1 Treatment with CB over 18 hour period.

After incubation of cells with CB for up to 18 hours immunofluorescence microscopy showed that NFκB expression could be stimulated in two phases, early and late (Fig 62). The early phase occurred in the first hour peaking at 30 minutes before a decrease at around 3 hours. A further increase occurred at around 6 hours which continues to increase up to 18 hours. The staining pattern is predominantly cytoplasmic although there is some evidence to suggest that NFκB is localised around the nuclear membrane at 18 hours. Immunofluorescent intensities were analysed and showed that there were indeed two phases of expression of NFκB (Fig 63).

Western blots were carried out on the cell lysate of untreated and treated cells (Fig 64). The Western blots showed that there was an increase in NFκB within the first hour similar to that shown by the immunofluorescence results before returning to a level similar to the untreated cells. However unlike the immunofluorescence data there was no increase detected after the initial rise and fall.

4.4.2 Treatment with ufCB over 18 hour period.

Cells were treated for up to 18 hours with 100μg/ml ufCB prior to immunofluorescent staining. Photographs taken reveal that NFκB was activated in the first 30 minutes (Fig 65) causing nuclear localisation of NFκB which persists for the first hour before the levels of NFκB are reduced by 3 hours of treatment. At the 6 and 18 hour time points the levels of NFκB are increased again and there appears to be mainly cytoplasmic staining with some localisation around the nuclear membrane. Intensity analysis (Fig 66) showed that there were peaks at ½ and at 6-18 hours of treatment with a decrease between 1 and 4 hours of treatment, however these could be a result of variation in the data since neither of these peaks are statistically significant.

Western blots were carried out for the cell lysate of untreated and treated cells (Fig 67). These indicate that the levels of NFκB within the cells vary over the time course with a reduction within the first hour, followed by and increase at 2 hours, a decrease at 4 hours followed by and increase at 6 and 18 hours.

4.4.3 Treatment with PM10 over 18 hour period.

Cells were treated for up to 18 hours with 80μg/ml PM10 from Wolverhampton. Immunofluorescence microscopy showed that NFκB was present in the untreated cells in the cytoplasm and by 30 minutes of PM10 treatment NFκB had entered the nucleus of the cells (Fig 68). The nuclear localisation of NFκB persisted until 2 hours before being detectable only in the cytoplasm at 3 hours of treatment. After 3 hours the levels of NFκB would appear to decrease back to the levels of the untreated cells by 18 hours. Average immunofluorescence intensities show that there is a decrease in the intensity at the 30 minute time point this is due to the nuclear localisation of NFκB when little protein is found in the cytoplasm of the cells (Fig 69). Cytoplasmic staining tends to give high intensities whereas nuclear staining tends to be punctated which leads to underestimation of the intensity values. Once more cytoplasmic staining is present at 1 hour, the intensities increase up to about 3 hours until decreasing at 6 and 18 hours when the NFκB levels return to the untreated levels.

Western blots were carried out on the cell lysates of the cells (Fig 70). This shows that there are higher levels of NFκB in the cell at 30 minutes and 4 hours. The higher level at 30 minutes is likely to be due to increased availability of NFκB binding sites for antibody binding when NFκB isn't bound to IκB. At 4 hours the high levels are more likely to be the result of high cytoplasmic levels of NFκB before returning to the untreated level of NFκB at 6 hours.

4.4.4 Treatment with $FeCl_3$ over 4 hour period.

Cells were treated with $FeCl_3$ over the 4 hour time period. This time period was chosen instead of the 18 hour time course since most effects on NFκB occur early in the time series of the other treatments and since $FeCl_3$ is soluble in the culture medium it might be expected to have a quicker effect on the cells than non-soluble, particulate, treatments. Immunofluorescence staining revealed that there was NFκB staining in the cytoplasm of the untreated cells as expected; however, the levels remained fairly constant over the four hour time course, with no nuclear localisation occurring, suggesting that no activation of NFκB occurred (Fig 71). The average immunofluorescence intensities of the cells revealed that the level of NFκB increased

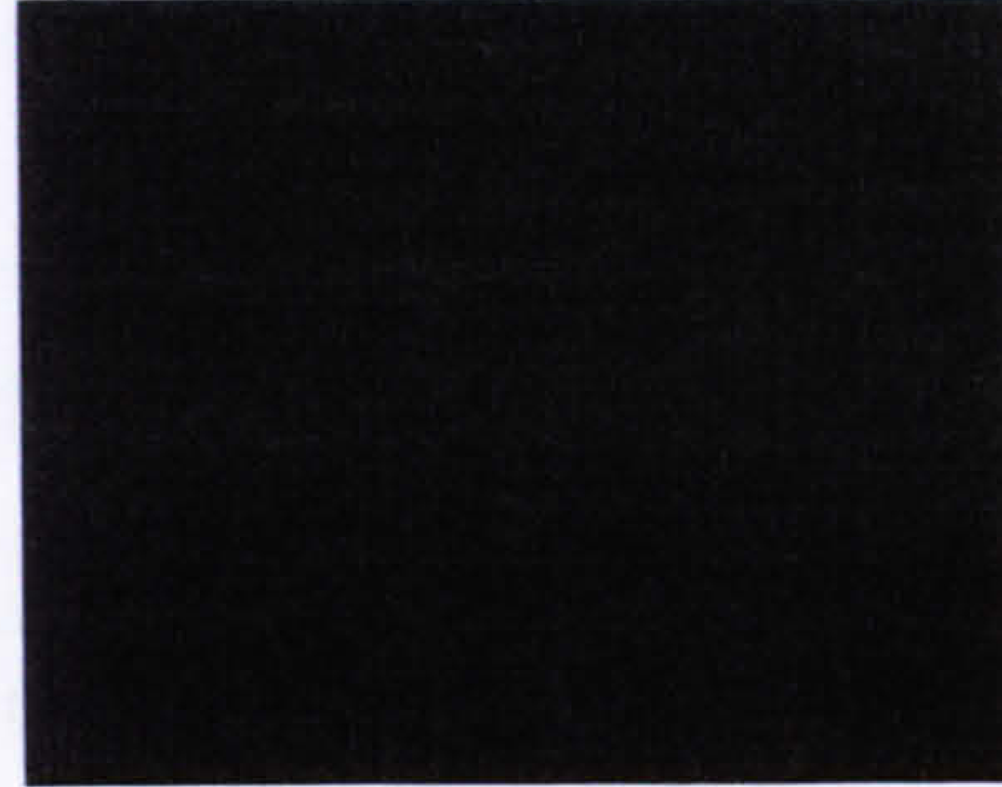
after ½ an hour of treatment which it may be the result of a minor stress response or may be a normal factor in the cell cycle (Fig 72).

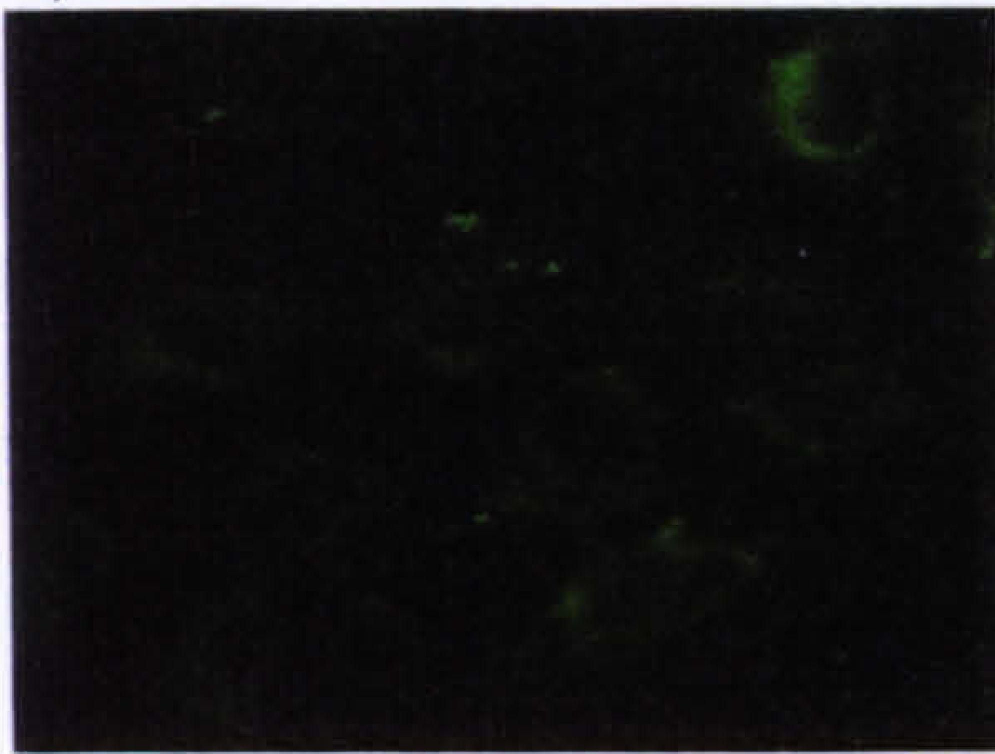
Western blots were carried out on the cells lysates of the cells over 18 hours of treatment. The cell lysate Western blot revealed that NFκB was present at high levels in the cells at all time points and that little increase was seen with the exception of the 6 hour time point where no NFκB was present (Fig 73). This may be the result of NFκB entering the nucleus at this time point, but it seems to occur at a later time point than expected and no evidence of this was seen in preliminary experiments to identify the best time series.

a) untreated

b) ½ hour

c) 1 hour


d) 3 hours


d) 6 hours

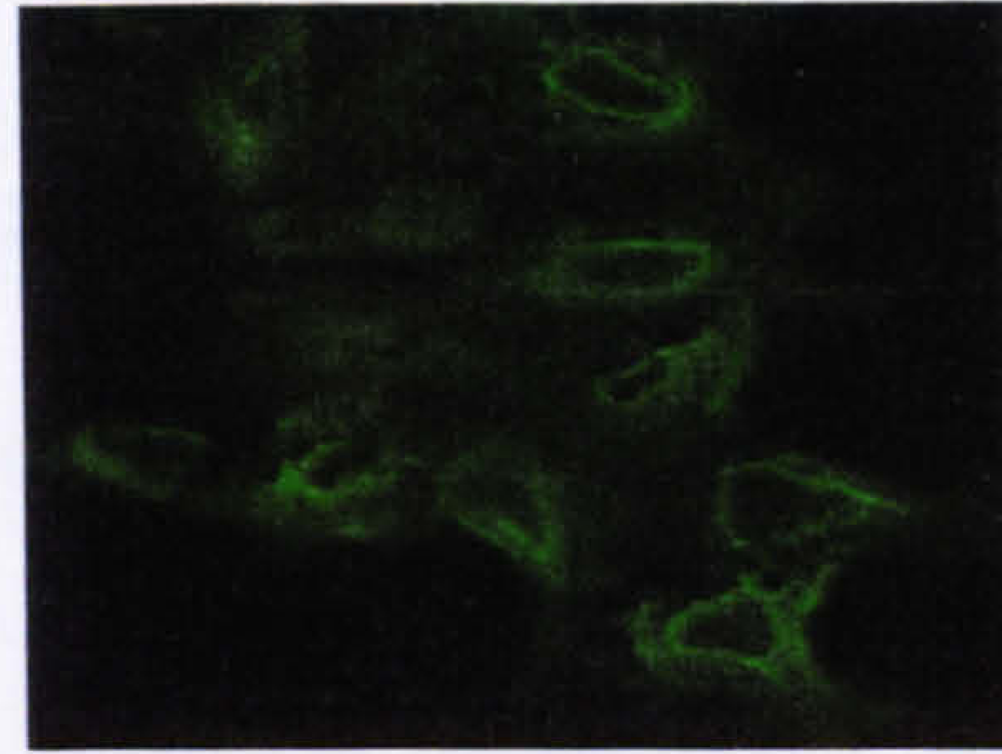

e) 18 hours

Figure 62: Expression of NFκB in A549 cells treated with 100μg/ml CB, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

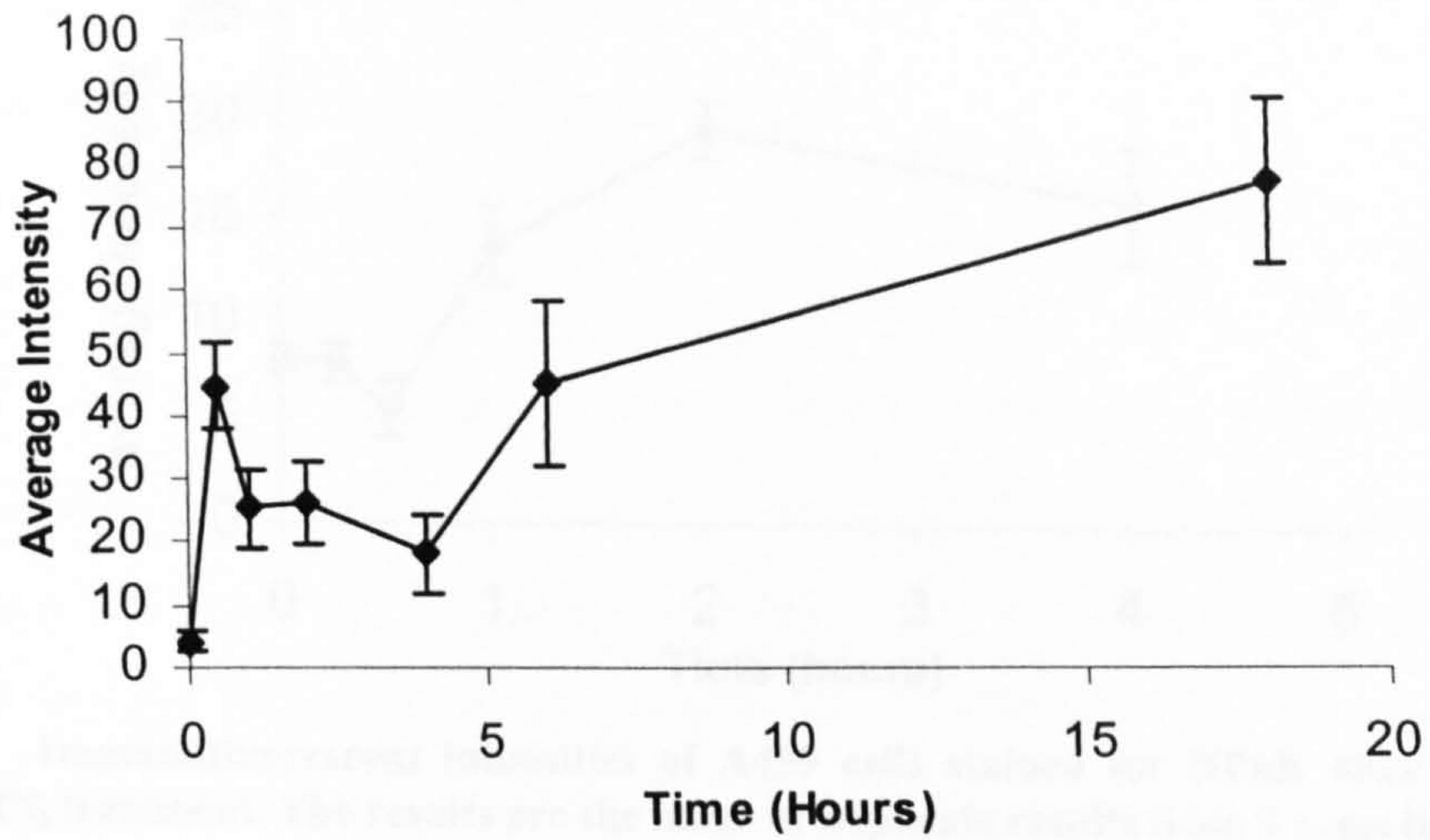


Figure 63: Immunofluorescent intensities of cells stained for NFκB (p65) after 18 hours of 100μg/ml CB treatment. The results are the mean of triplicate results from 3 experiments ± SE.

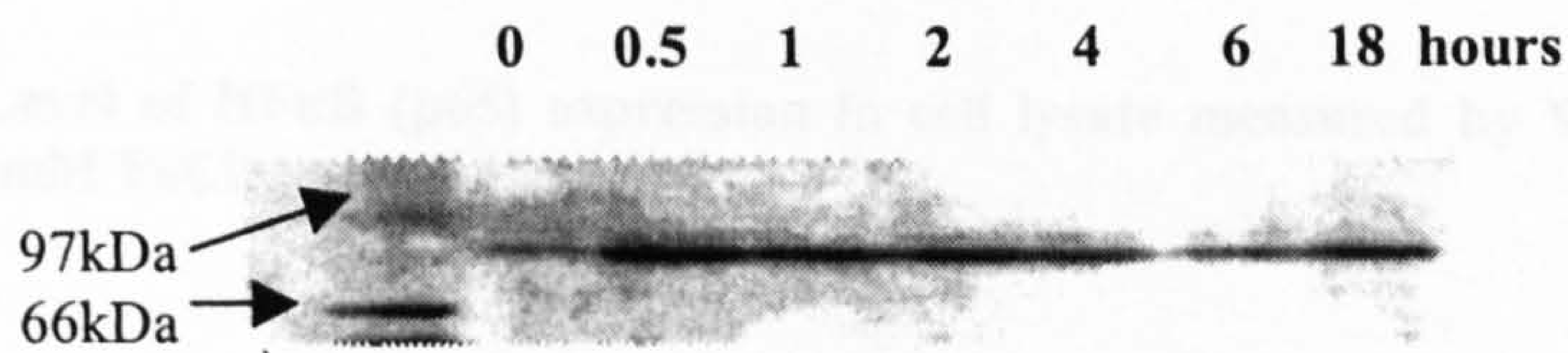


Figure 64: Level of NFκB (p65) expression in cell lysate measured by Western blot after 18 hours of 100μg/ml CB treatment.

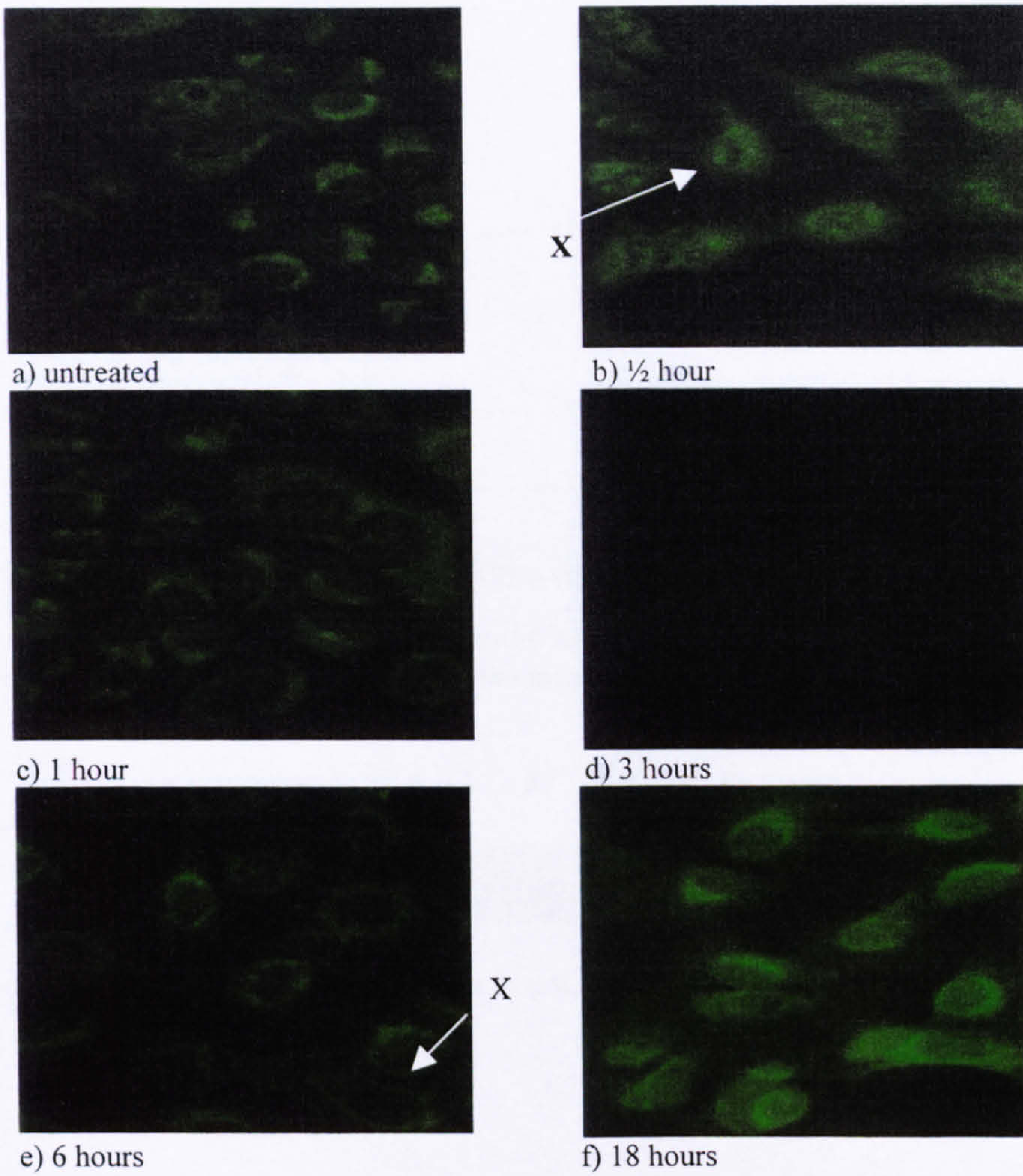


Figure 65: Expression of NFκB in A549 cells treated with 100μg/ml ufCB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation of NFκB. For controls see Appendix I.

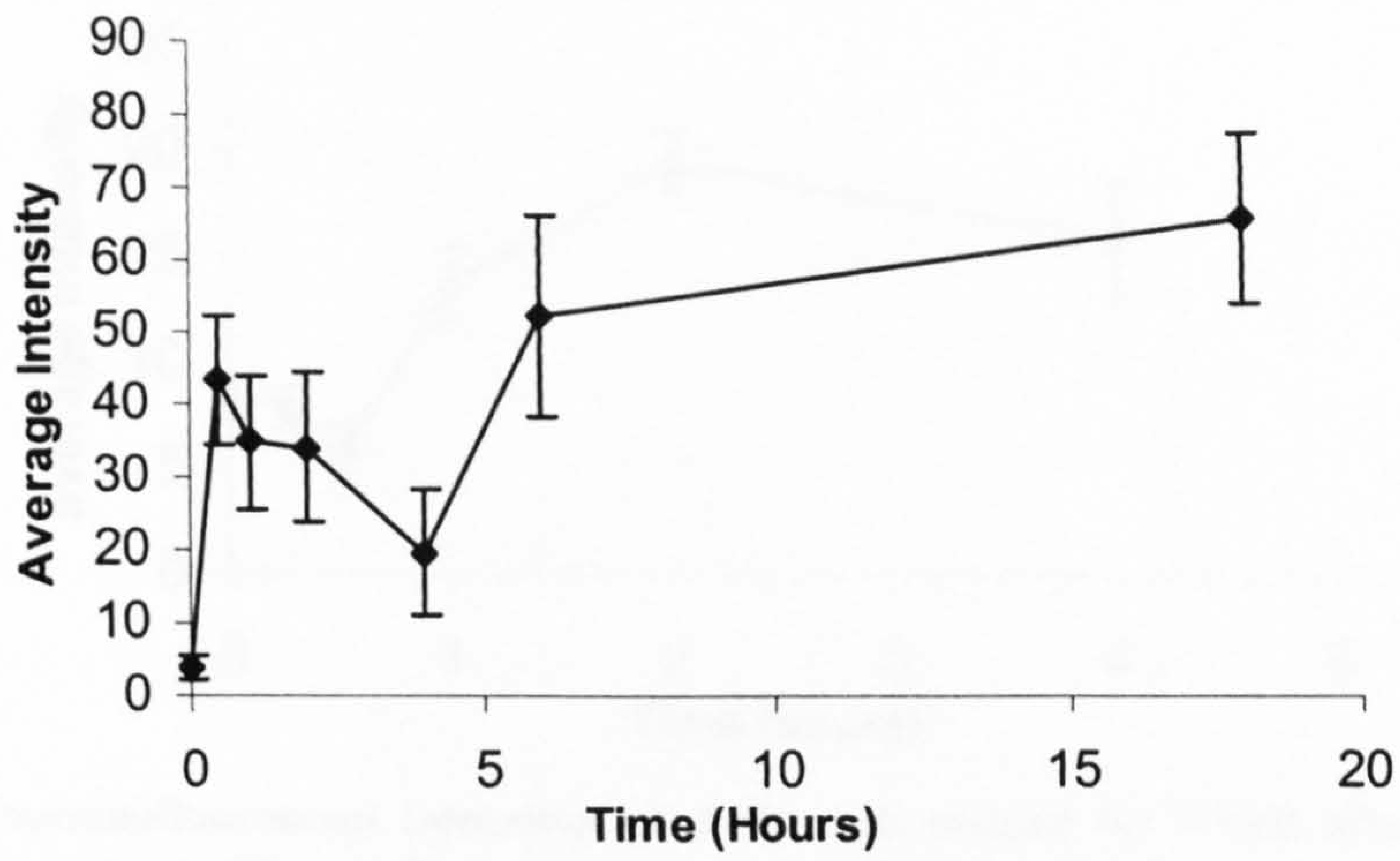


Figure 66: Immunofluorescent intensities of A459 cells stained for NFκB after 18 hours of 100μg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE.

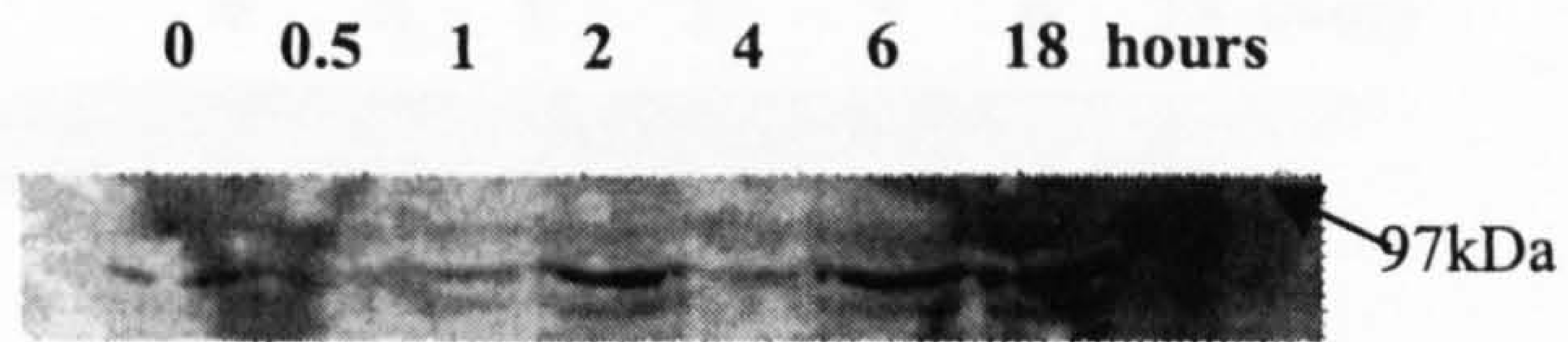


Figure 67: Level of NFκB (p65) expression in cell lysate measured by Western blot after 18 hours of 100 μg/ml ufCB treatment.

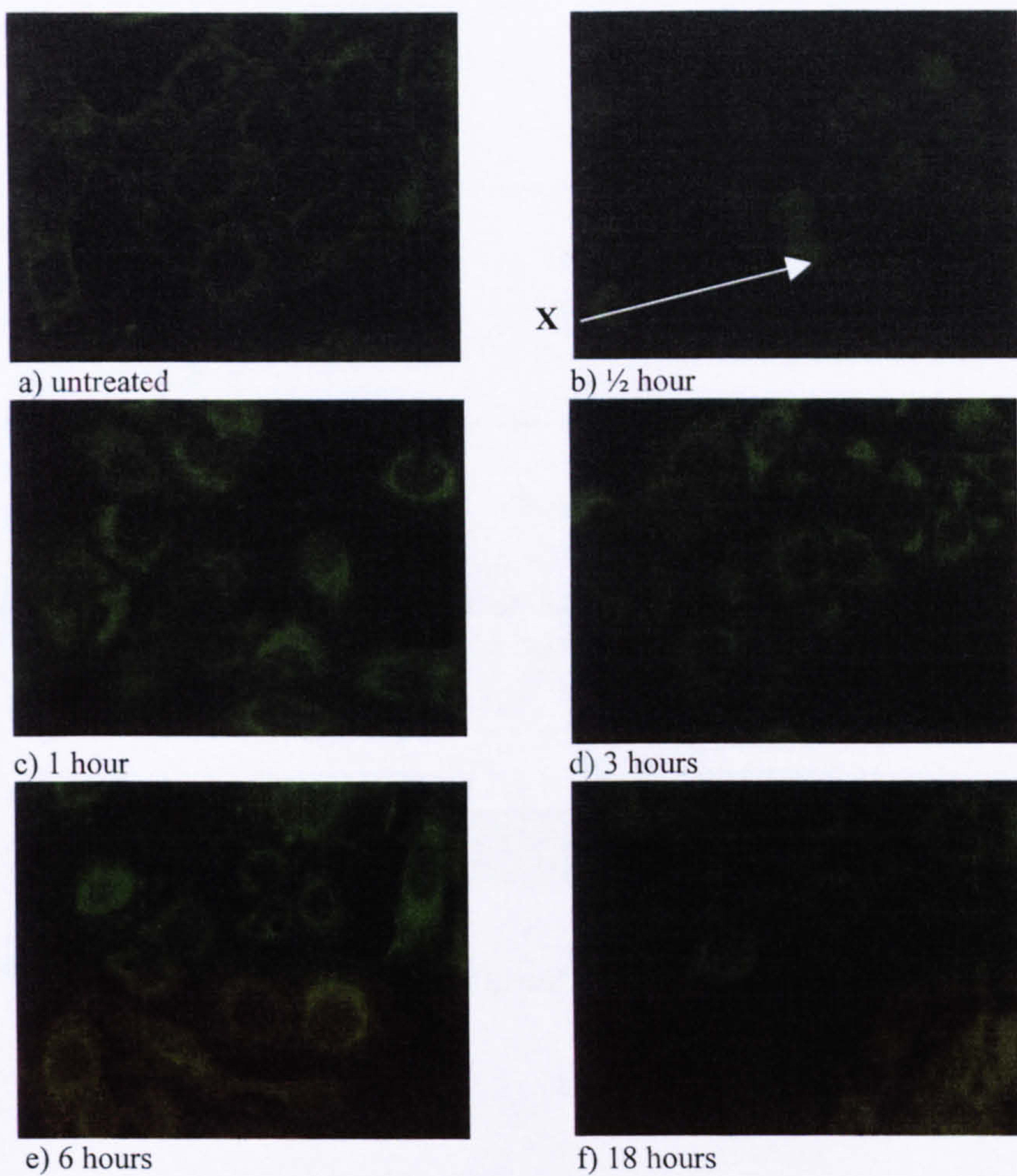


Figure 68: Expression of NFκB in A549 cells treated with 80μg/ml PM10, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation of NFκB. For controls see Appendix I.

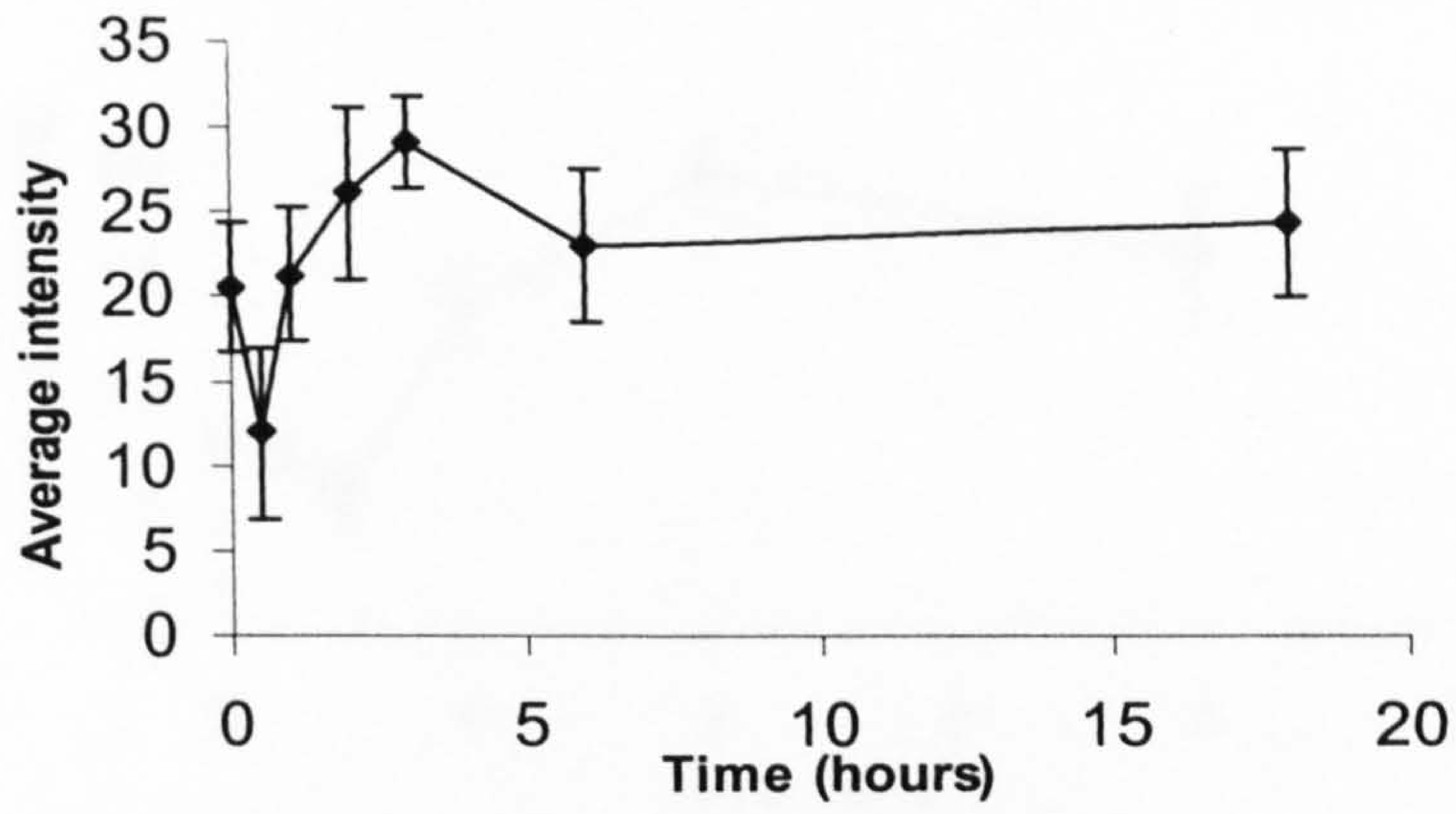


Figure 69: Immunofluorescent intensities of A459 cells stained for NFκB after 18 hours of 80μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE.

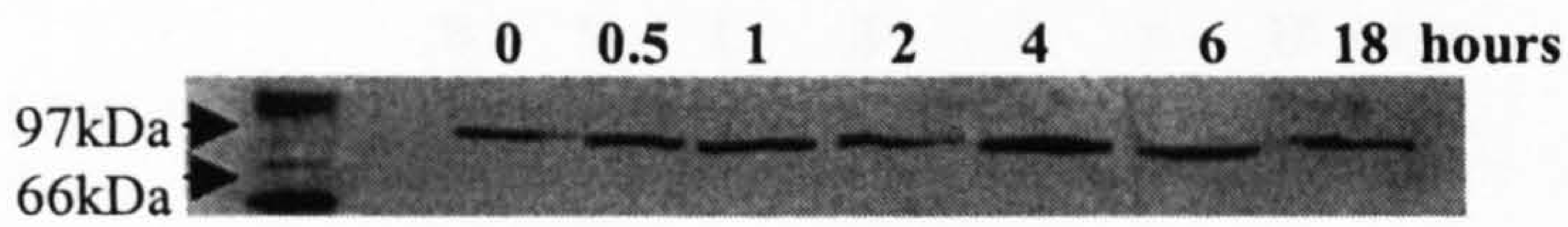
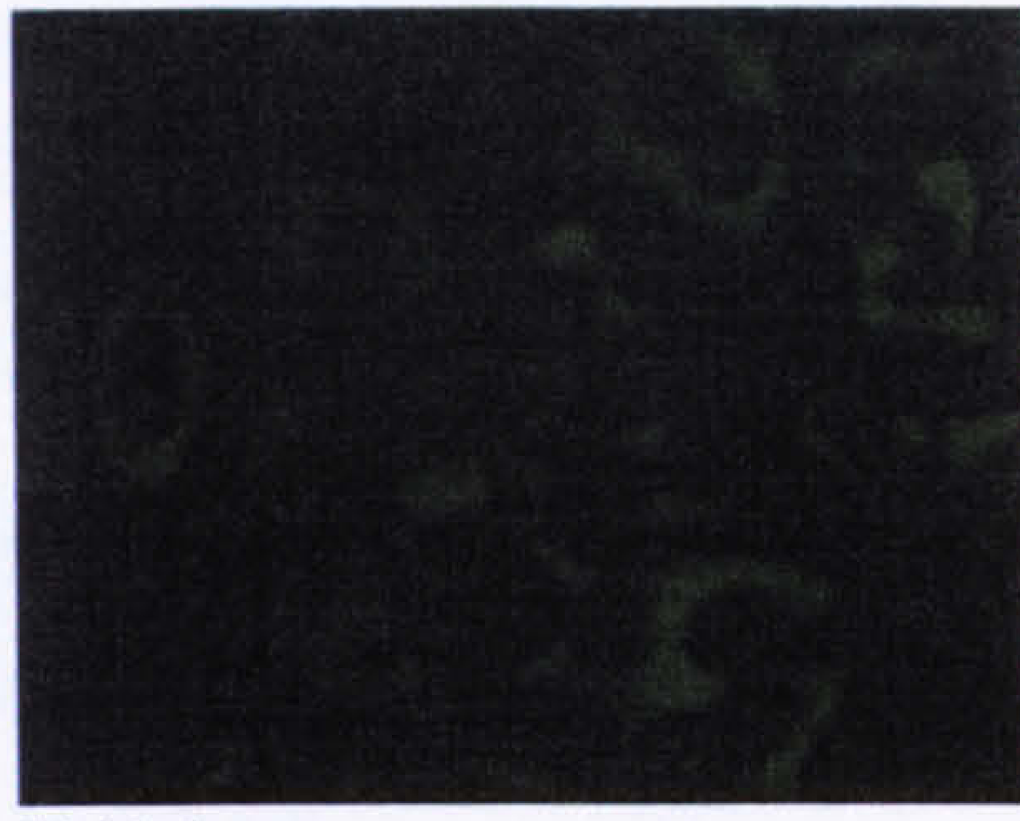


Figure 70: Level of NFκB (p65) expression in cell lysate measured by Western blot after 18 hours of 80μg/ml PM10 treatment.

a) untreated



b) ¼ hour

c) ½ hour

d) 1hour

e) 2 hour

f) 4 hour

Figure 71: Expression of NFκB in A549 cells treated with 100mM $FeCl_3$, for various time points up to 4 hours as shown by indirect immunofluorescence. For controls see Appendix I.

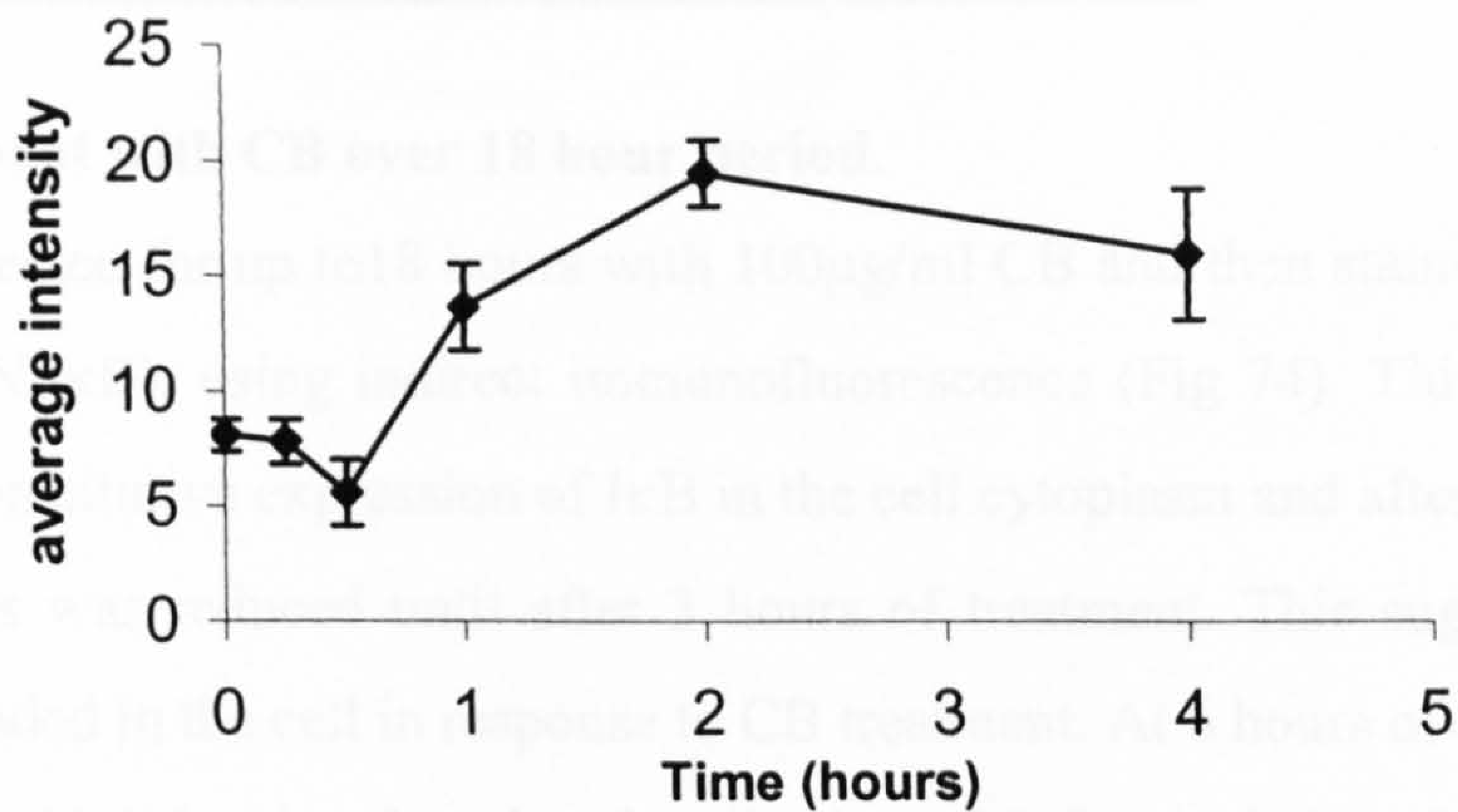


Figure 72: Immunofluorescent intensities of A459 cells stained for NFκB after 18 hours of 100mM $FeCl_3$ treatment. The results are the mean of triplicate results from 3 experiments ± SE.

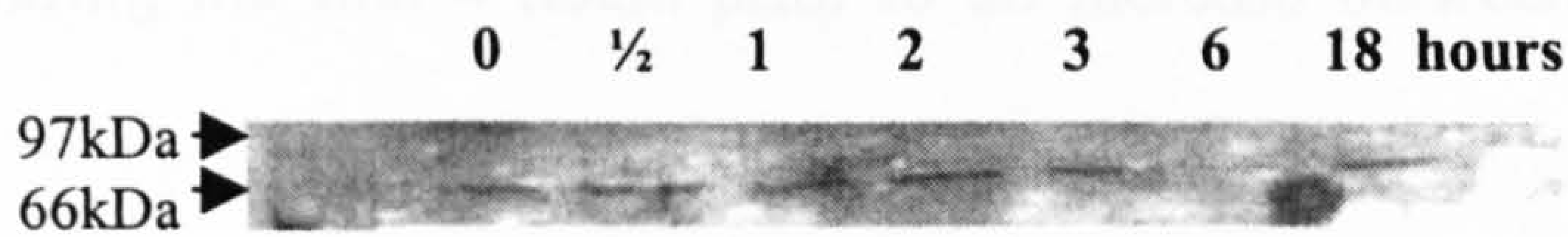


Figure 73: Level of NFκB (p65) expression in cell lysate measured by Western blot after 18 hours of 100mM $FeCl_3$ treatment.

4.5.0 Effect of CB and ufCB treatment on IκB expression.

4.5.1 Treatment with CB over 18 hour period.

Cells were treated for up to18 hours with 100μg/ml CB and then stained for IκB (the inhibitor of NFκB), using indirect immunofluorescence (Fig 74). This revealed that there were constitutive expression of IκB in the cell cytoplasm and after 30 minutes of treatment this was reduced until after 3 hours of treatment. This suggests that IκB may be degraded in the cell in response to CB treatment. At 6 hours of treatment there appears to be high levels of nuclear localisation of IκB which is reduced to mainly cytoplasmic staining around the nucleus of the cells at 18 hours. Immunofluorescence intensities were calculated (Fig 75) and this showed that there was a low level of IκB in the cells during the first 4 hours prior to an increase between 4 and 6 hours of treatment.

Western blots were carried out on the cell lysates of the untreated and treated cells (Fig 76). The Westerns blots also indicated that there is a decrease in the levels of IκB in the cells during the first four hours of treatment. The increase and decrease in IκB appear to be less pronounced in the Western blots than the immunofluorescence and this is likely to be the result of different sensitivities of the techniques.

4.5.2 Treatment with ufCB over 18 hour period.

Cells were treated with 100 μg/ml ufCB for various time points and analysed by indirect immunofluorescence (Fig 77). The findings show that there is a decrease in the levels of IκB after 30 minutes of treatment followed by high levels of IκB in the cell nucleus after 1 hour which persists until 3 hours of treatment. This suggests that there is degradation of IκB in the cell followed by an increase of IκB which translocates to the nucleus. This is indicative of a typical stress response where IκB is degraded in the cytoplasm allowing NFκB to enter the nucleus (see Fig 78) and bind to the DNA signalling the production of pro-inflammatory molecules including IκB. The newly synthesised IκB can then enter the nucleus and bind to NFκB and inhibit DNA binding resulting in the translocation of the IκB-NFκB complex back into the cell cytoplasm. Intensity analysis of the fluorescence images shows that there is a

peak in IκB levels at 1 hour of treatment and that this decreases until 3 hours before increasing to 18 hours of treatment.

Western blots of the cell lysate revealed that IκB was present at high concentrations in the untreated cells and this level was decreased after 30 minutes of treatment an that the level of IκB rose thereafter (Fig 79).

4.5.3 Treatment with PM10 over 18 hour period.

Cells were treated for up to 18 hours with PM10 from Wolverhampton. Immunofluorescence microscopy showed that IκB was present in the untreated cells in the cytoplasm (Fig 80). There appeared to be no nuclear localisation of IκB even though NFκB was present in the nucleus from ½ - 2 hours of treatment. Average immunofluoresence intensities show that there is some fluctuation in the levels of IκB over the time course but at no time are the levels significant from the untreated cells (Fig 81).

Western blots were carried out on the cell lysates of the cells (Fig 82). This shows that the levels of IκB in the cell fluctuate over the time course similarly to what was shown in the immunofluorescence results.

4.5.4 Treatment with $FeCl_3$ over 4 hour period.

Cells were treated with $FeCl_3$ over the 4 hour time period. This time period was chosen instead of the 18 hour time course since this was the time point chosen for NFκB. Immunofluorescence staining revealed that there was staining in the cytoplasm of the untreated cells, as expected however the levels remained fairly constant of the four hour time course with no nuclear localisation, suggesting no activation was occurring (Fig 83).There was a decrease in the levels of IκB at ½ an hour of treatment, this may be due to degradation of IκB due to activation however, no NFκB nuclear localisation occurred for this treatment making degradation unlikely. The average immunofluorescence intensities of the cells revealed that the level of IκB in the cells decreases after half an hour of treatment before rising slightly with time (Fig 84).

Western blots were carried out on the cell lysates of the cells over 18 hours of treatment. The cell lysate Western blots revealed that IκB was present at high levels of the cells at all time points and that little change occurred with the exception of a decrease the half hour time point (Fig 85), which supports the immunofluorescence results.

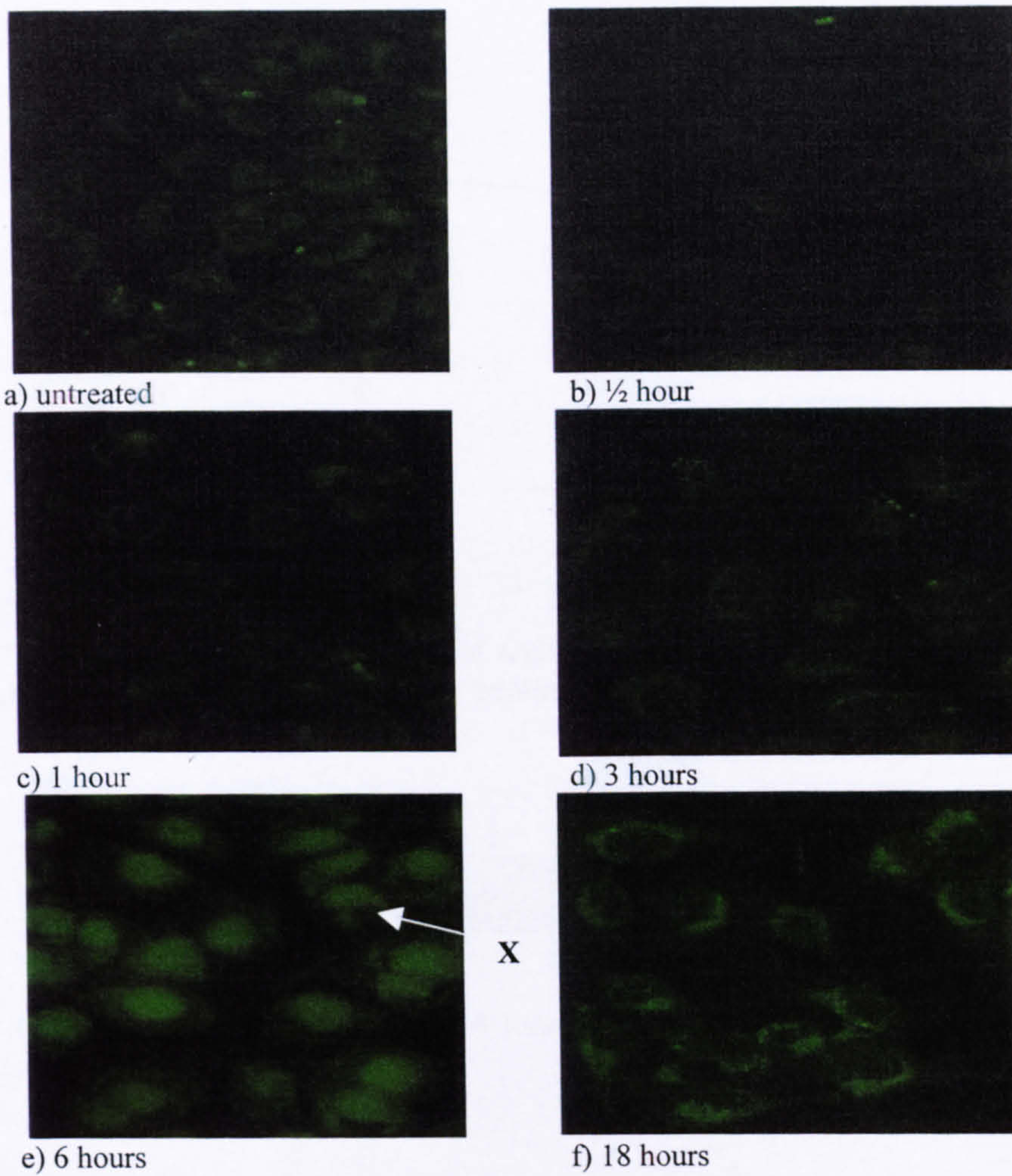


Figure 74: Expression of IκB in A549 cells treated with 100μg/ml CB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation of IκB. For controls see Appendix I.

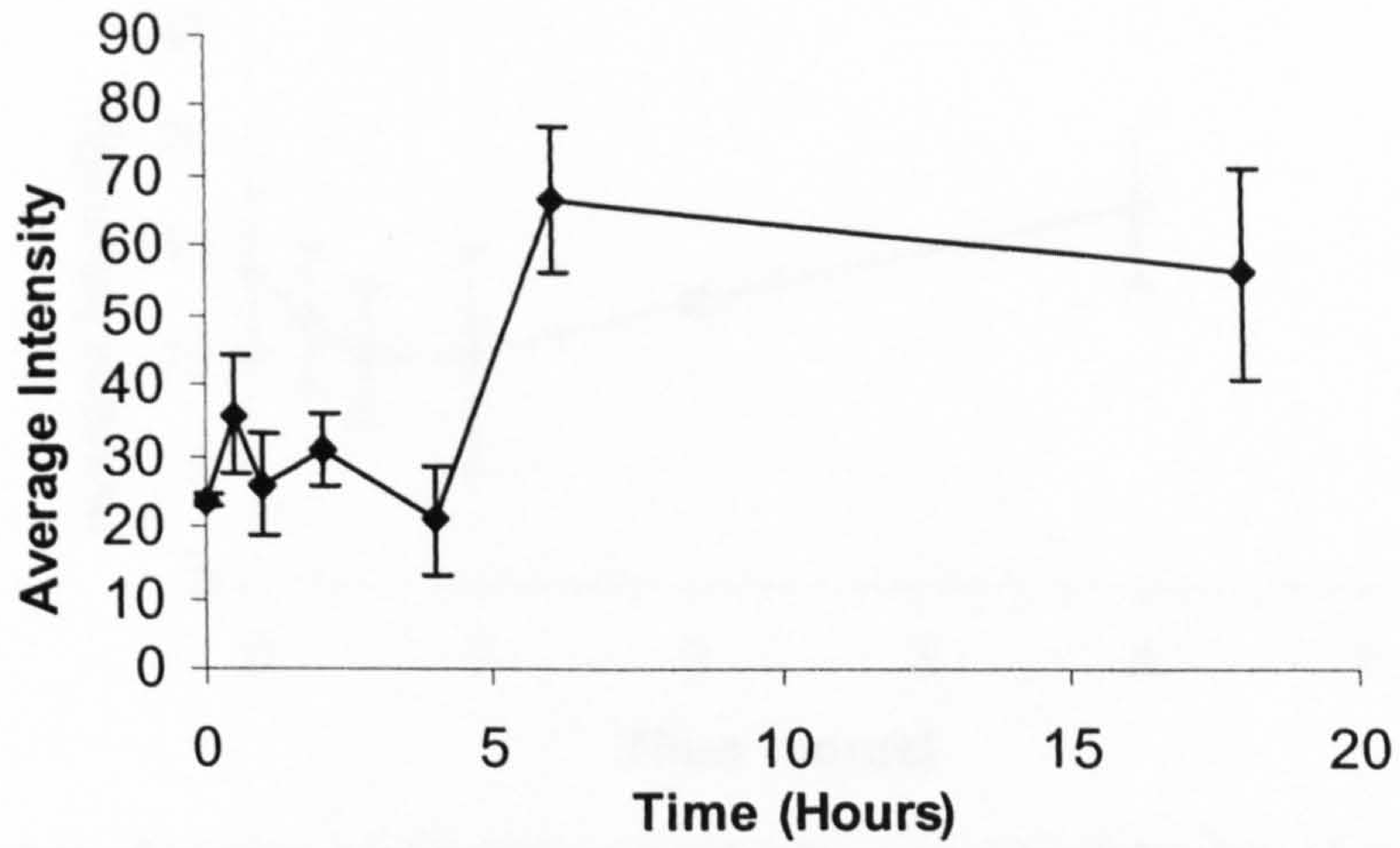


Figure 75: Immunofluorescent intensities of A459 cells stained for IκB after 18 hours of 100μg/ml CB treatment. The results are the mean of triplicate results from 3 experiments ± SE.

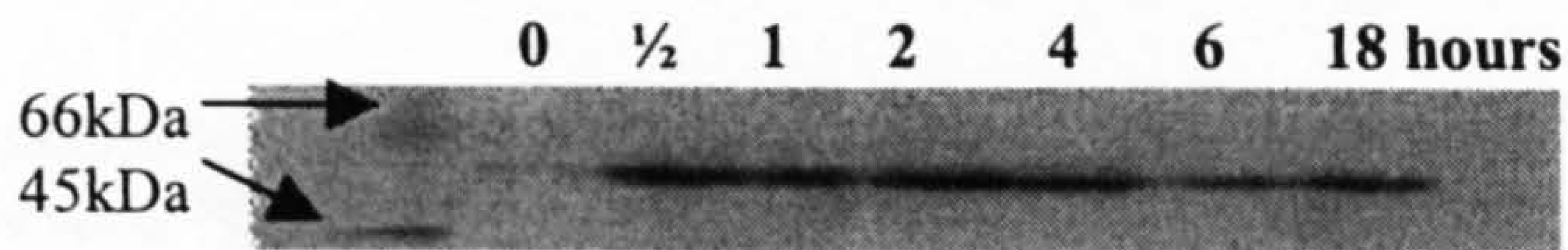


Figure 76: Level of IκB expression in cell lysate measured by Western blot after 18 hours of 100μg/ml CB treatment.

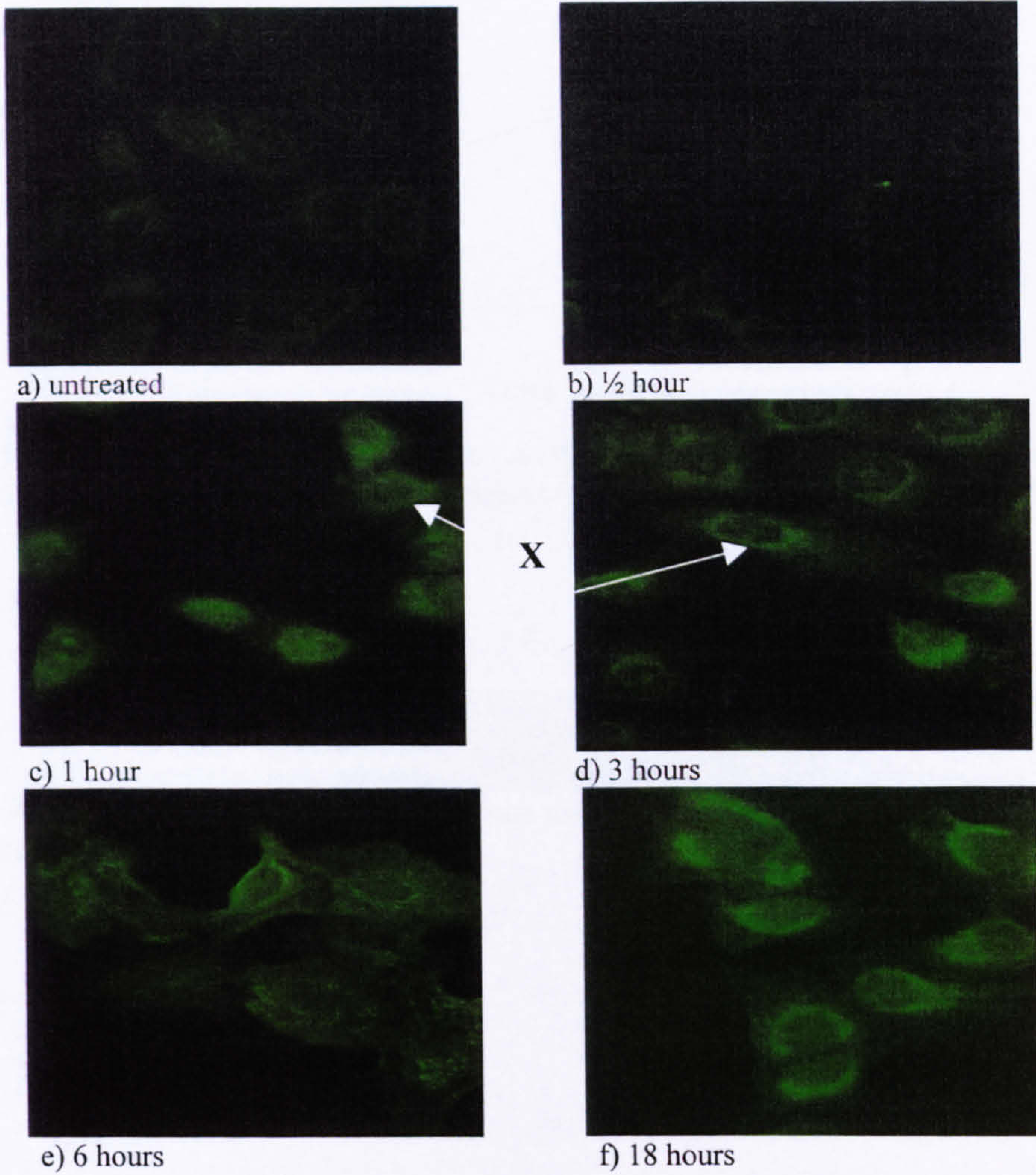


Figure 77: Expression of IκB in A549 cells treated with 100μg/ml ufCB, for various time points up to 18 hours as shown by indirect immunofluorescence. X indicates nuclear localisation of IκB. For controls see Appendix I.

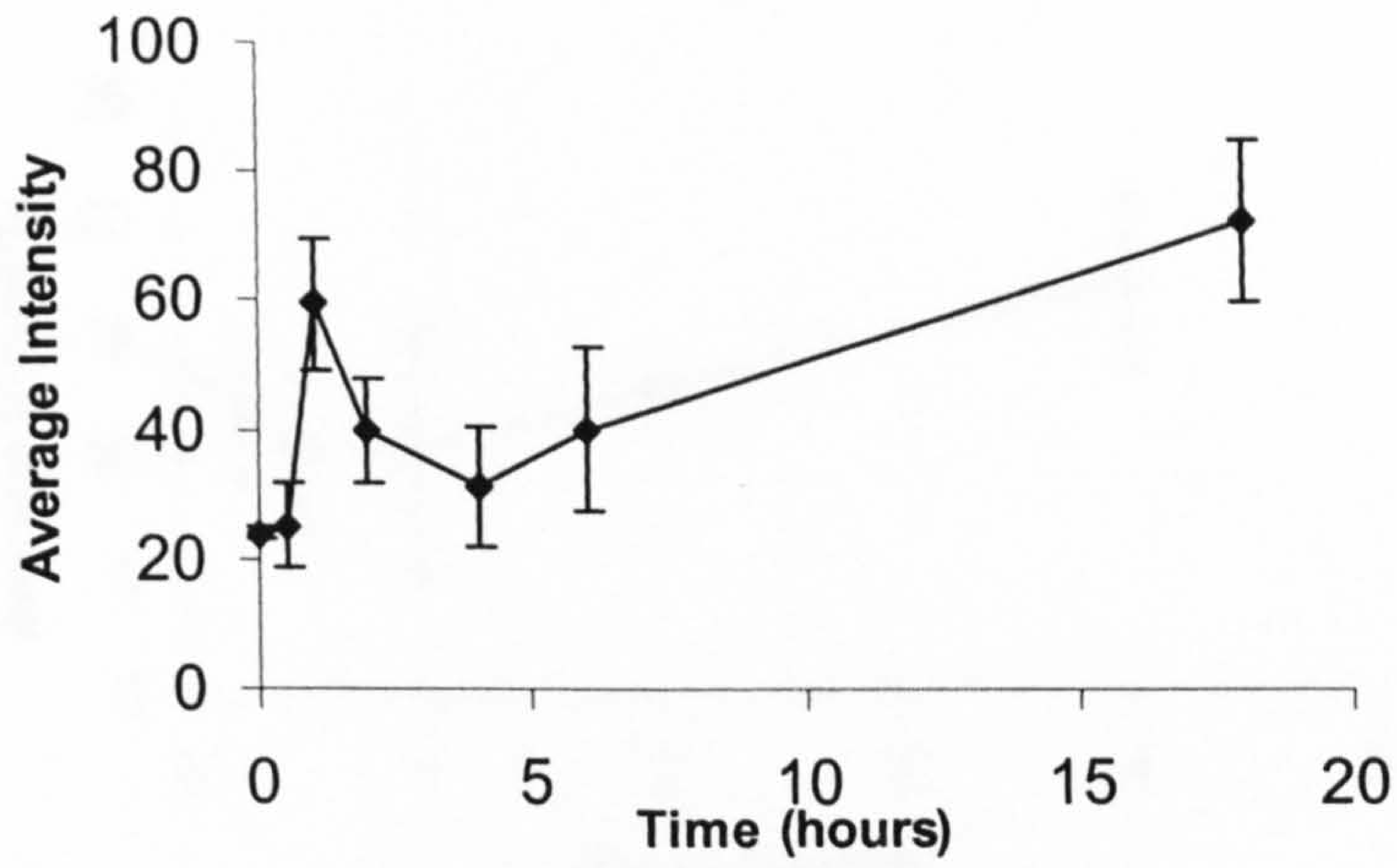


Figure 78: Immunofluorescent intensities of A459 cells stained for IκB after 18 hours of 100 μg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE.

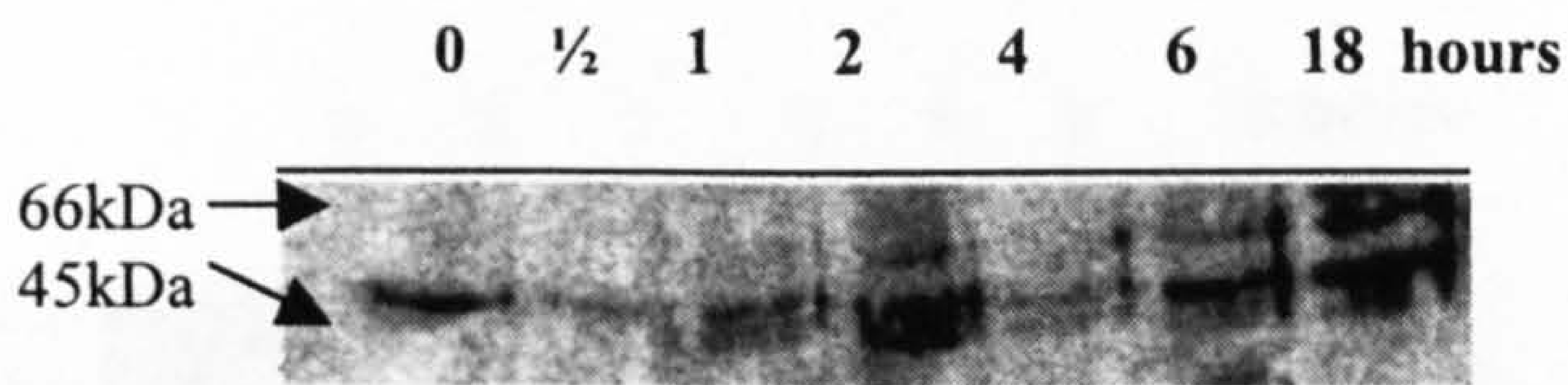
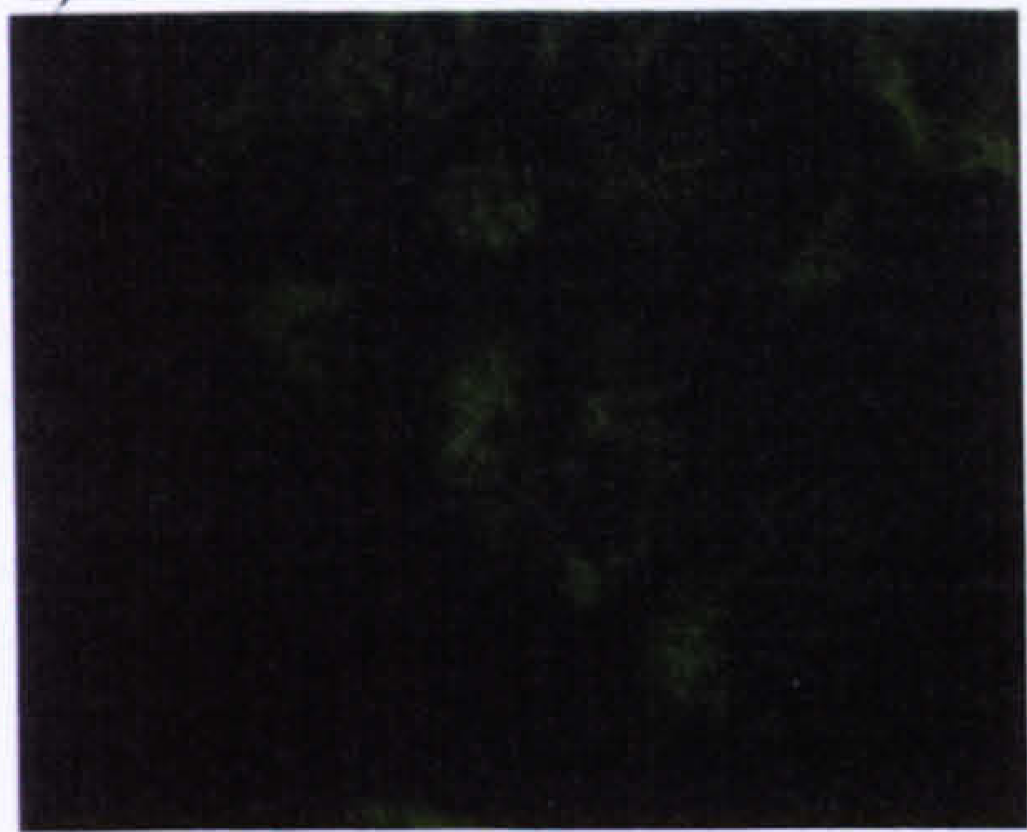


Figure 79: Level of IκB expression in cell lysate measured by Western blot after 18 hours of 100 μg/ml ufCB treatment.

a) untreated

b) ½ hour


c) 1 hour

d) 3 hours

e) 6 hours

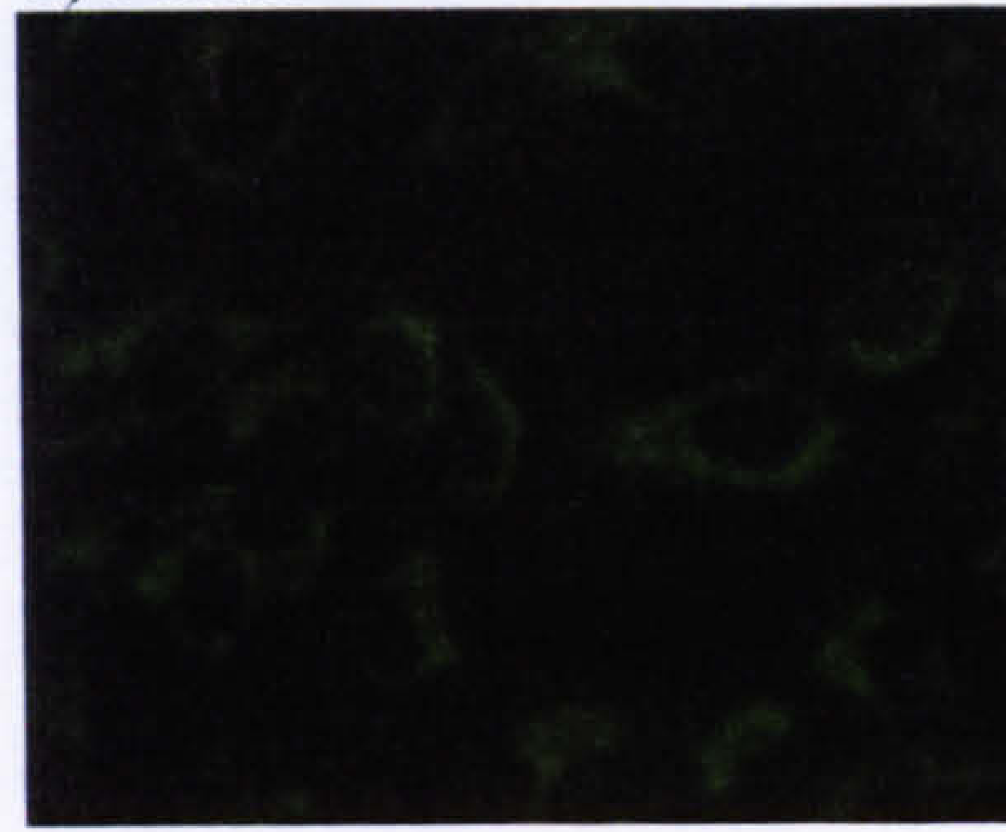

f) 18 hours

Figure 80: Expression of IκB in A549 cells treated with 80μg/ml PM10, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

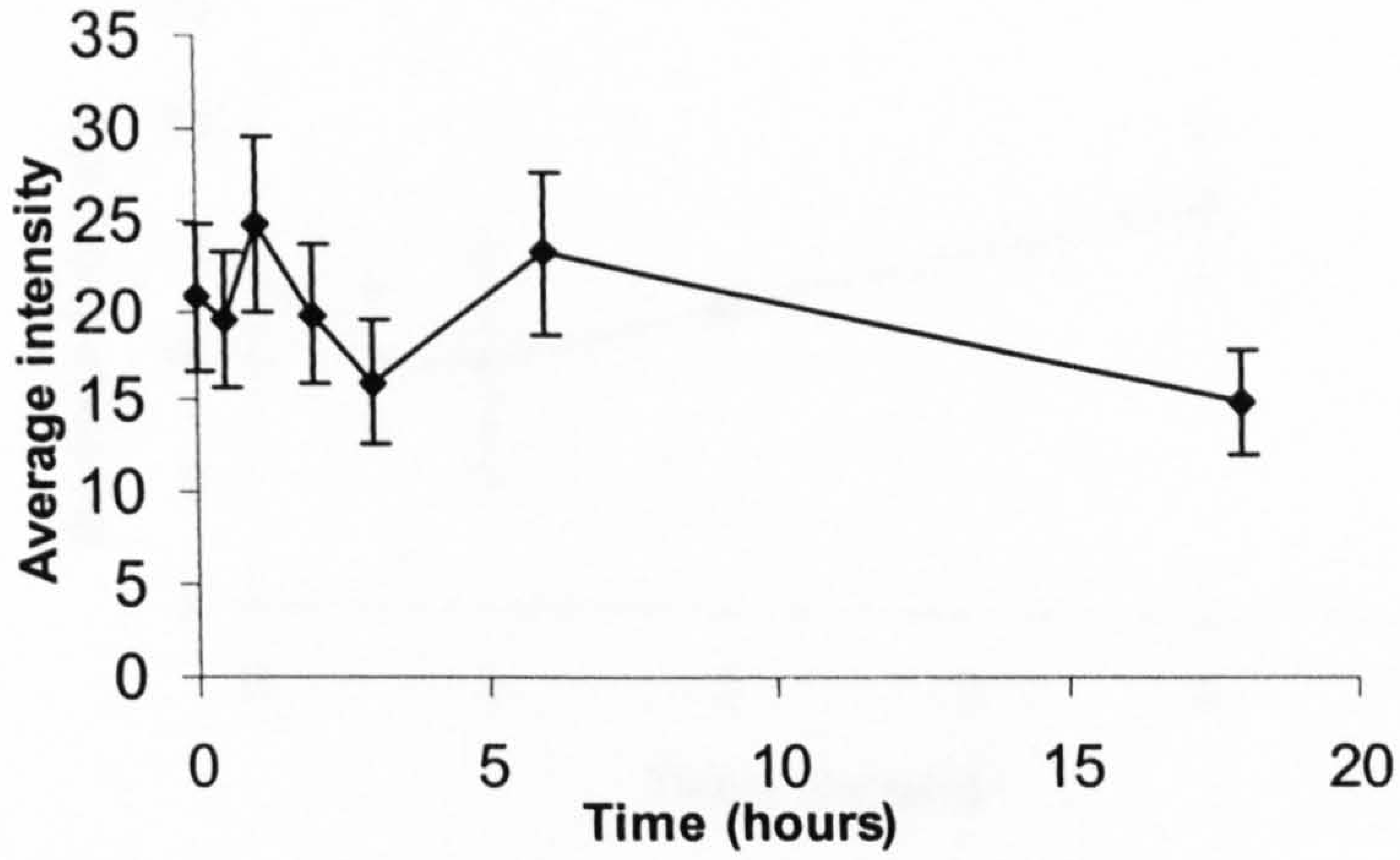


Figure 81: Immunofluorescent intensities of A459 cells stained for IκB after 18 hours of 80μg/ml PM10 treatment. The results are the mean of triplicate results from 3 experiments ± SE.

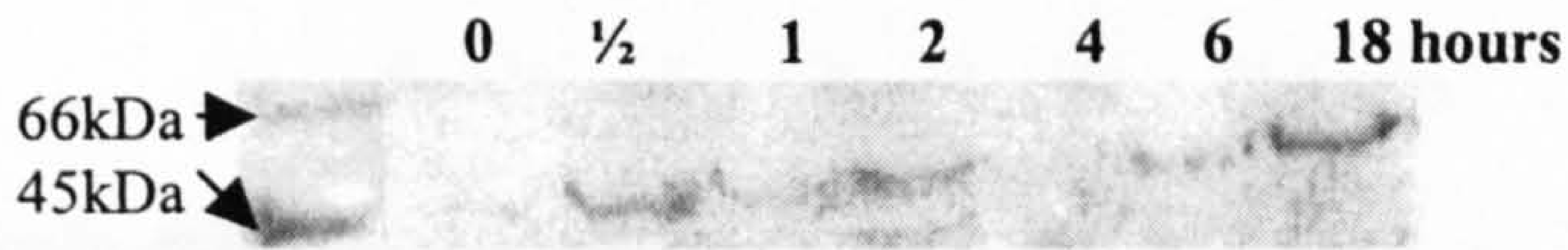
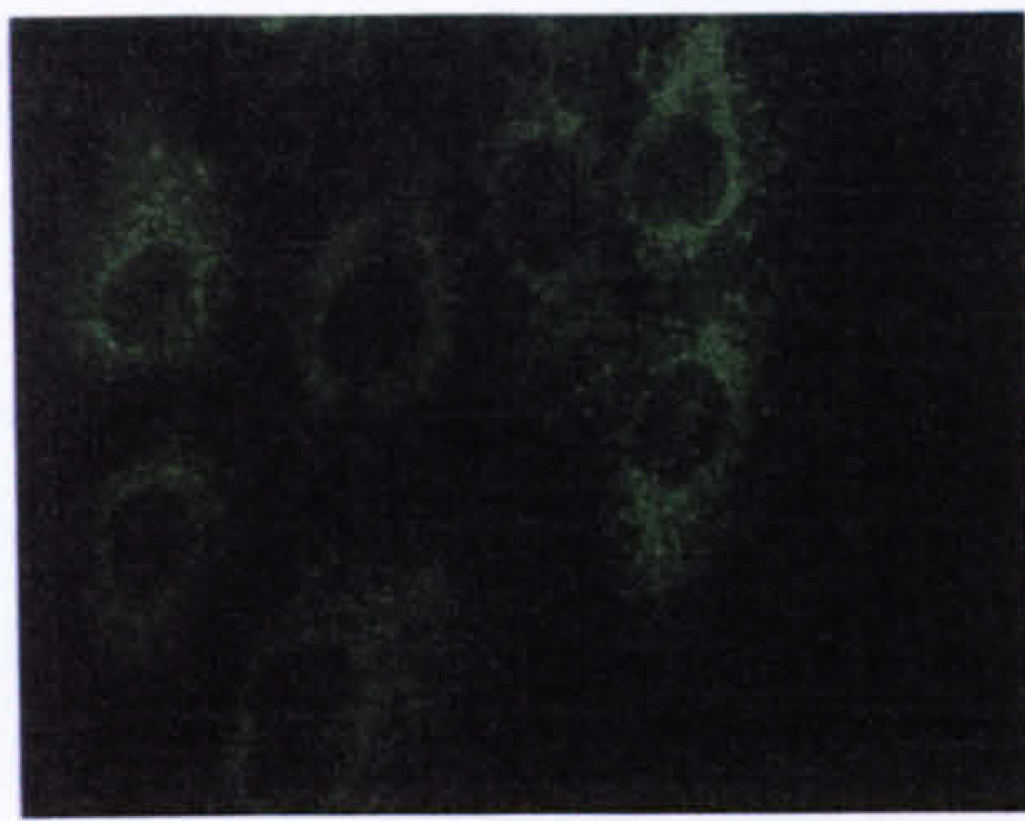
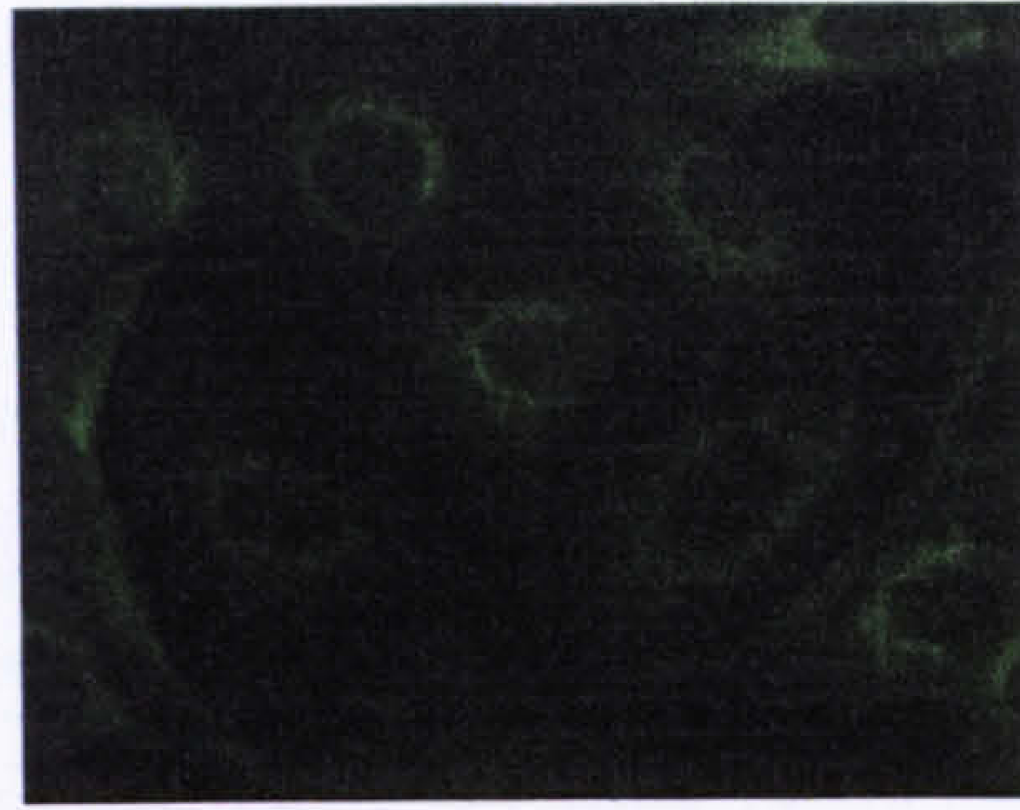


Figure 82: Level of IκB expression in cell lysate measured by Western blot after 18 hours of 80μg/ml PM10 treatment.

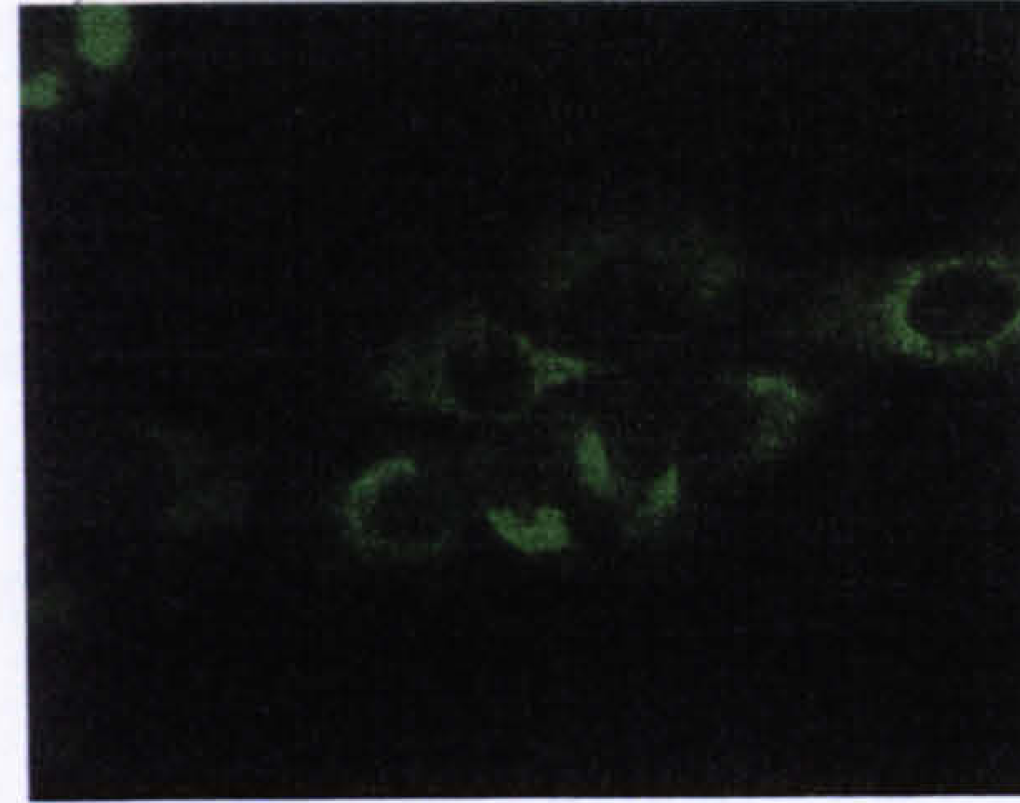


a) untreated

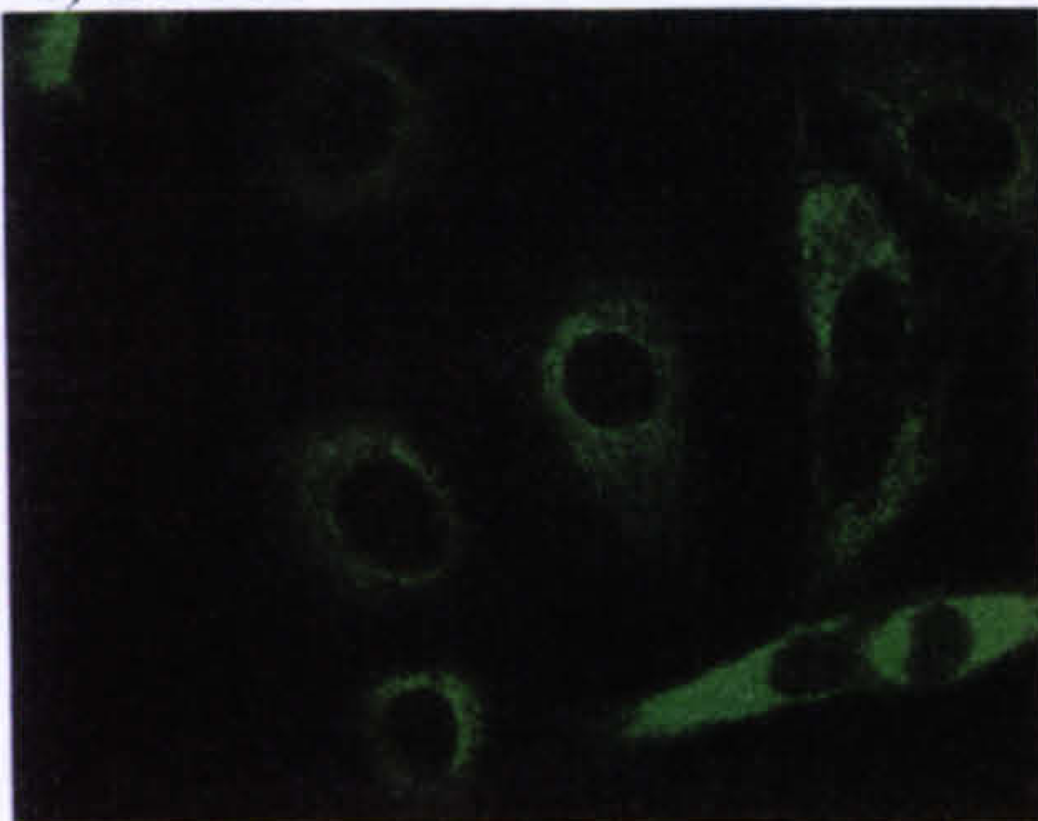


b) ¼ hour

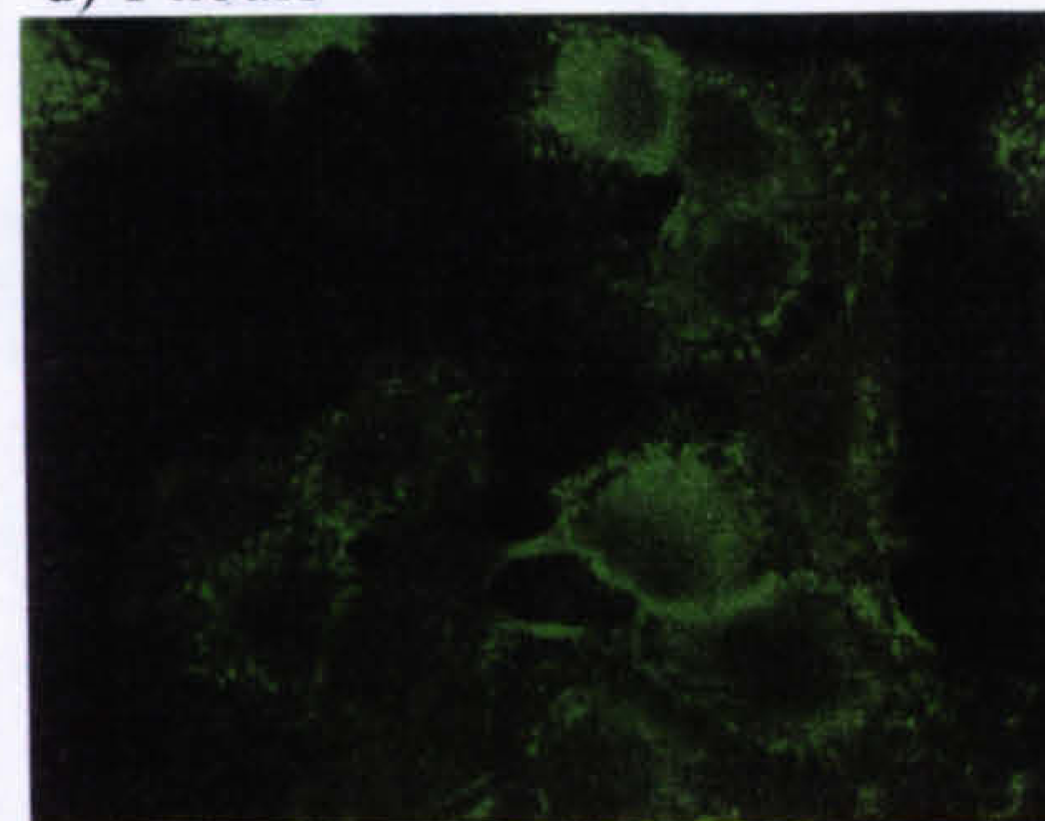
c) 1/2 hour



d) 1 hours



e) 2 hours



f) 4 hours

Figure 83: Expression of IκB in A549 cells treated with 100μM $FeCl_3$, for various time points up to 18 hours as shown by indirect immunofluorescence. For controls see Appendix I.

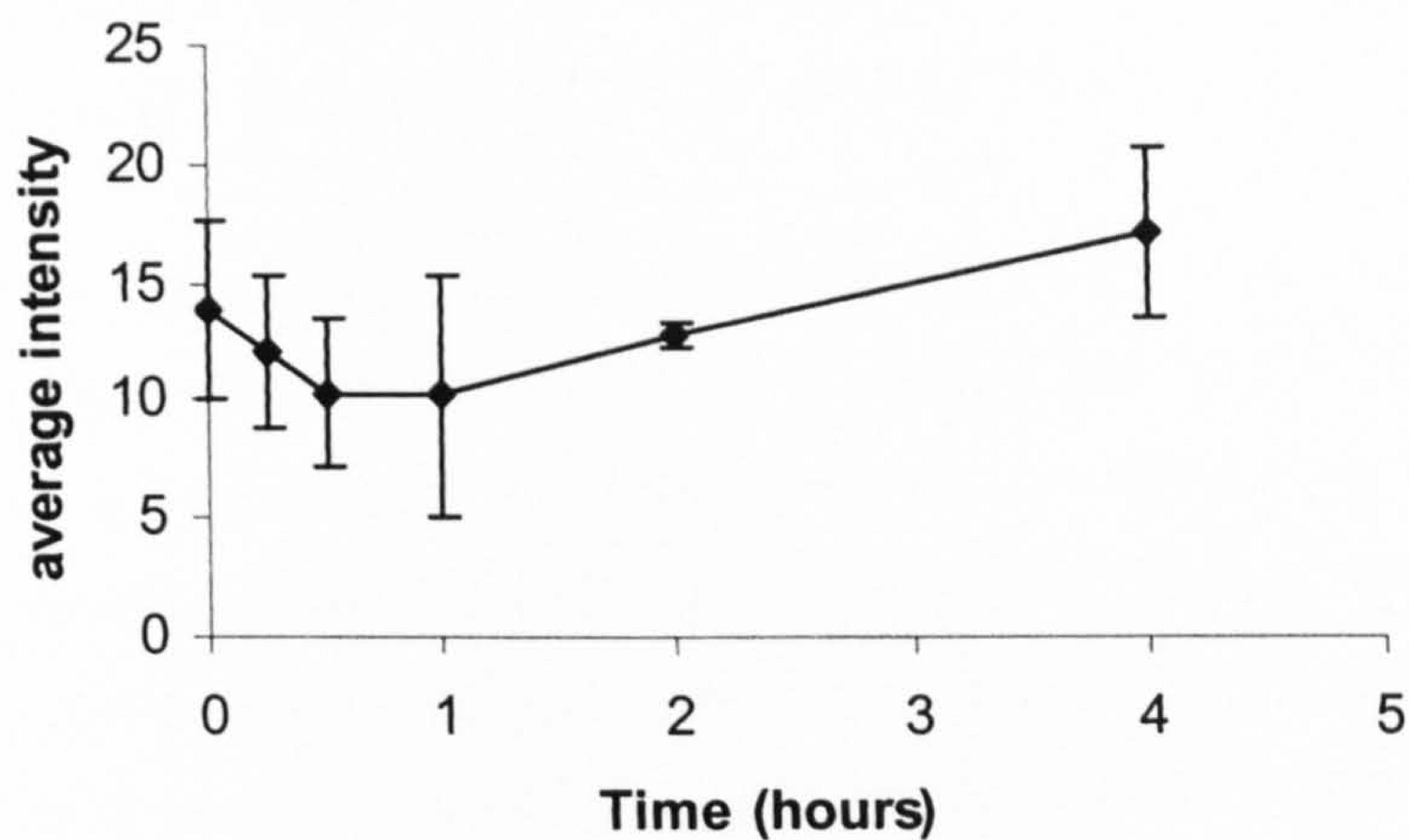


Figure 84: Immunofluorescent intensities of A459 cells stained for IκB after 18 hours of 100μM $FeCl_3$ treatment. The results are the mean of triplicate results from 3 experiments ± SE.

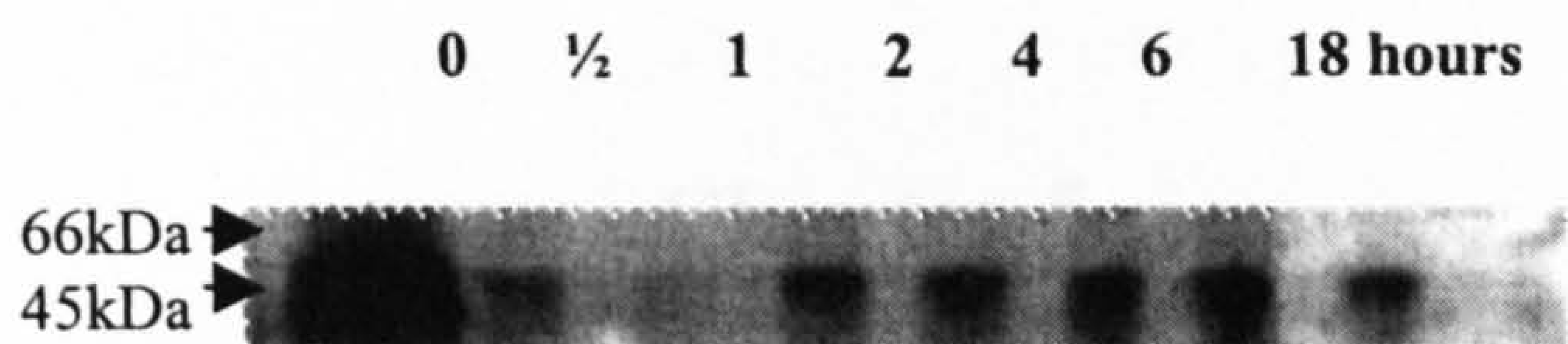


Figure 85: Level of IκB expression in cell lysate measured by Western blot after 18 hours of 100μM $FeCl_3$ treatment.

Chapter 5

Possible mechanisms of inflammatory protein expression and secretion in lung epithelial cells in response to particle treatments.

5.1.0 Are inflammatory proteins actively secreted from the cells into the culture supernatant or released as a result of membrane damage?

5.1.1. Is the cell membrane damaged during cell treatment?

To determine if CRP, fibrinogen and Hsp70 present in the cell supernatant were present in the cell supernatant as a result of membrane damage during cell treatment dye exclusion studies were carried out. Due to the limited availability of Wolverhampton PM10, investigations of particle mechanisms were carried out using ufCB which showed similar results to the PM10 treatments in previous experiments. Cells were grown on glass coverslips and treated with ufCB as shown previously before incubation with propidium iodide. If the cell membrane was damaged due to particle exposure the propidium iodide would be able to enter the cell and stain the cell nuclei. Cells were viewed under the UV microscope and the percentage viability was calculated (Fig 88). This showed that for ufCB that there was only a small decrease in cell viability which was not statistically significant suggesting that the cells remained viable after treatment.

5.1.2 Treatment of A549 cells with particles and Brefeldin A.

To identify if CRP, fibrinogen and Hsp70 present in the cell supernatant were as a result of active secretion from the cell via the Golgi, cells were co-treated with either CB or ufCB with Brefeldin A. Brefeldin A is an antiviral antibiotic which can inhibit the transport of newly synthesised proteins from the endoplasmic reticulum to the Golgi complex (Misumi *et. al.,* 1986). ELISAs were carried out for CRP and fibrinogen and Western blots were carried out for Hsp70 on the cell supernatants with particle and Brefeldin A co-treatments and for particle treatments alone. Levels of CRP in the supernatants was found to be decreased for all treatments (untreated control, 100μg/ml CB and 100μg/ml ufCB) when Brefeldin A was present (Fig 87). Both the particle treatments with Brefeldin A showed a decrease in CRP in the supernatant which was statistically significant ($p<0.05$) from the particle treatment alone, suggesting that secretion was responsible for CRP being present in the supernatant.

The concentration of fibrinogen in the supernatants were analysed using ELISA for the Brefeldin A treatment. Fibrinogen secretion was found to be prevented for all treatments (untreated control, 100μg/ml CB and 100μg/ml ufCB) when Brefeldin A was present (Fig 88). In the untreated control cells, there was a large decrease in the amount of fibrinogen present; however, this result was not statistically significant. Both the particle treatments showed a decrease with Brefeldin A treatment with only ufCB being statistically significant ($p<0.05$) from the particle treatment alone. This suggests that secretion was responsible for fibrinogen being present in the supernatant for ufCB treatment and that it is likely to be responsible for CB.

The amount of Hsp70 secreted from the cells with particle and Brefeldin A treatment was determined using Western blotting. Secretion of Hsp70 was found to be prevented for all treatments (untreated control, 100μg/ml CB and 100μg/ml ufCB) when Brefeldin A was present (Fig 89). This indicates that Hsp70 is actively secreted from A549 cells in response to both CB and ufCB exposure.

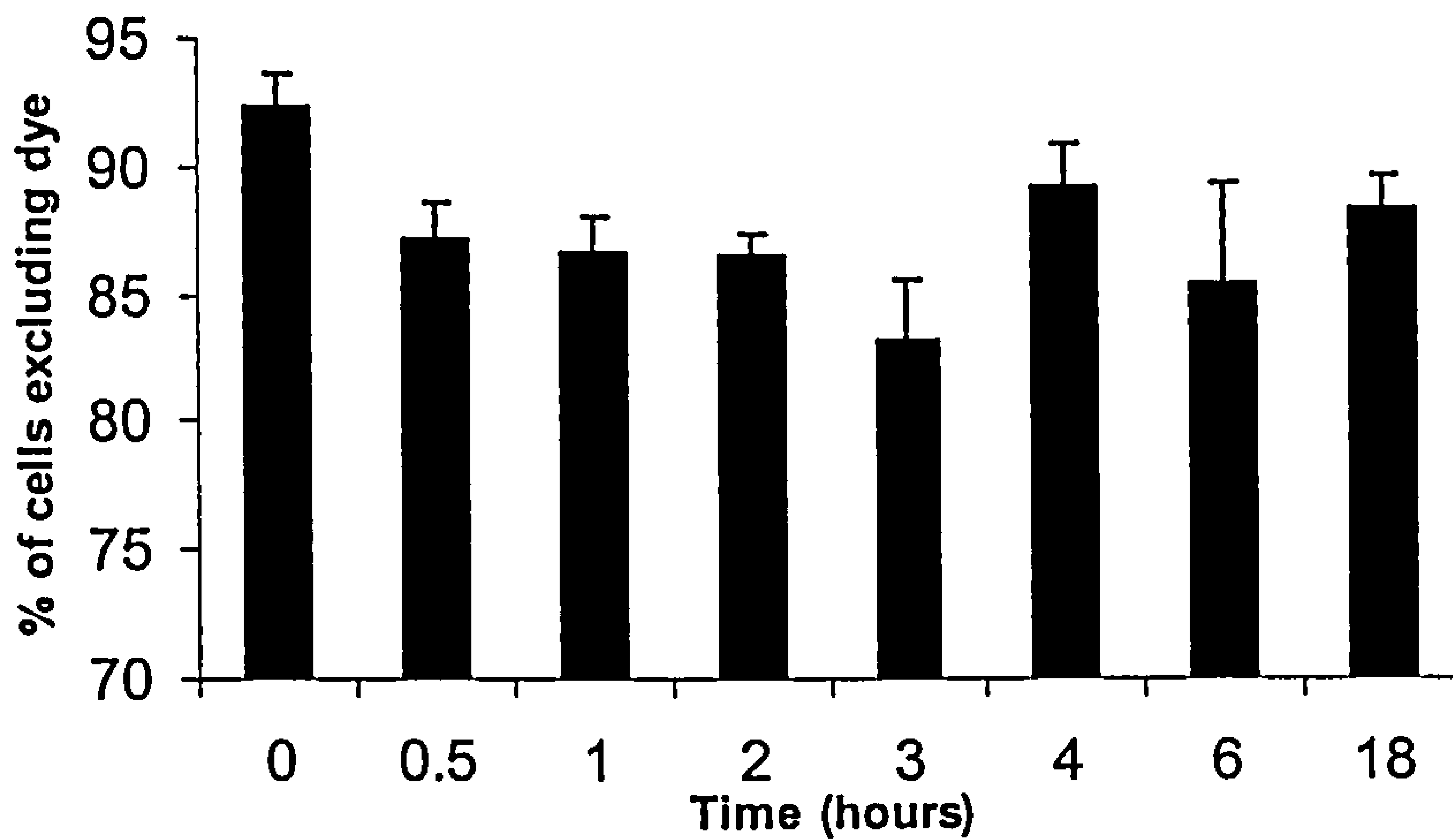


Figure 86: Percentage of propidium iodide dye exclusion in A549 cells with 100µg/ml ufCB treatment. The results are the mean of triplicate results from 3 experiments ± SE.

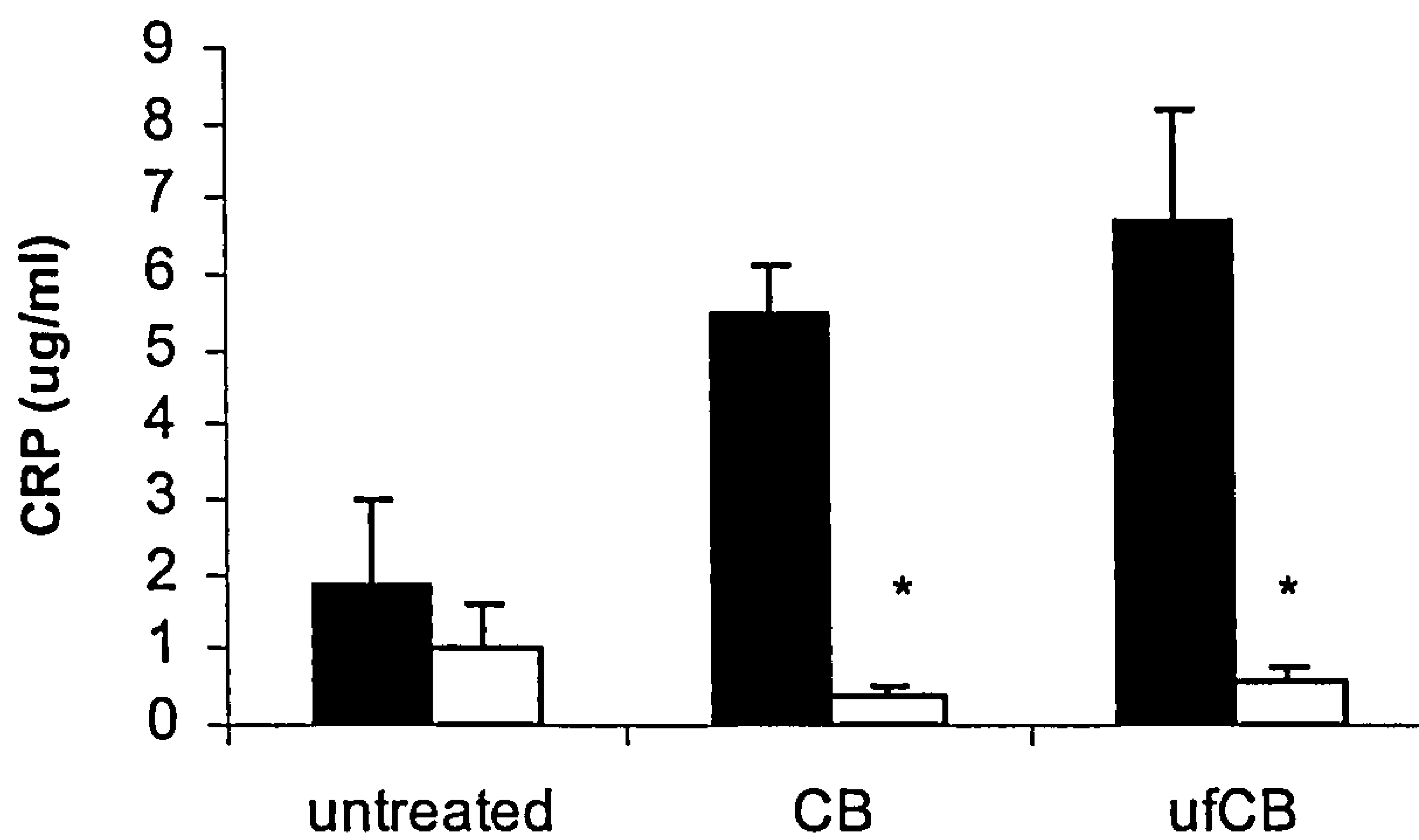


Figure 87: Concentration of CRP in cell supernatant measured by ELISA after 6 hours of Brefeldin A (10µg/ml) and particle treatment (100µg/ml) of A549 cells. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from particle only treatments, $p < 0.05$.

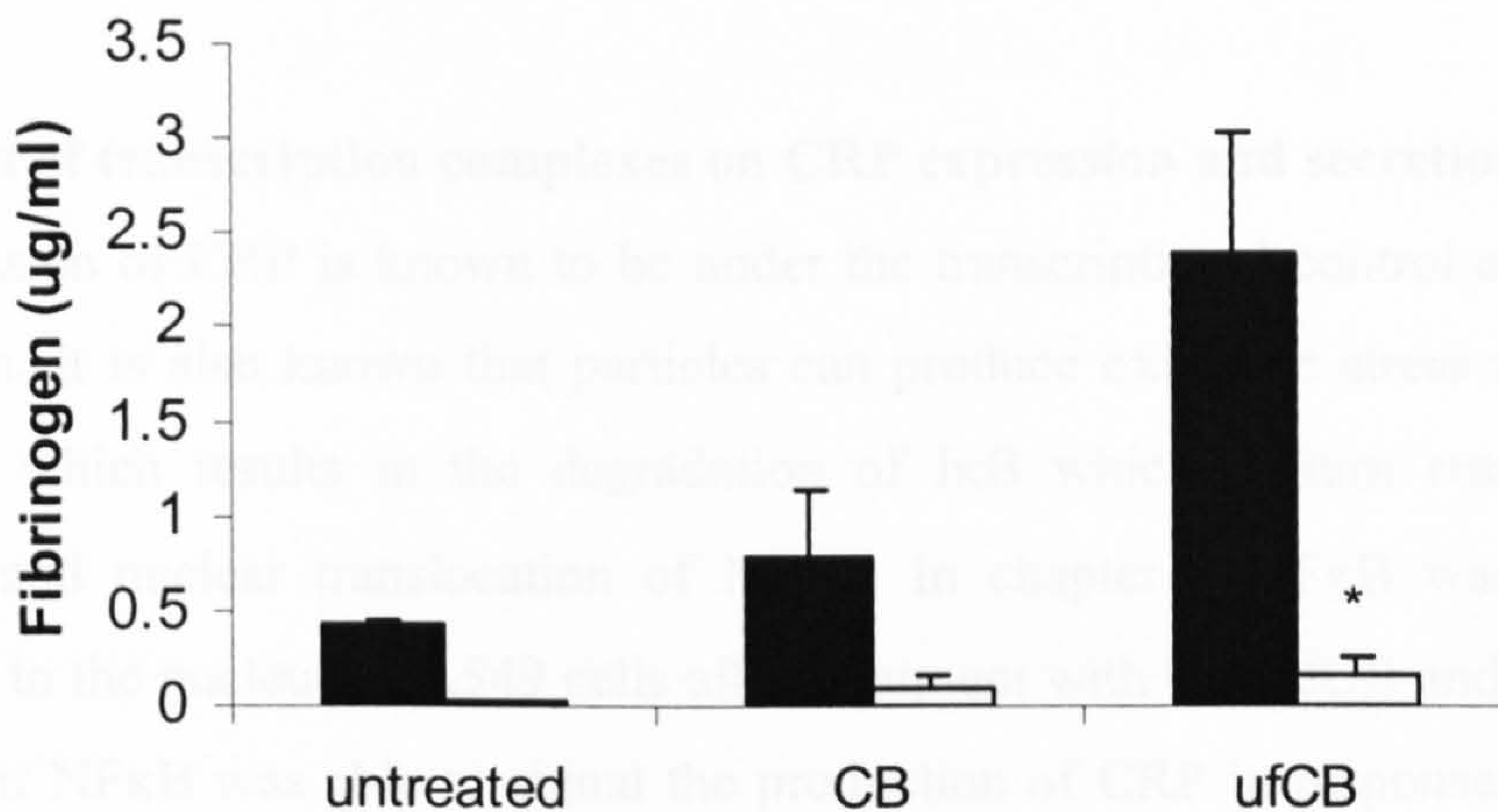
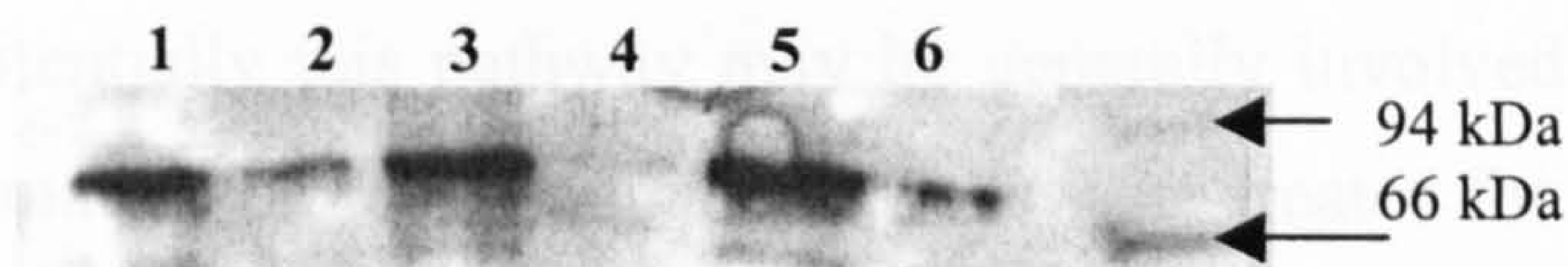
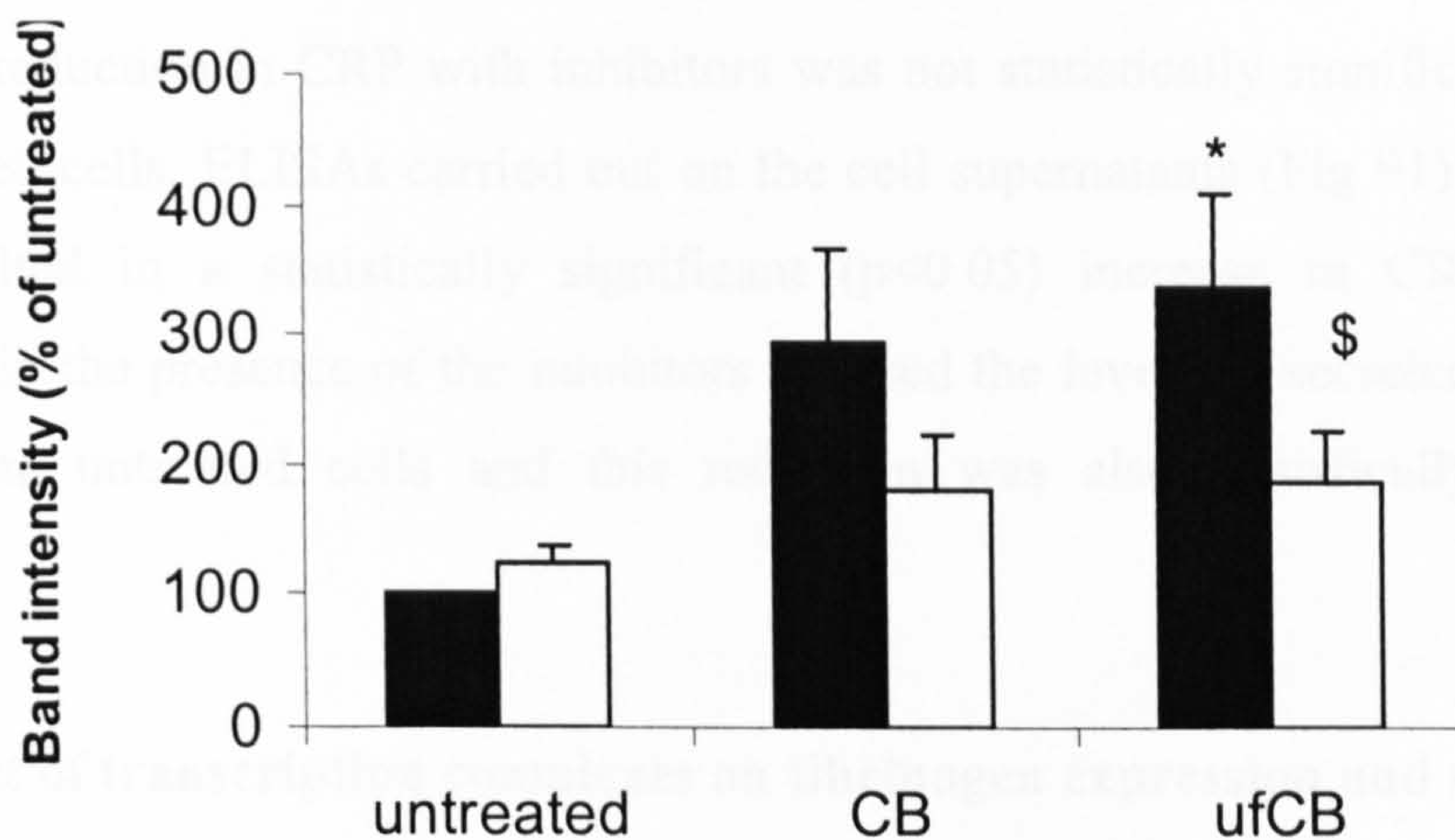


Figure 88: Concentration of fibrinogen in cell supernatant measured by ELISA after 6 hours of Brefeldin A (10μg/ml) and particle treatment (100 μg/ml) of A549 cells. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from particle only treatments, p< 0.05.



a) Western blot of particle and Brefeldin A treatments, stained for Hsp70. Where 1) untreated, 2) untreated + Brefeldin A, 3) CB treated, 4) CB + Brefeldin A treated, 5) ufCB treated, and 6) ufCB and Brefeldin A treated.



b) Average band intensities of Hsp70

Figure 89: Level of Hsp70 in cell supernatant measured by Western blot band intensities using Metamorph software after 6 hours of Brefeldin A and particle treatment of A549 cells. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated control cells, p< 0.05 and $ denotes significant changes from particle only treatments, p< 0.05.

5.2 How do particles induce the expression of CRP, Fibrinogen and Hsp70?

5.2.1 Effect of transcription complexes on CRP expression and secretion.

The expression of CRP is known to be under the transcriptional control of NFκB in hepatocytes. It is also known that particles can produce oxidative stress in the lung epithelium which results in the degradation of IκB which in turn results in the activation and nuclear translocation of NFκB. In chapter 2 NFκB was found to translocate to the nucleus of A549 cells after treatment with CB, ufCB and PM10. To determine if NFκB was able to signal the production of CRP in response to particle treatments, cells were treated with ufCB (100μg/ml) in the presence of sn50 (50μg/ml) an inhibitor of NFκB nuclear translocation. Cells were also treated with another inhibitor Ag490 (50μM), which inhibits the Jak family tyrosine kinase, preventing its phosphorylation. This inhibitor was used since it was shown to inhibit the Jak/STAT transcription complex involved in the production of Hsp70 in response to oxidative stress. Potentially this pathway may be generally involved in oxidative mechanisms of inflammatory protein production. Cells were treated for 6 hours with ufCB in the presence and absence of these inhibitors. ELISAs carried out on the cell lysates (Fig 90) showed that there was a statistically significant increase ($p<0.05$) in CRP with ufCB treatment and that both inhibitors reduced the level of CRP in the cells. The reduction in CRP with inhibitors was not statistically significant from the ufCB treated cells. ELISAs carried out on the cell supernatants (Fig 91) showed that ufCB resulted in a statistically significant ($p<0.05$) increase in CRP secretion. Treatment in the presence of the inhibitors reduced the levels of secreted CRP to the level of the untreated cells and this reduction was also statistically significant ($p<0.05$).

5.2.2 Effect of transcription complexes on fibrinogen expression and secretion.

To investigate the effect of transcription events of fibrinogen production A549 cells were treated with ufCB (100μg/ml) and the transcription inhibitors sn50 (50μg/ml) and Ag490 (50μM), as shown for the effects on CRP production. ELISAs carried out for fibrinogen showed that ufCB induced a statistically significant increase ($p<0.05$) in the levels of fibrinogen in the cells compared to untreated cells (Fig 92). The cells treated with ufCB in the presence of Ag490 appeared to have no inhibitory effects of

the expression of fibrinogen induced by ufCB. ELISAs carried out on the cell supernatants (Fig 93) showed that there was an increase in fibrinogen secretion with ufCB and the levels of secretion were reduced by both inhibitors. The cells treated with sn50 (the inhibitor of NFκB nuclear translocation) showed a reduction in fibrinogen secretion which was lower than the levels of secretion in untreated cells; however, this reduction was not statistically significant from the ufCB treated cells.

5.2.3 Effect of transcription complexes on Hsp70 expression and secretion.

To investigate the effect of transcription events of the expression of Hsp70 with ufCB exposure the same inhibitors were used as for CRP and fibrinogen. Immunofluorescence microscopy was carried out on the treated cells and the average intensities of the cells carried out (Fig 94). This shows that ufCB (100μg/ml) increased the levels of Hsp70 in the cells and that Ag490 reduced the level of Hsp70 while sn50 had little effect. Western blots were carried out on the cell supernatants (Fig 95). This showed that ufCB induced an increase in secreted Hsp70. The Western blots also showed that the inhibitors reduced the amount of secreted Hsp70, particularly in the Ag490 treated cells.

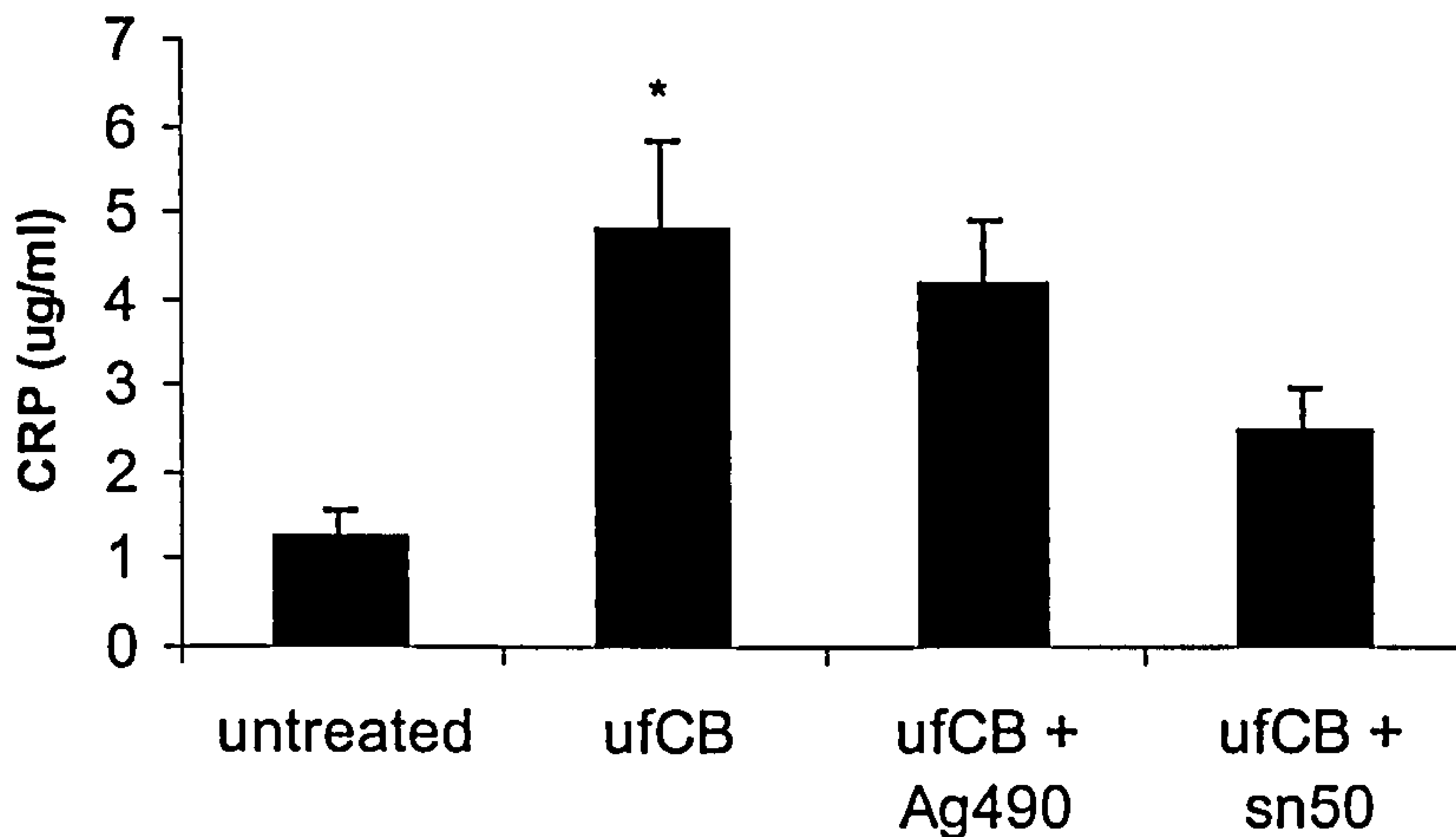


Figure 90: Concentration of CRP in cell lysate measured by ELISA after 6 hours of ufCB (100µg/ml) and transcription inhibitor treatment of A549 cells. Cells were treated with 50µM Ag490 and 50µg/ml sn50. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$.

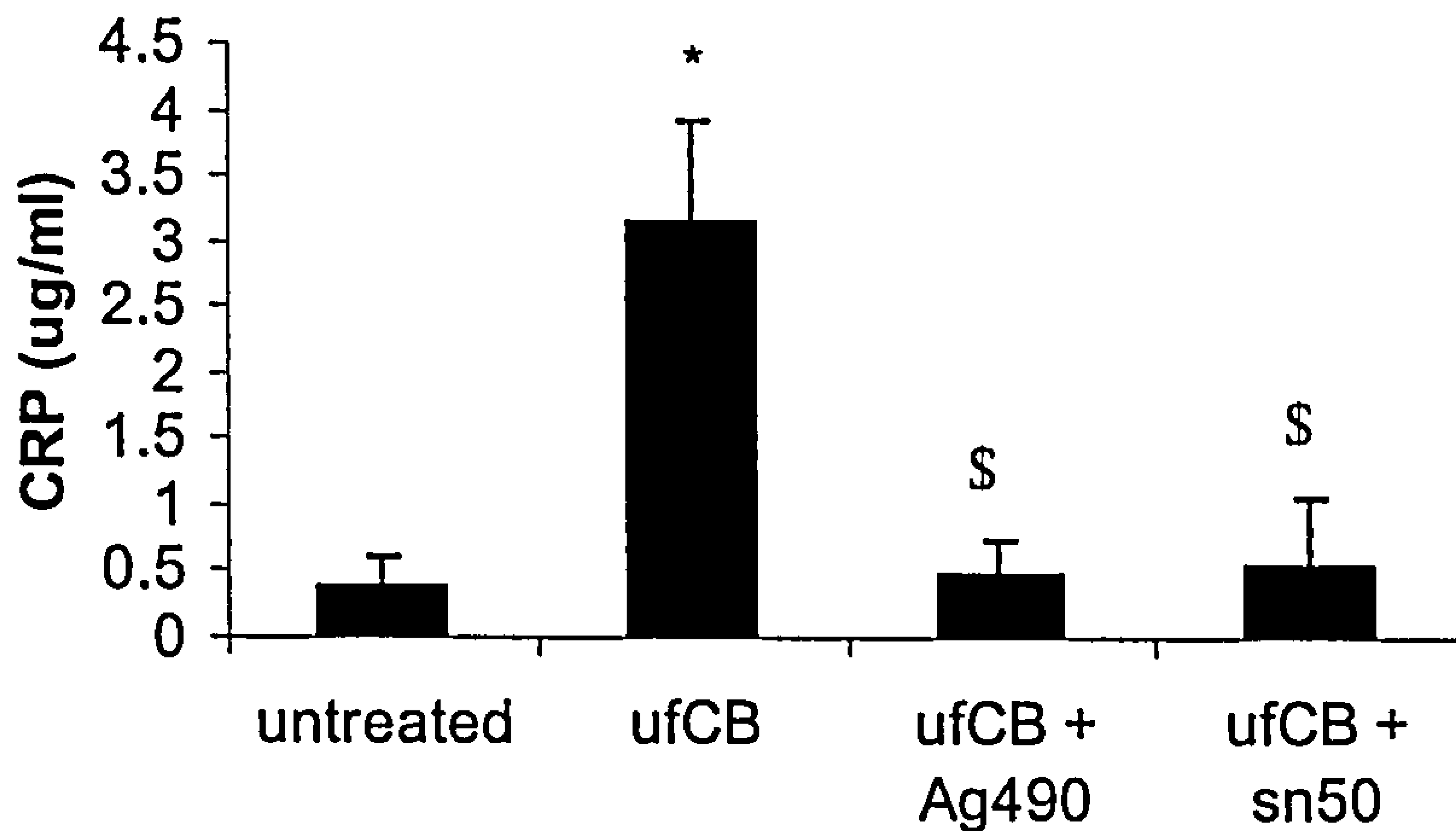


Figure 91: Concentration of CRP in cell supernatant measured by ELISA after 6 hours of ufCB (100µg/ml) and transcription inhibitor treatment of A549 cells. Cells were treated with 50µM Ag490 and 50µg/ml sn50. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$ and $ denotes significant decrease from ufCB treated cells.

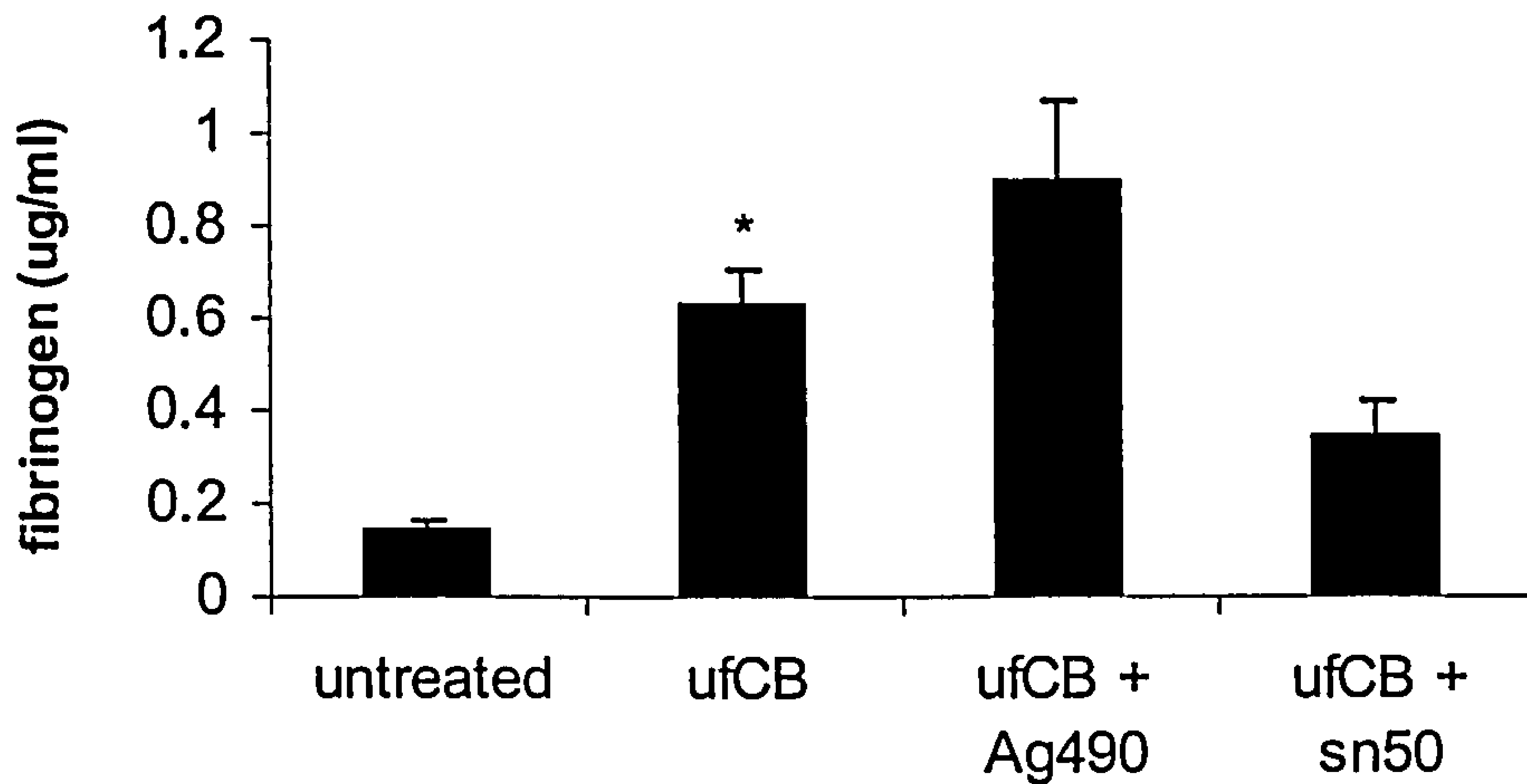


Figure 92: Concentration of fibrinogen in cell lysate measured by ELISA after 6 hours of ufCB (100μg/ml) and transcription inhibitor treatment of A549 cells. Cells were treated with 50μM Ag490 and 50μg/ml sn50. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$.

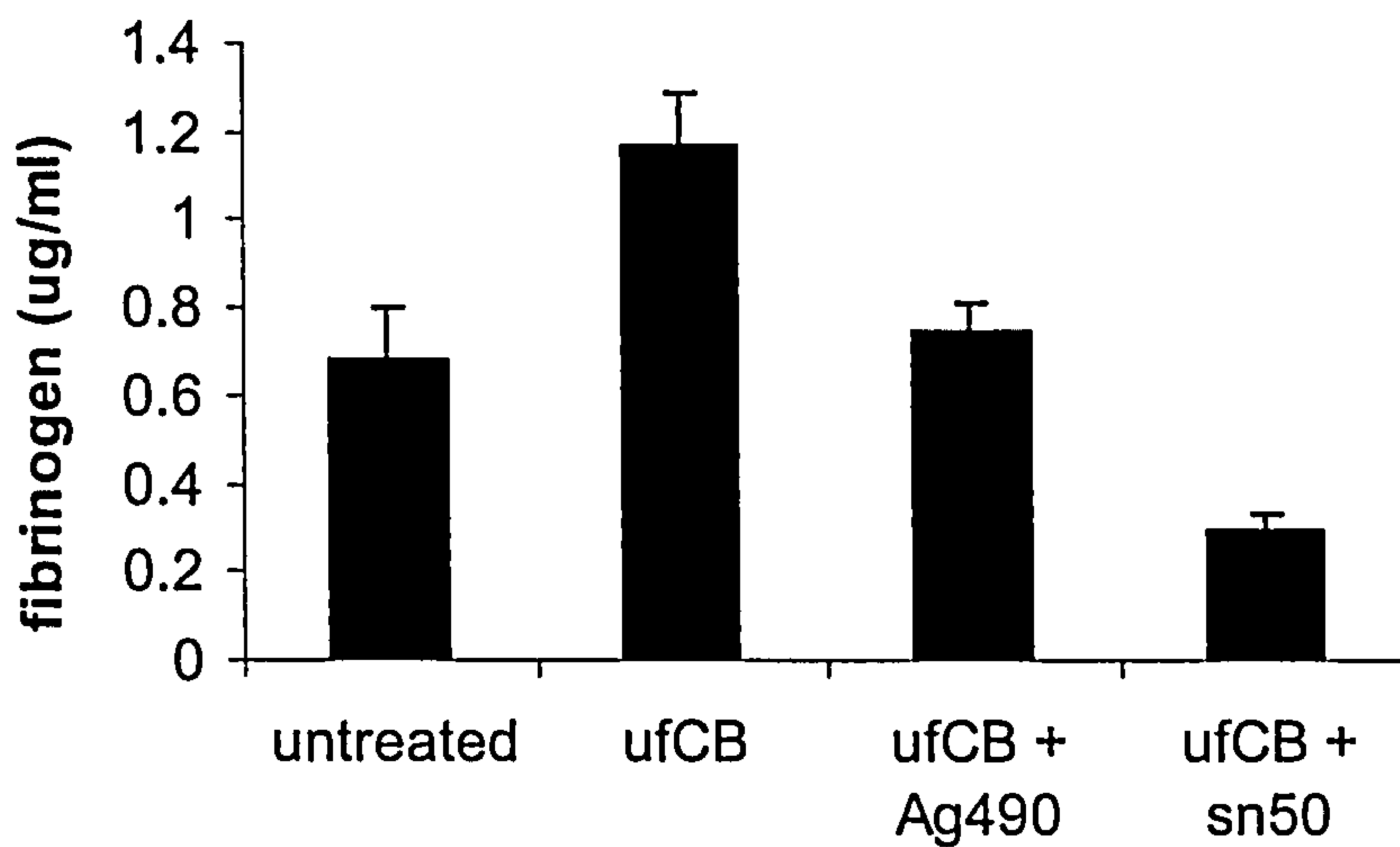


Figure 93: Concentration of fibrinogen in cell supernatant measured by ELISA after 6 hours of ufCB (100μg/ml) and transcription inhibitor treatment of A549 cells. Cells were treated with 50μM Ag490 and 50μg/ml sn50. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE.

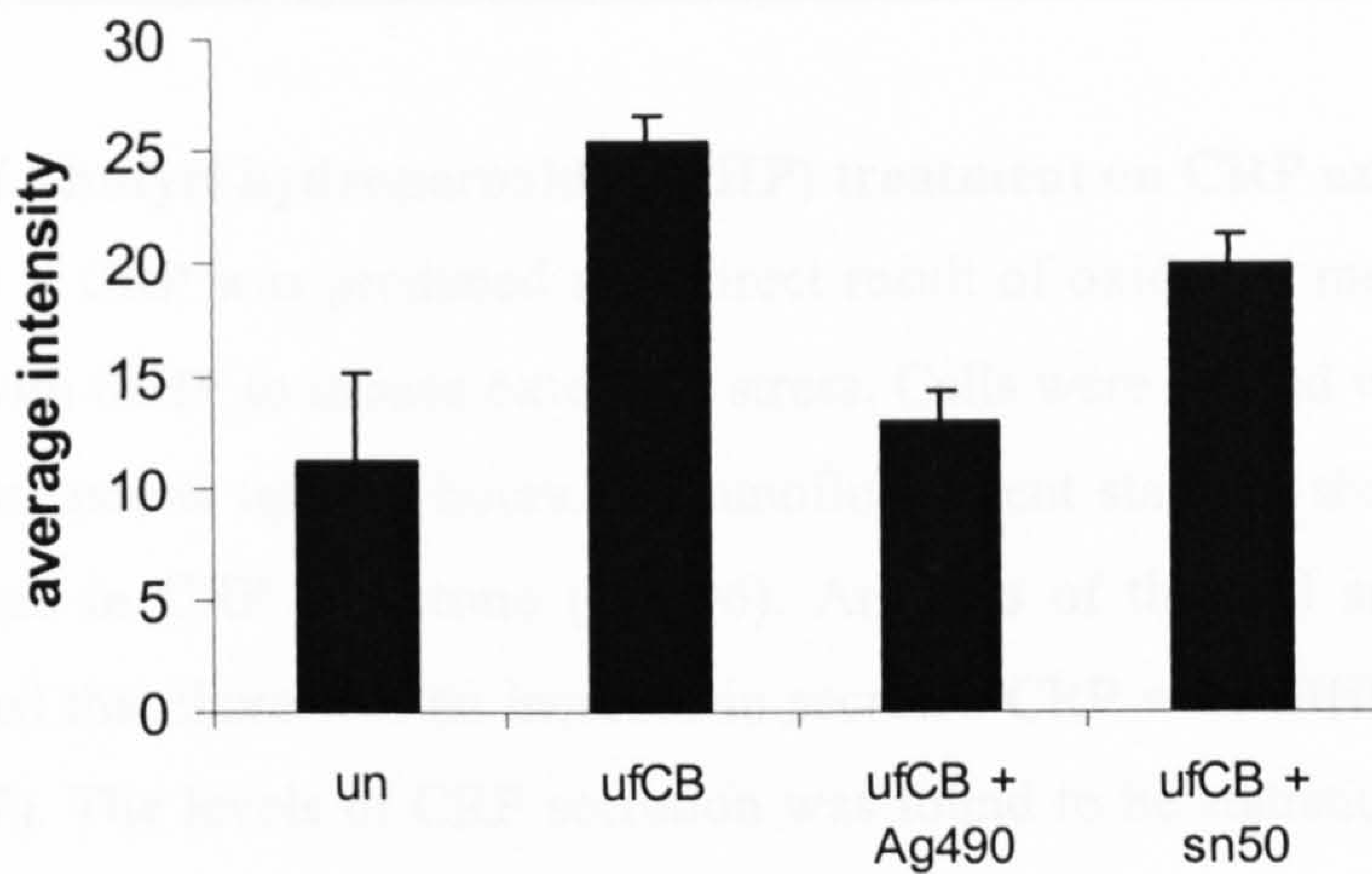


Figure 94: Immunofluorescent intensities of A459 cells stained for Hsp70 after 6 hours of ufCB (100μg/ml) and transcription inhibitor treatment of A549 cells. Cells were treated with 50μM Ag490 and 50μg/ml sn50. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE.

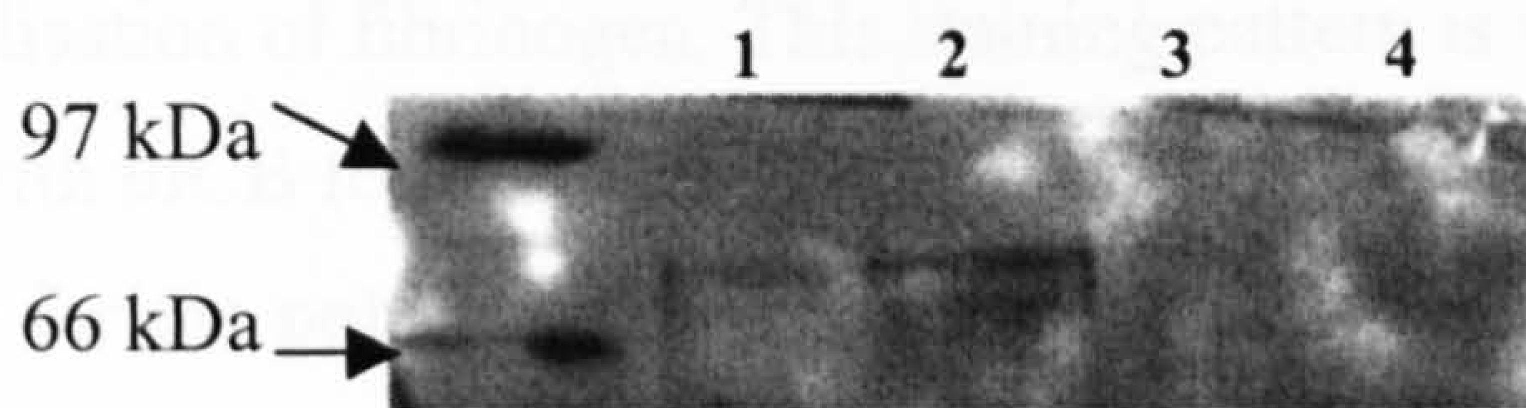


Figure 95: Western blot of A459 cells stained for Hsp70 after 6 hours of ufCB (100μg/ml) and transcription inhibitor treatment of A549 cells. Where lane 1 is untreated cells, 2 is ufCB treated, 3 is cells treated with 50μM Ag490 and ufCB and lane 4 is cells treated with 50μg/ml sn50 and ufCB. Where Ag490 is the inhibitor of the JAK/STAT transcription events and sn50 is an inhibitor of NFκB nuclear translocation. The results are the mean of triplicate results from 3 experiments ± SE.

5.3.0 Are CRP, Fibrinogen and Hsp70 produced in response to oxidative stress?

5.3.1 Effect of t-butyrl hydroperoxide (tBHP) treatment on CRP expression.

To investigate if CRP was produced as a direct result of oxidative mechanisms cells were treated with tBHP to induce oxidative stress. Cells were treated with 5μM tBHP for various time points up to 6 hours. Immunofluorescent staining showed that there was an increase in CRP with time (Fig 96). Analysis of the cell supernatants, by ELISA, showed that there was an increase in secreted CRP with tBHP over the time course (Fig 97). The levels of CRP secretion was found to be statistically significant after 6 hours of treatment ($p<0.05$).

5.3.2 Effect of t-butyrl hydroperoxide (tBHP) treatment on fibrinogen expression.

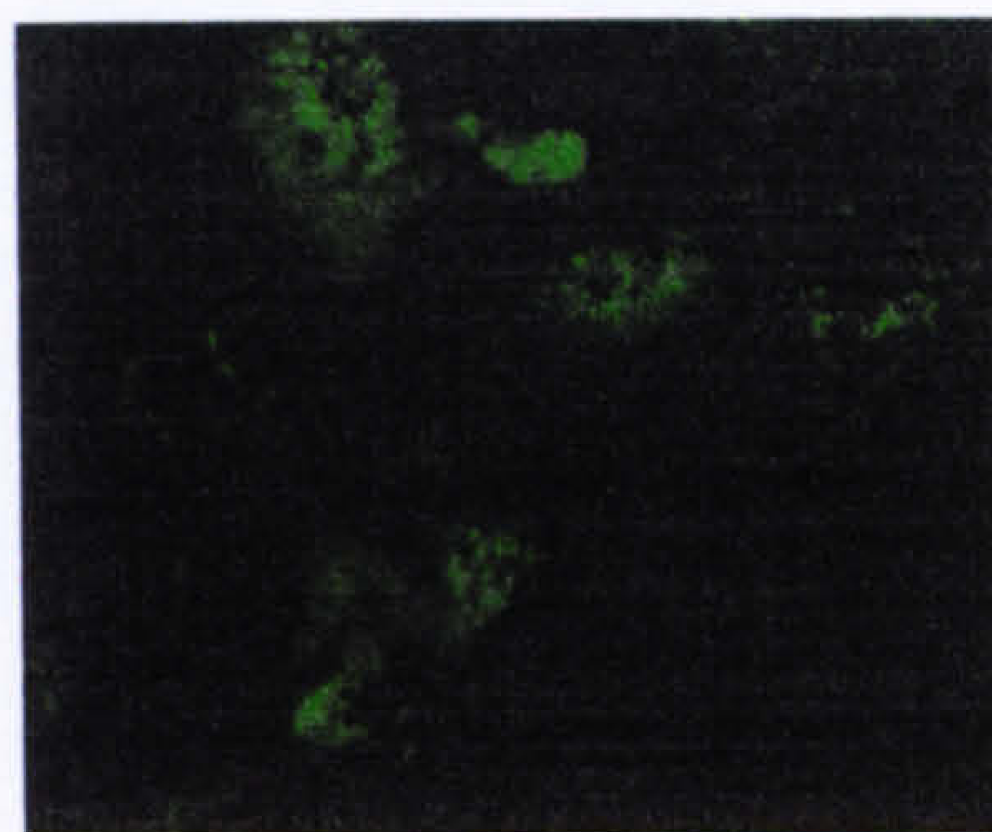
Cells were treated with tBHP similarly to that for CRP expression. Immunofluorescence staining showed that fibrinogen expression was increased over the time course (Fig 98). The staining pattern at the 6 hour time point shows some polarised localisation of fibrinogen. This staining pattern is very similar to that seen in cells treated with ufCB for 6 and 18 hours. This may suggest that a similar mechanism is responsible for the polarisation of fibrinogen for these treatments. Analysis of the cell supernatants, by ELISA, shows that there is an increase in the secretion of fibrinogen with time but differences between the experimental results are too great to achieve any statistical significance (Fig 99).

5.3.3 Effect of t-butyrl hydroperoxide (tBHP) treatment on Hsp70 expression.

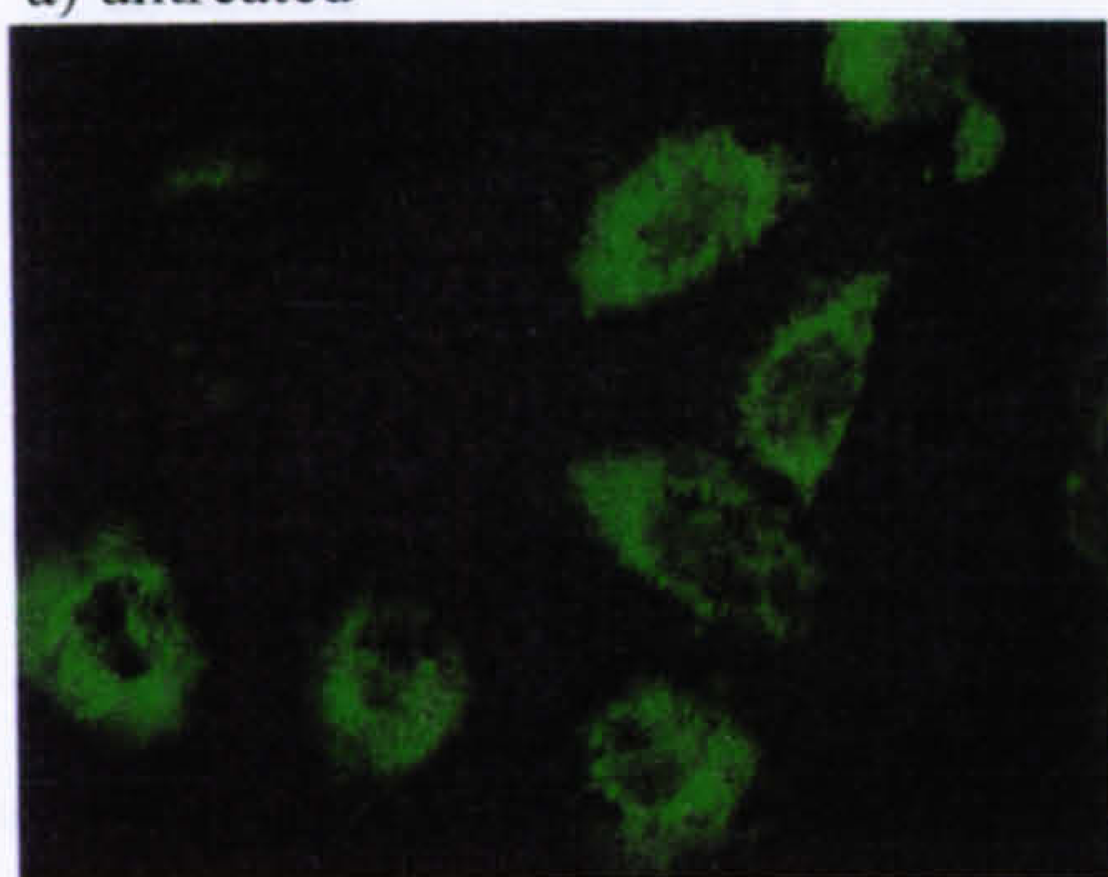
Cells treated with tBHP for up to 6 hours showed that there was an increase in the levels of Hsp70. Immunofluorescence analysis shows that there is some nuclear localisation of Hsp70 by 30 minutes of treatment (Fig 100). At the 6 hour time point there are several different staining patterns present including nuclear and peri-nuclear staining patterns. Some of the cells would appear to be activated and producing projections which have some Hsp70 staining. Hsp70 may be distributed evenly along the cytoplasm or there may be membrane staining. The perinuclear staining shows a slight polarised effect which may suggest morphological changes in the cells. Analysis of the cell supernatants was carried out using Western blotting (Fig 101).

This shows that there is a large increase in Hsp70 by half an hour of treatment and this continues to be increased up to the 6 hour time point.

a) untreated



b) ½ hour treatment



c) 6 hour treatment

Figure 96: Expression of C-reactive protein (CRP) in A549 cells treated with 5μM tBHP, for various time points up to 6 hours as shown by indirect immunofluorescence.

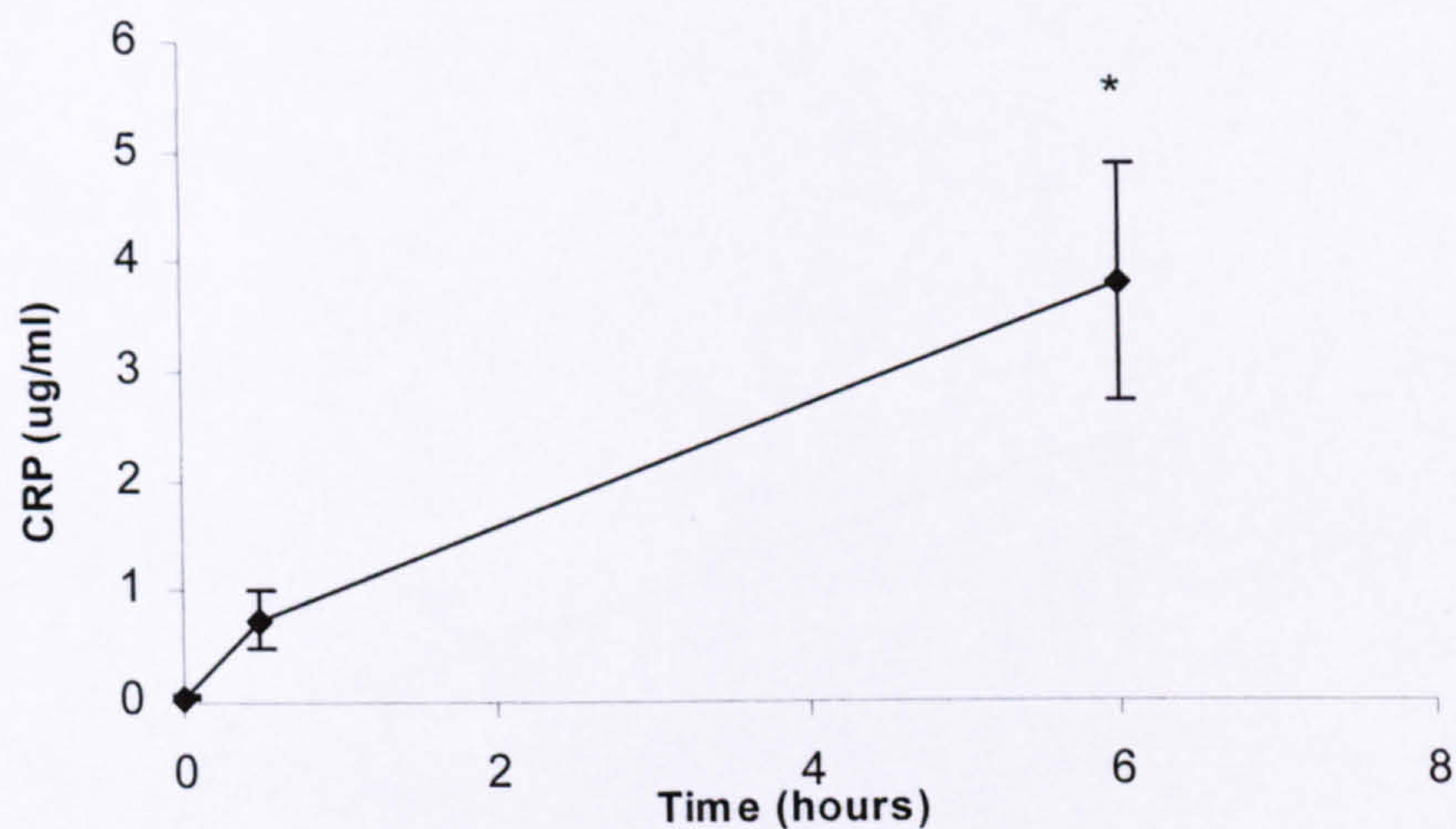
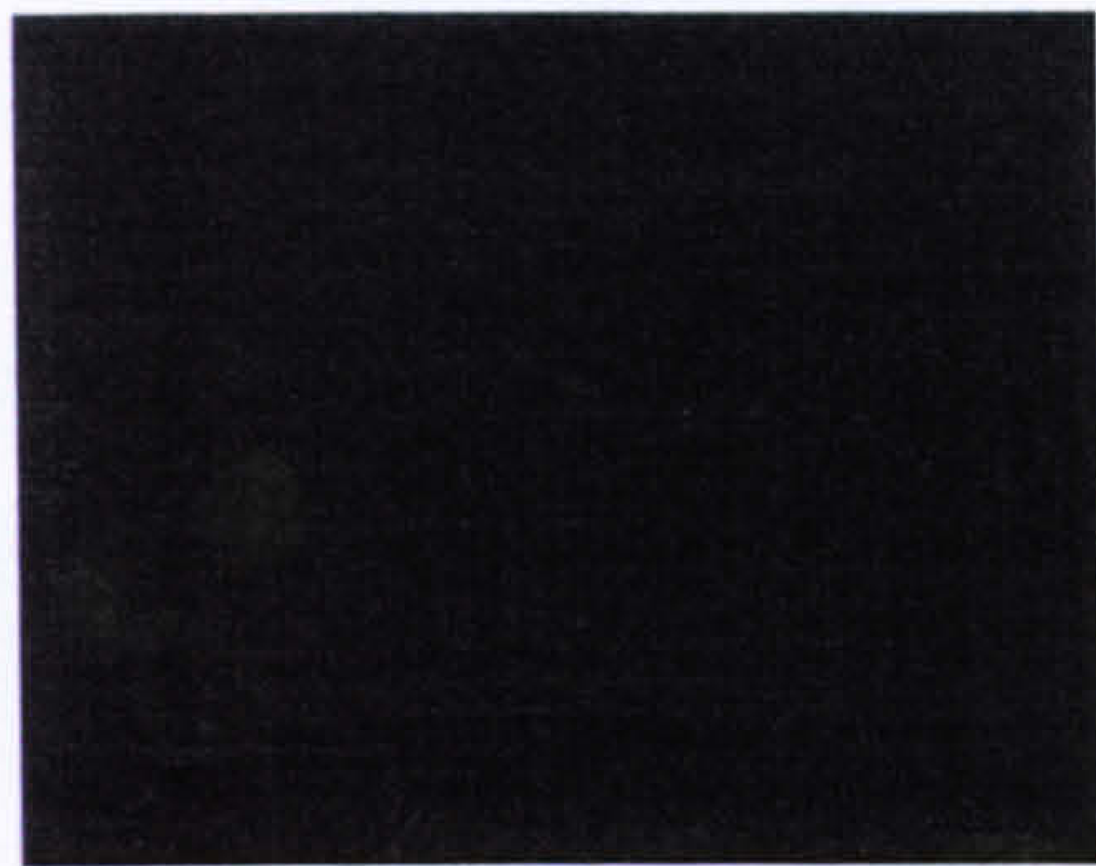
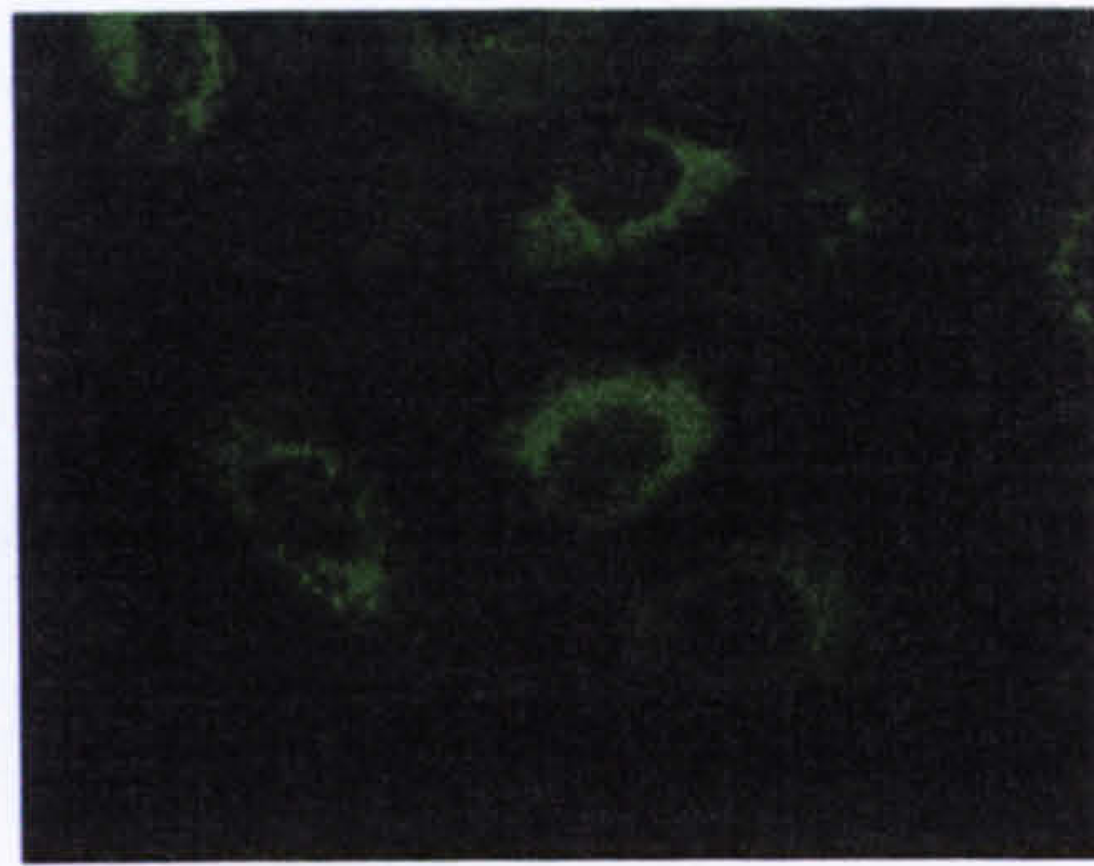


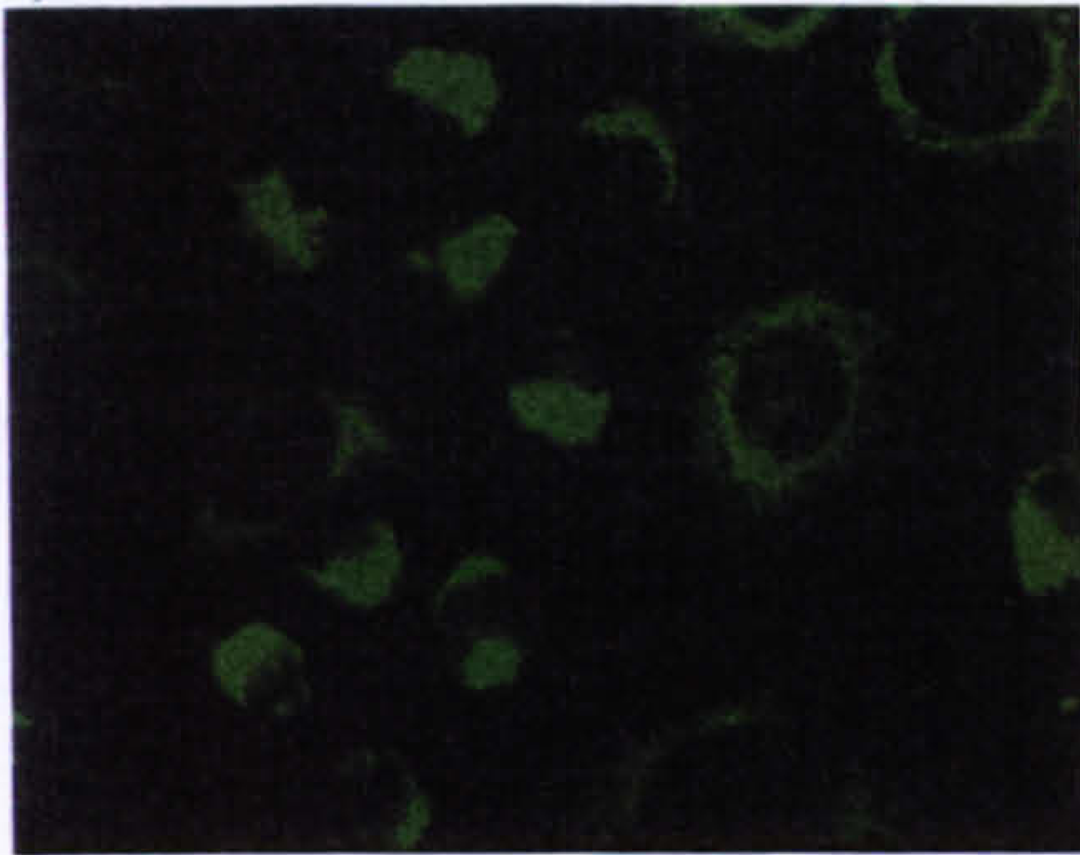
Figure 97: Concentration of CRP in cell supernatant measured by ELISA after 6 hours of 5μM tBHP treatment of A549 cells. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$.



a) untreated



b) ½ hour treatment



c) 6 hour treatment

Figure 98: Expression of fibrinogen in A549 cells treated with 5μM tBHP, for various time points up to 6 hours as shown by indirect immunofluorescence.

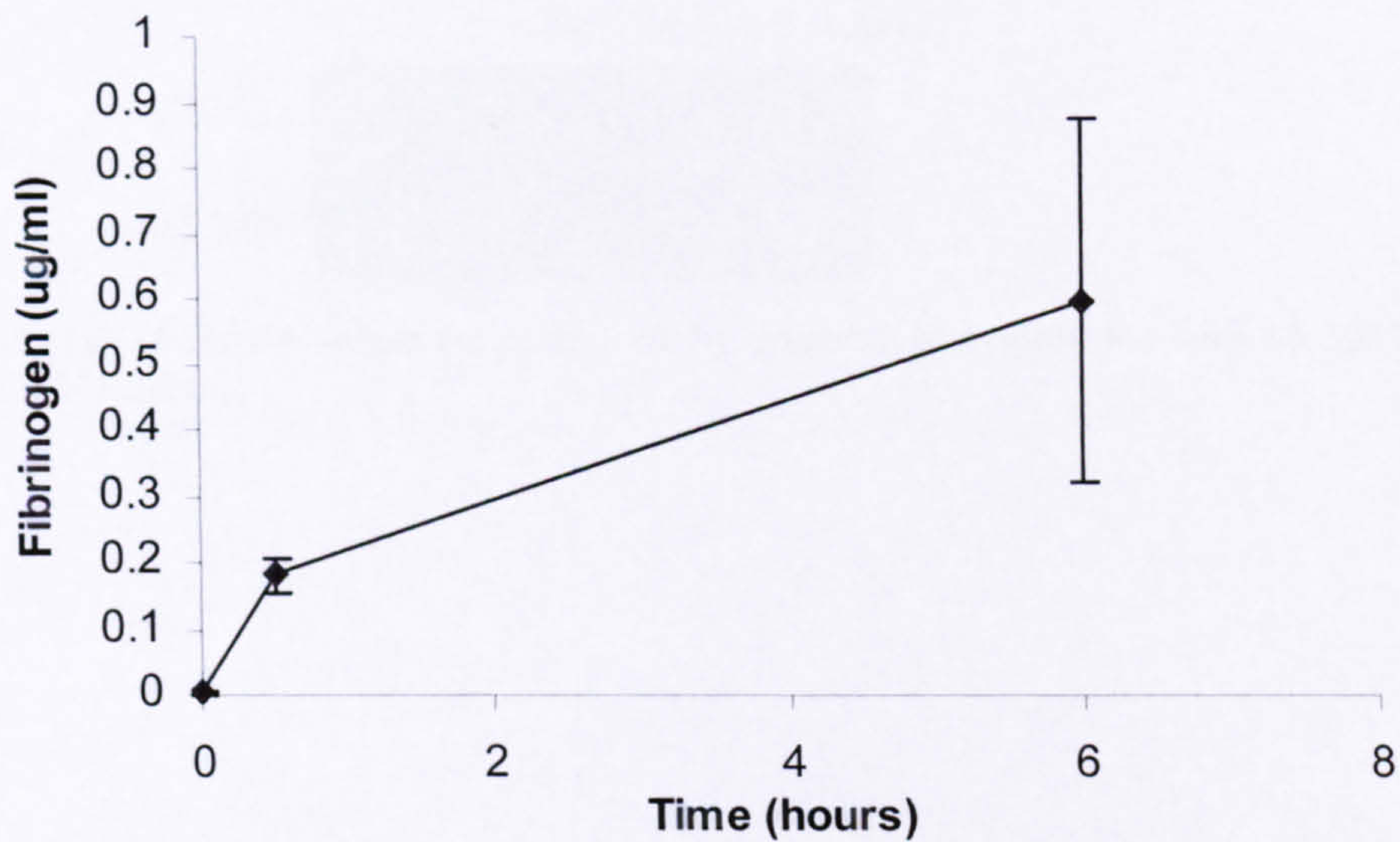


Figure 99: Concentration of fibrinogen in cell supernatant measured by ELISA after 6 hours of 5μM tBHP treatment of A549 cells. The results are the mean of triplicate results from 3 experiments ± SE.

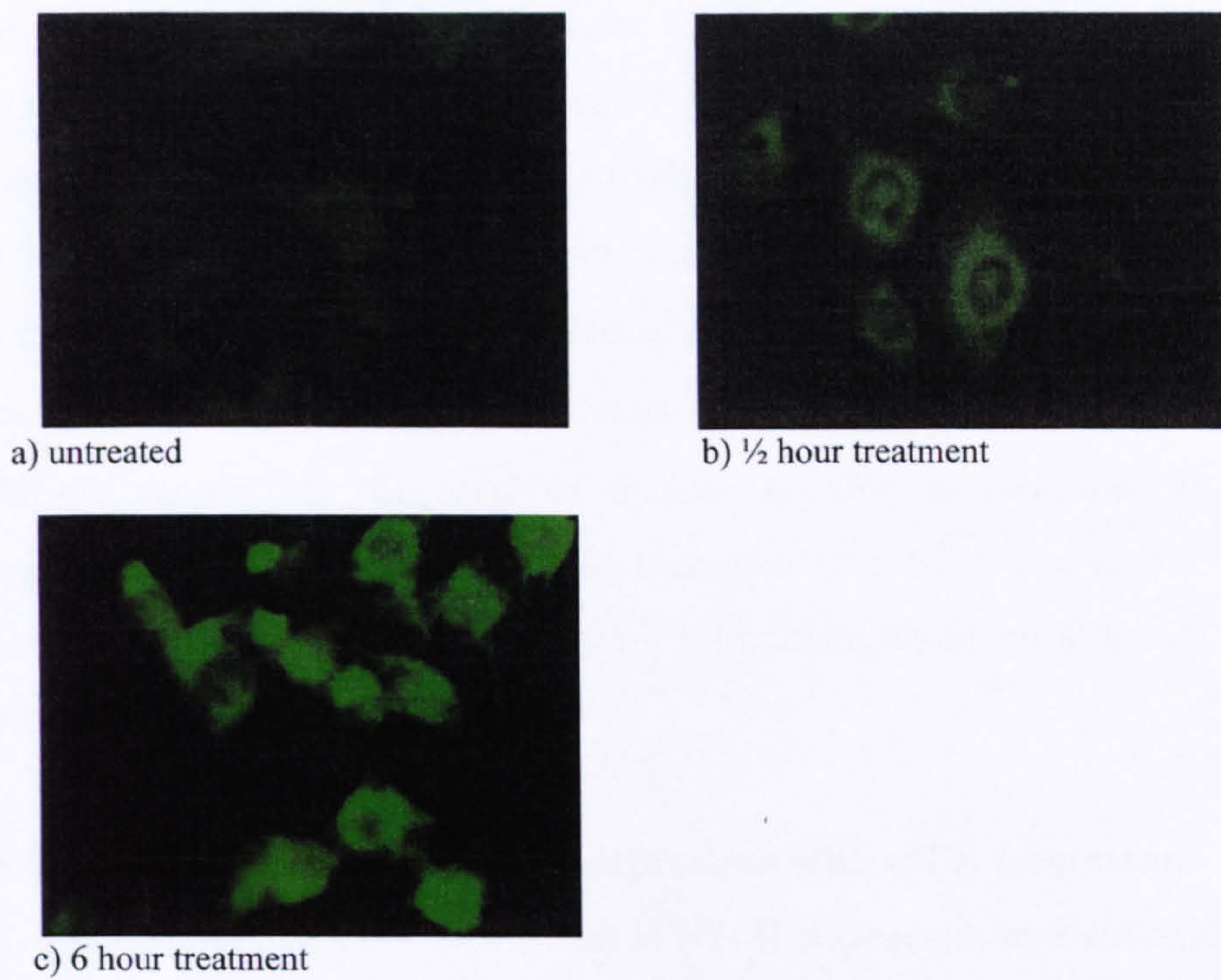


Figure 100: Expression of Hsp70 in A549 cells treated with 5μM tBHP, for various time points up to 6 hours as shown by indirect immunofluorescence.

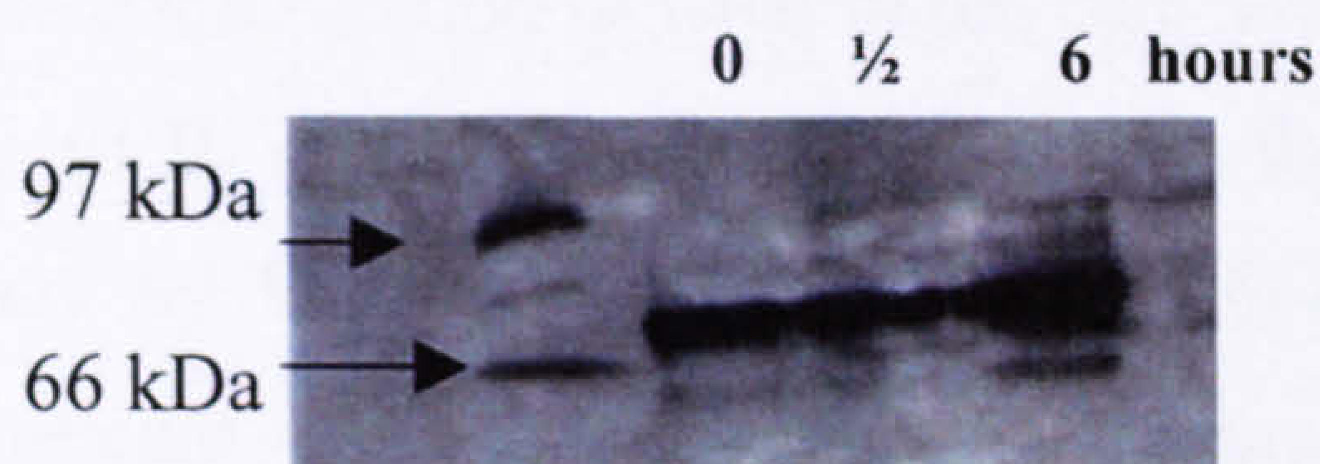


Figure 101: Level of Hsp70 secretion measured by western blot after 6 hours of 5μM tBHP treatment of A549 cells.

5.3.4 Effect of antioxidants on NFκB expression with ufCB treatment.

To investigate if the increase in cellular NFκB and its nuclear translocation was a result of oxidative stress cells were treated with ufCB in the presence of antioxidants. The antioxidants used were Nacystelin (NAL) and Trolox. Antioxidants have been shown to prevent the nuclear translocation of NFκB by mopping up free radicals, thus reducing the signalling and degradation of IκB. Cells treated with ufCB show some nuclear localisation of NFκB after 6 hours of treatment. Immunofluorescent staining for NFκB shows that the level of nuclear staining is decreased in cells with antioxidants simultaneously with ufCB treatment (Fig 102). The amount of nuclear staining is not completely eliminated with antioxidant treatment although it is greatly reduced.

5.3.5 Effect of antioxidants on CRP expression with ufCB treatment.

After establishing that CRP expression is NFκB dependent, and antioxidants reduce the translocation of NFκB, the effect of antioxidants on CRP expression was carried out. Cells were treated for 6 hours in the presence of ufCB and antioxidants. ELISAs were carried out (Fig 103) on the cell lysates of treated cells. This showed that ufCB increased the level of CRP and this increase was statistically significant from untreated cells. Treatments with both the antioxidants in the presence of ufCB showed a reduction in the concentration of CRP in the cell lysates. Although this result is not significant from ufCB, it is no longer different from the control cells. Analysis of the cells supernatants by ELISA (Fig 104) showed that once again ufCB induced a statistically significant increase in the amount of secreted CRP ($p<0.05$). When cells were treated with ufCB and antioxidants the level of secreted CRP was reduced to the level in the untreated cells for both NAL and Trolox and this reduction was statistically significant for both ($p<0.05$).

5.3.6 Effect of antioxidants on fibrinogen expression with ufCB treatment.

The effect of antioxidants on the expression of fibrinogen in A549 cells with ufCB was carried out as for CRP. ELISAs of the cell lysate showed that ufCB induced an increase in fibrinogen which was statistically significant ($p<0.05$) from untreated cells (Fig 105). The cells treated with antioxidants simultaneously with ufCB showed a small decrease in the level of fibrinogen however when the degree of error is taken

into consideration this decrease is small. Analysis of the cell supernatants using ELISAs (Fig 106) showed that ufCB increased the level of secreted fibrinogen in a statistically significant manner ($p<0.05$). The cells treated with ufCB and antioxidants simultaneously showed a decrease to the level in the untreated cells and this decrease was statistically significant ($p<0.05$).

5.3.7 Effect of antioxidants on Hsp70 expression with ufCB treatment.

Treatment of A549 cells with antioxidants and ufCB was carried out to identify the effects on the expression of Hsp70. Immunofluorescence staining of the cells for Hsp70 was carried out and the average fluorescent intensity of the cells was calculated (Fig 107). This showed that ufCB increased the level of Hsp70 in the cells and this was reduced by simultaneous treatment of ufCB with antioxidants. With the ufCB and NAL treatment inducing a decrease which is statistically significant ($p<0.05$). Western blots were carried out on the cell supernatants (Fig 108). This showed that ufCB increased the level of Hsp70 secreted from the cells. Treatment in the presence of antioxidants reduced the levels of secreted Hsp70 induced by ufCB.

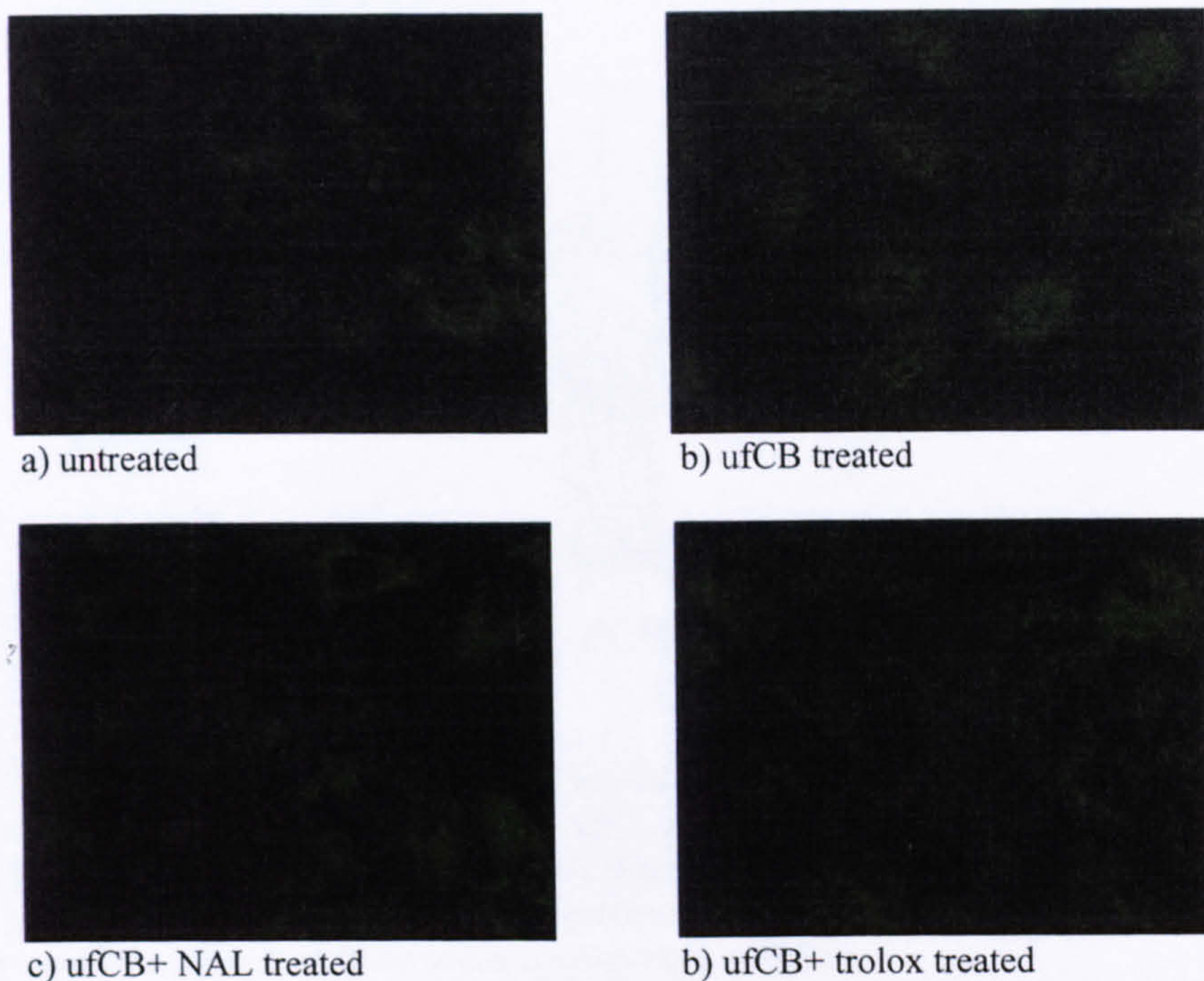


Figure 102: Expression of NFκB in A549 cells treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM) for 6 hours as shown by indirect immunofluorescence.

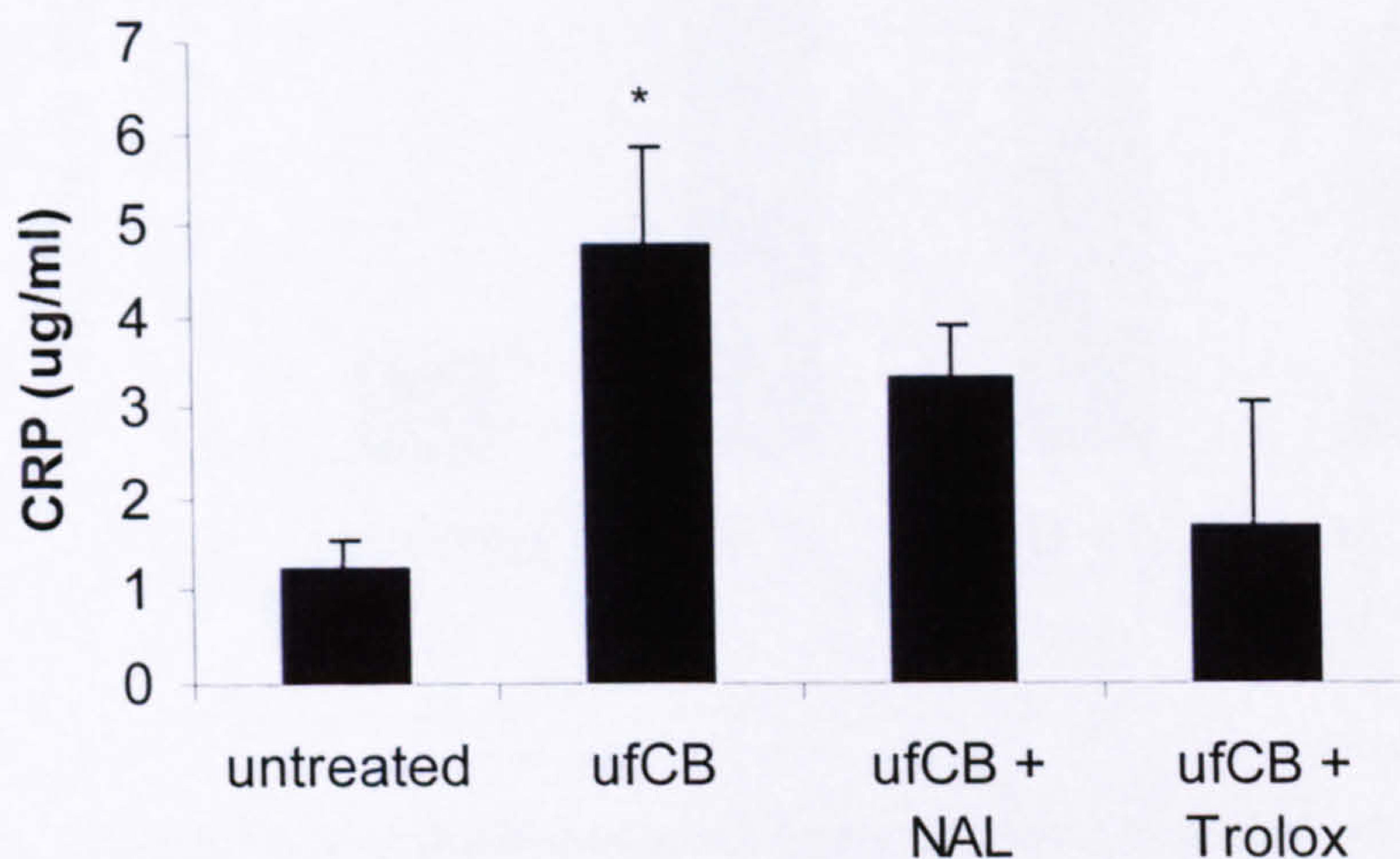


Figure 103: Concentration of CRP in cell lysate measured by ELISA after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$.

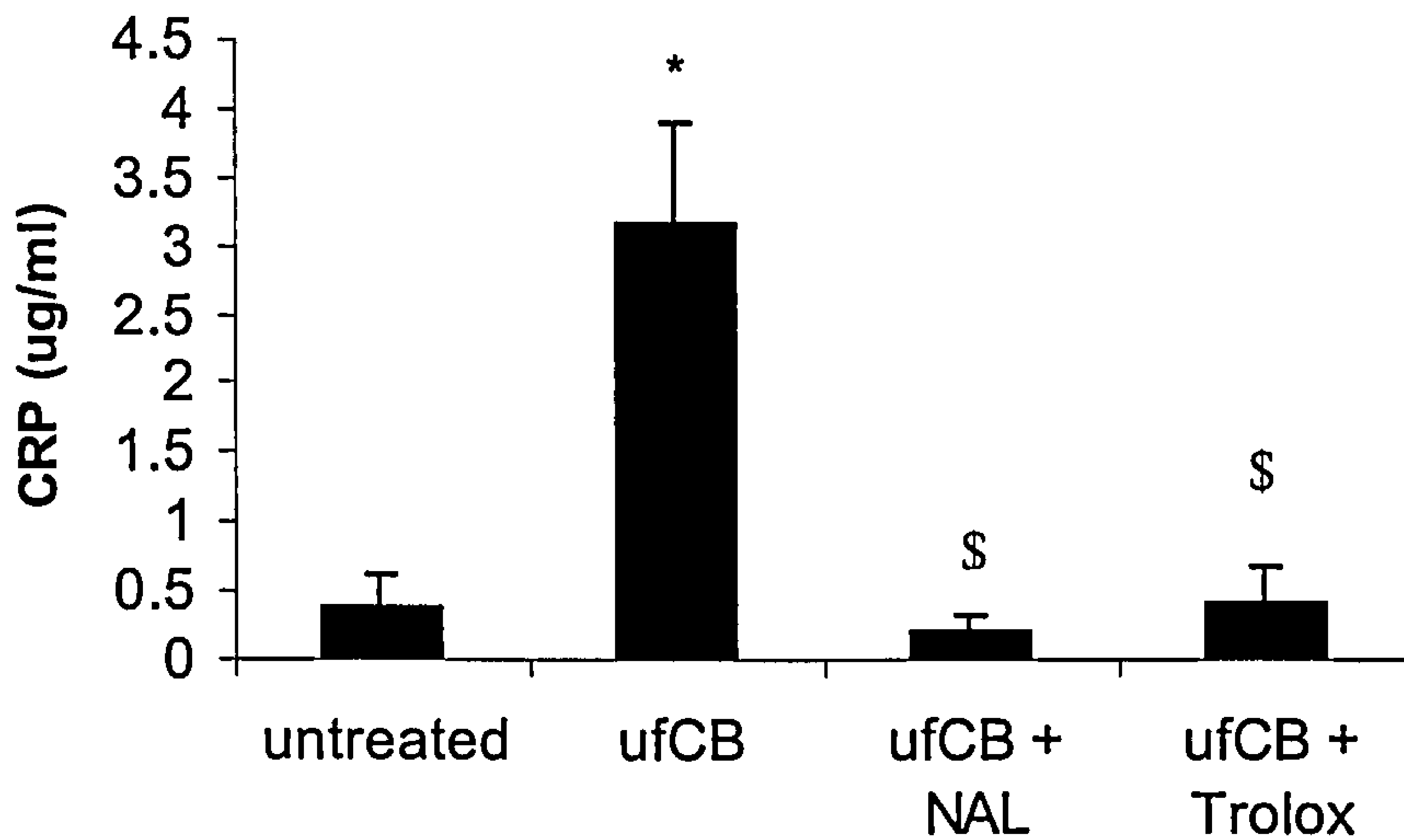


Figure 104: Concentration of CRP in cell supernatant measured by ELISA after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$ and $ denotes significant decrease from ufCB treated cells, $p<0.05$.

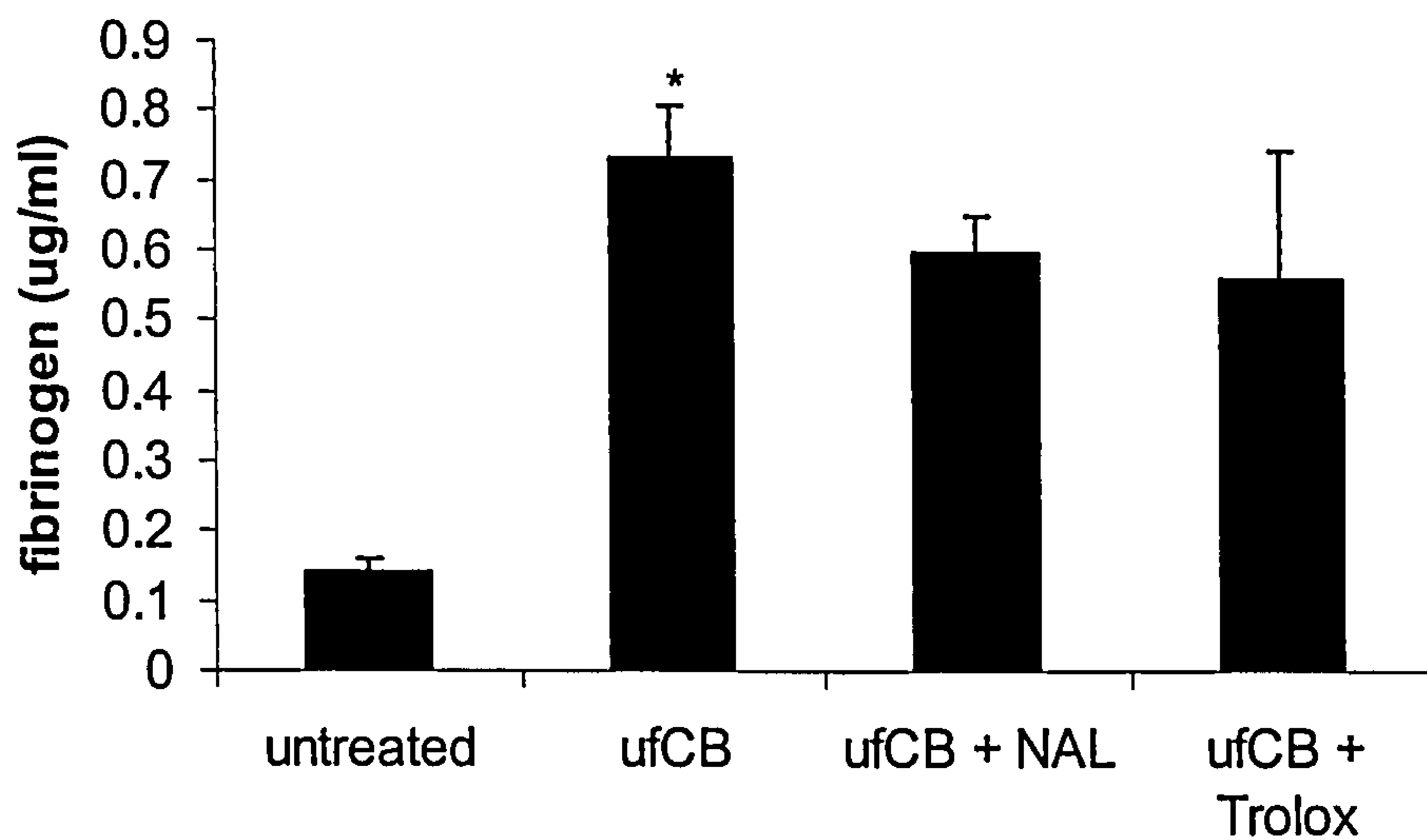


Figure 105: Concentration of fibrinogen in cell lysate measured by ELISA after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, $p < 0.05$.

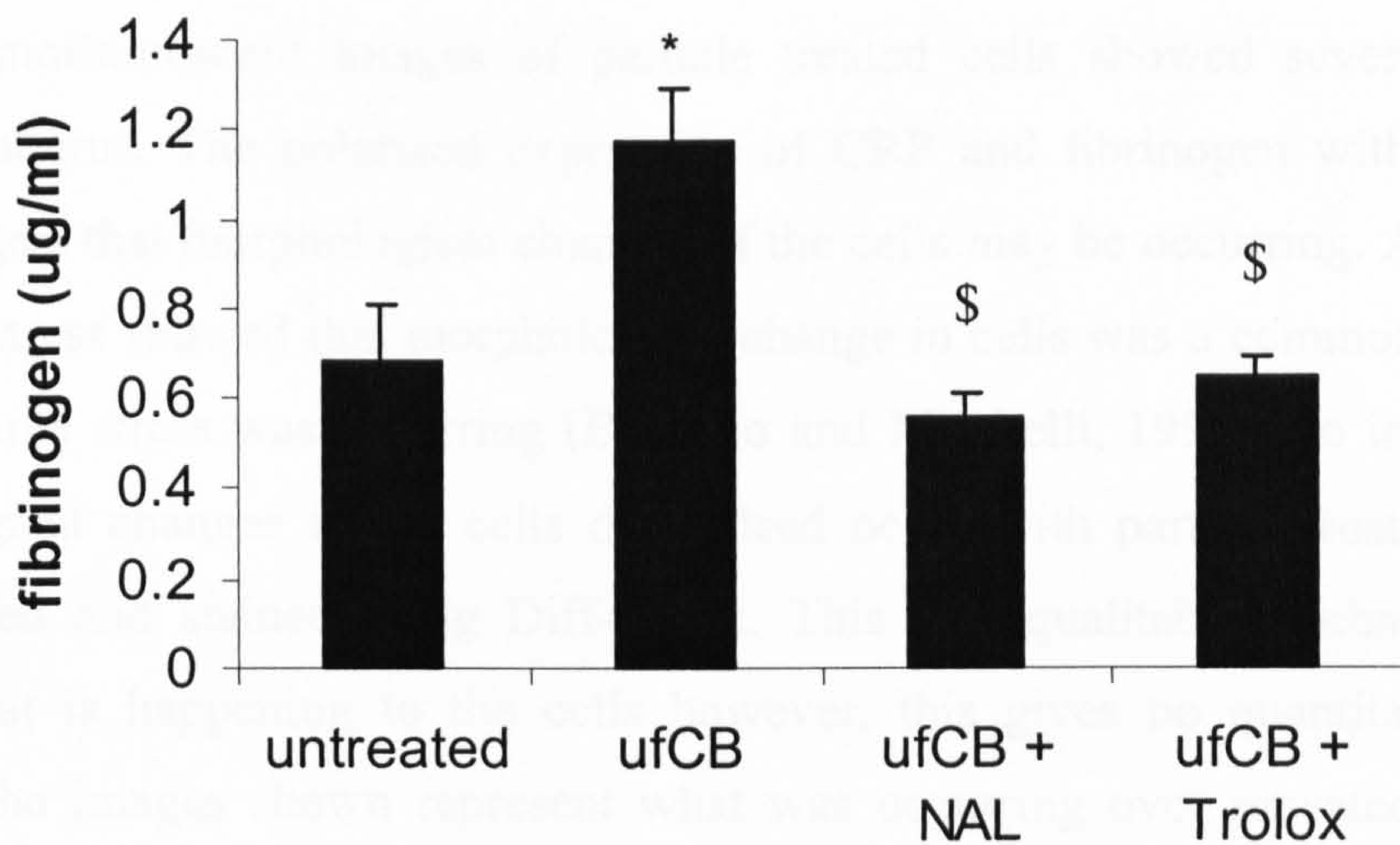


Figure 106: Concentration of fibrinogen in cell supernatant measured by ELISA after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, p< 0.05 and $ denotes significant changes from ufCB treated cells, p<0.05.

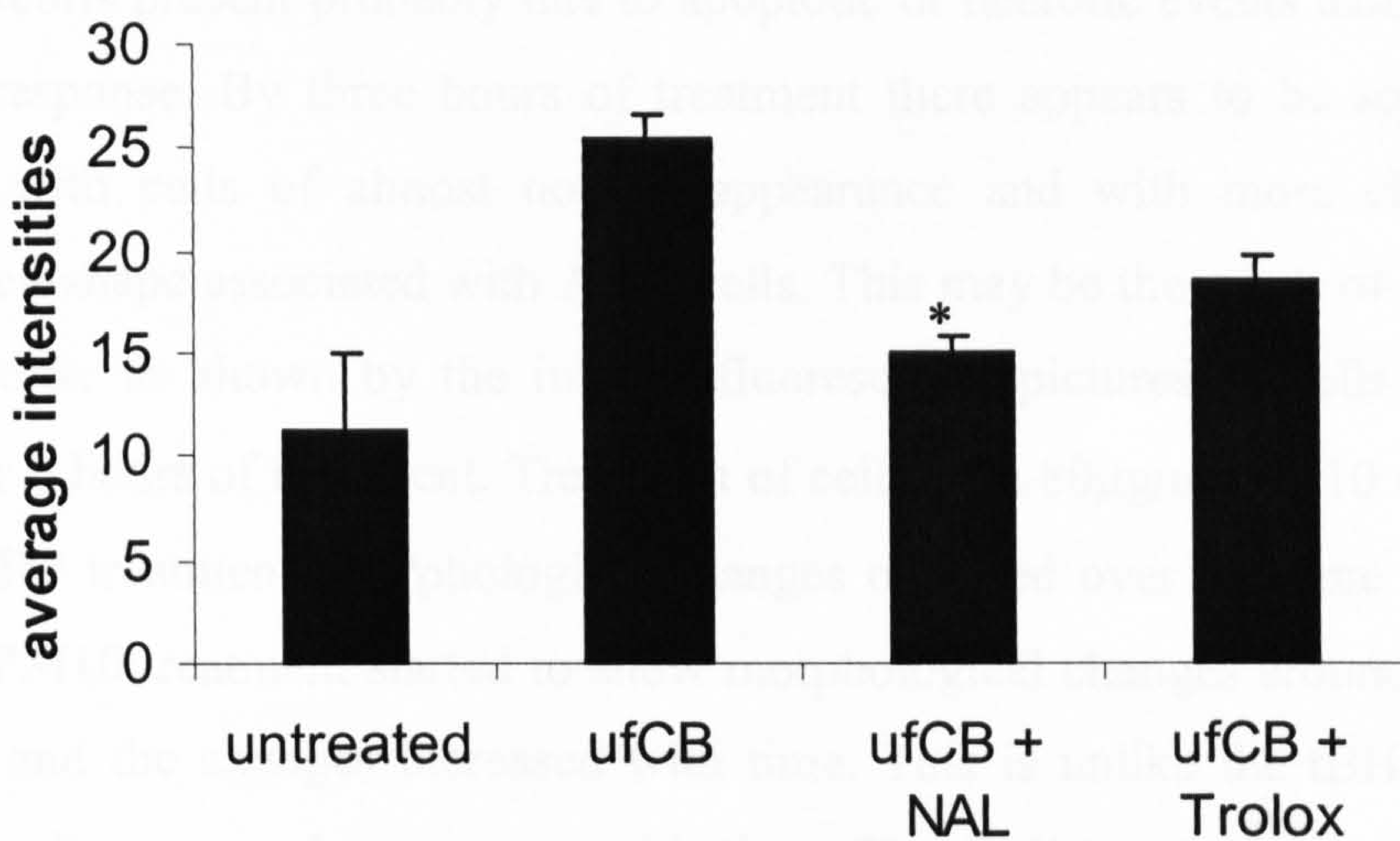


Figure 107: Level of Hsp70 expression measured by immunofluorescence after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated cells, p< 0.05.

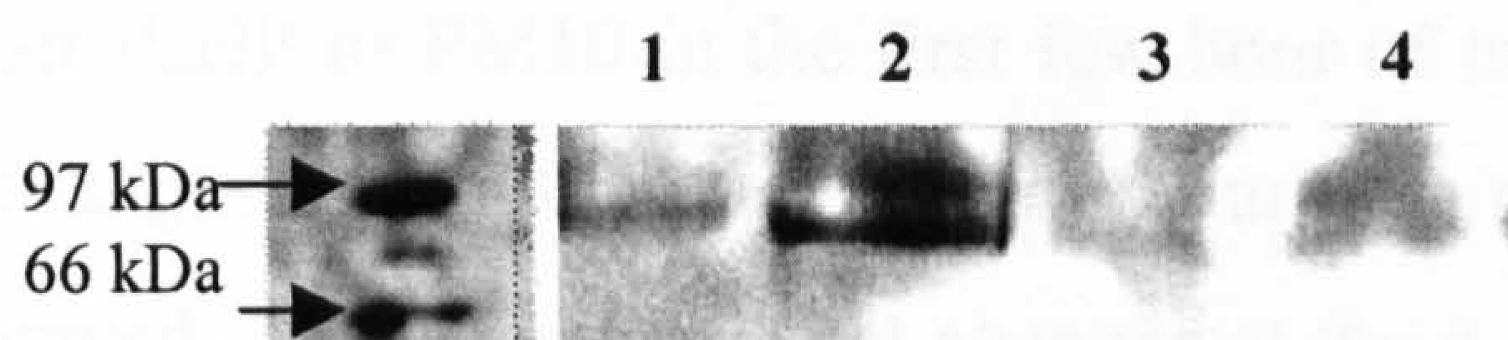
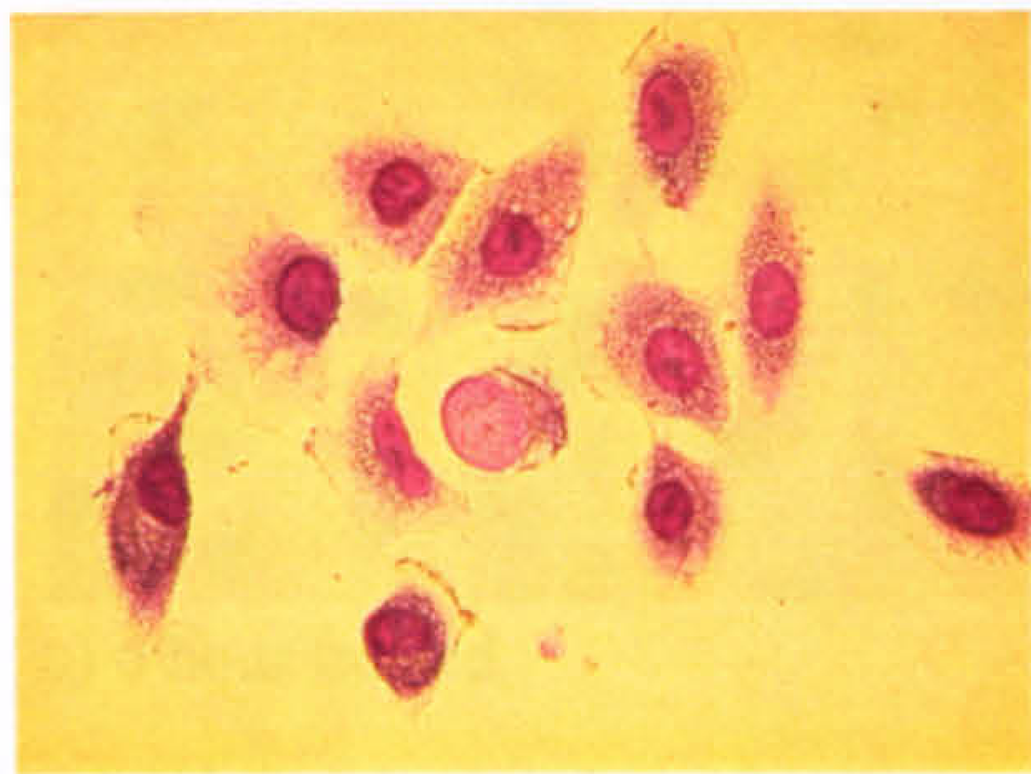


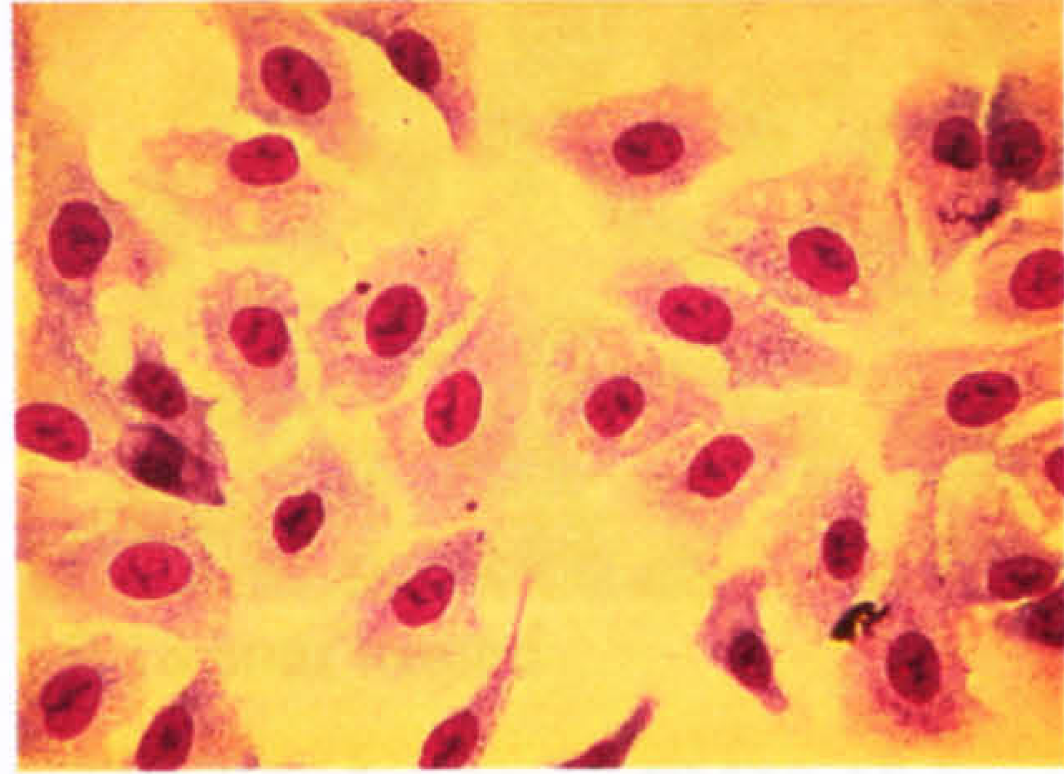
Figure 108: Level of Hsp70 secretion measured by western blot after 6 hours of ufCB (100μg/ml) and antioxidant treatment of A549 cells. Cells were treated with the antioxidants Nacystalin (NAL, 200μM) or Trolox (25μM). Where lane 1 is untreated cells, 2 is ufCB treated, 3 is NAL treated and 4 is Trolox treated cells. The results are the mean of triplicate results from 3 experiments ± SE.

5.3.8. Morphological changes in A549 cells with particle changes.

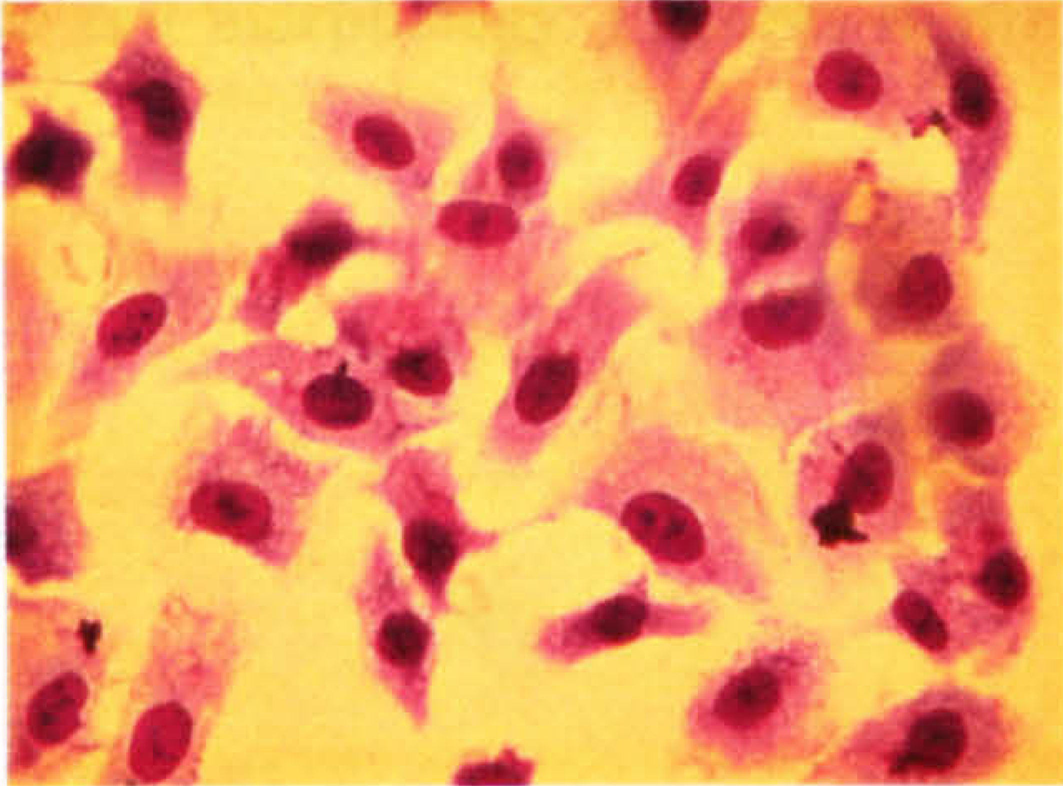
The immunofluorescent images of particle treated cells showed several different staining patterns. The polarised expression of CRP and fibrinogen with ufCB and tBHP suggest that morphological changes of the cells may be occurring. A review on oxidative stress showed that morphological change in cells was a common indication that oxidative stress was occurring (Bellomo and Mirabelli, 1992). To investigate if morphological changes in the cells did indeed occur with particle treatments cells were treated and stained using Diff-Quick. This is a qualitative technique which shows what is happening to the cells however, this gives no quantitative results although the images shown represent what was occurring over repeated measures. Cells treated with 5μM tBHP would be expected to be under going oxidative stress and when the Diff-Quick stained slides were analysed there did appear to be some morphological changes over the first 6 hours of treatment (Fig 109). By one hour of treatment there was elongation of the cells and after two hours there appeared to be some cell debris present probably due to apoptotic or necrotic events associated with the stress response. By three hours of treatment there appears to be some cellular adaptation with cells of almost normal appearance and with more characteristic spherical cell shape associated with A549 cells. This may be the result of a large heat shock response as shown by the immunofluorescence pictures of cells stained for Hsp70 after 6 hours of treatment. Treatment of cells with 80μg/ml PM10 showed that like the tBHP treatment, morphological changes occurred over the time course (Fig 110). The PM10 treatment started to show morphological changes around the 3 hour time point and the changes increased with time. This is unlike the tBHP treatment where the cells appeared to recover with time. The PM10 treatment appears to be activating the cells with increased cell projections present, particularly after 18 hours of treatment. Treatment with 100μg/ml ufCB showed morphological changes from half an hour of treatment (Fig 111). These changes appeared to be greater than those produced by either tBHP or PM10 in the first few hour of treatment, although ufCB showed similar changes as PM10 at the 6 and 18 hour time points. Cells exposed to 100μg/ml CB showed some morphological changes at the 6 and 18 hour time points although they were not as great as the changes shown with PM10 of ufCB treatment (Fig 112). Cells exposed to 100mM $FeCl_3$ showed very few morphological changes over the 18 hour time course (Fig 113).



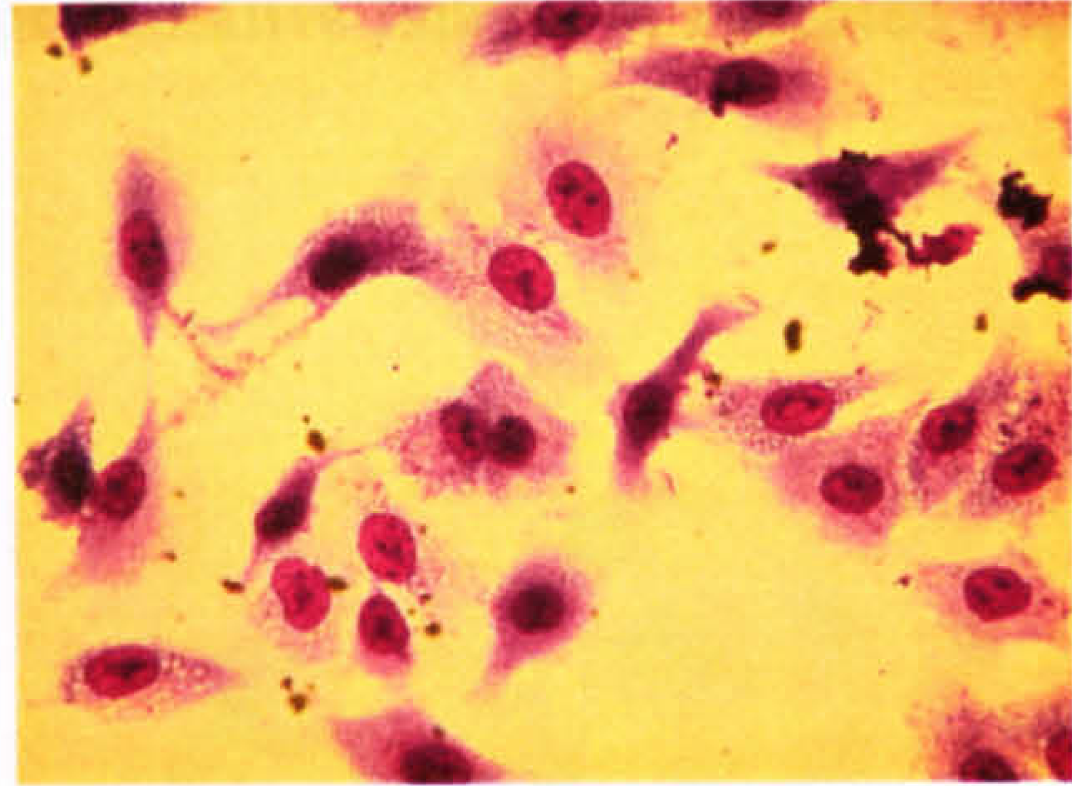
a) untreated



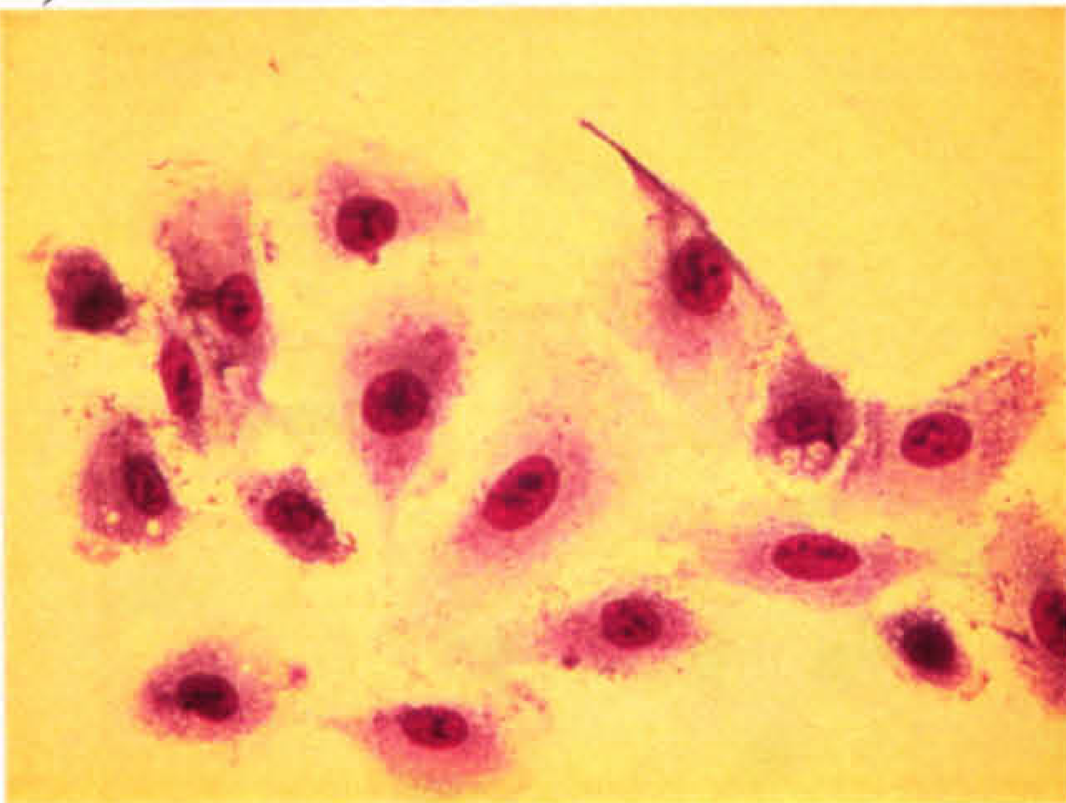
b) ½ treated



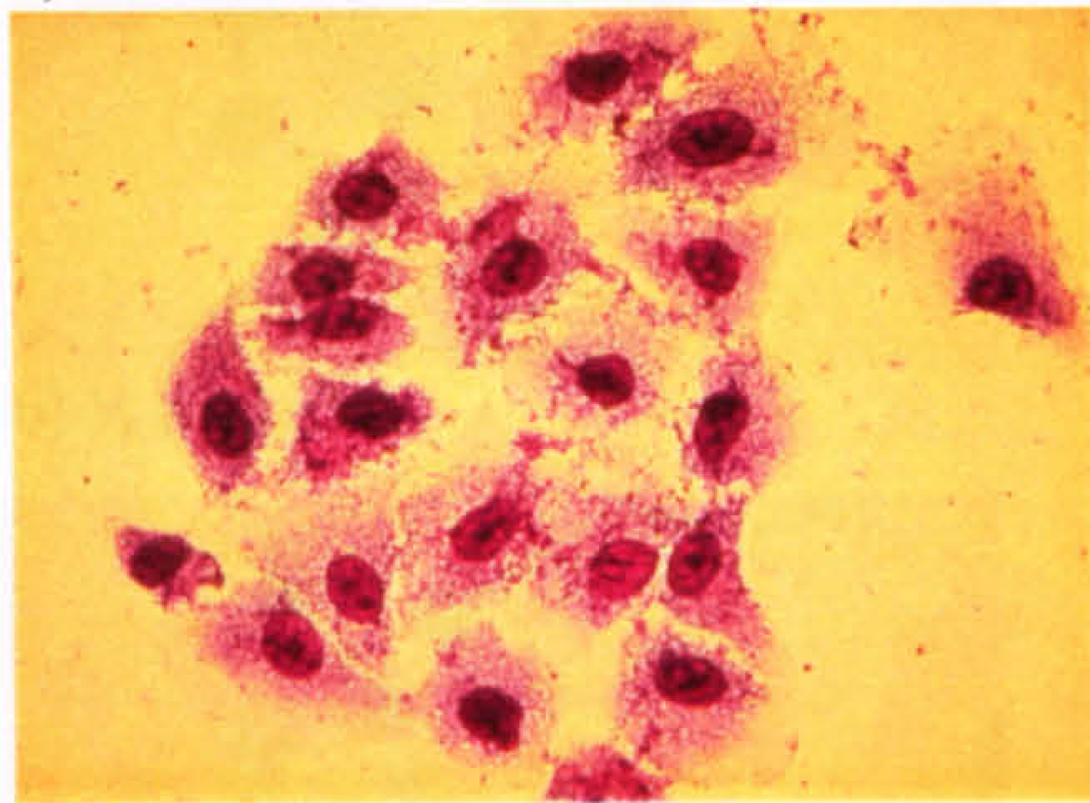
c) 1 hour treated



d) 2 hour treated

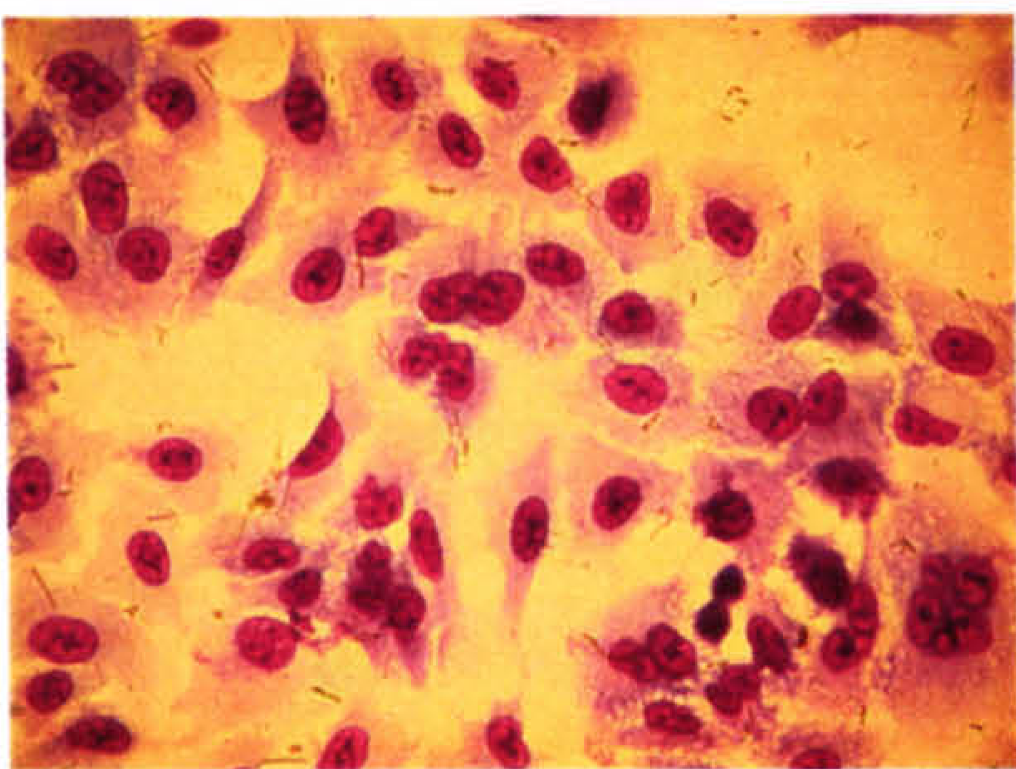


e) 3 hour treated

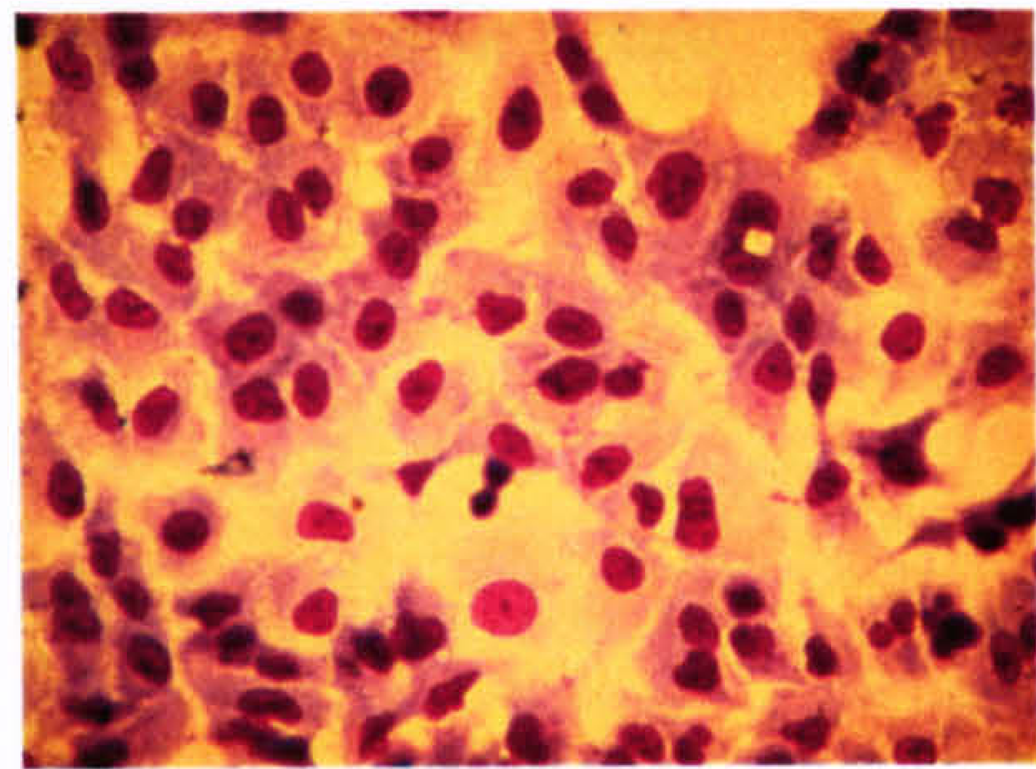


f) 4 hour treated

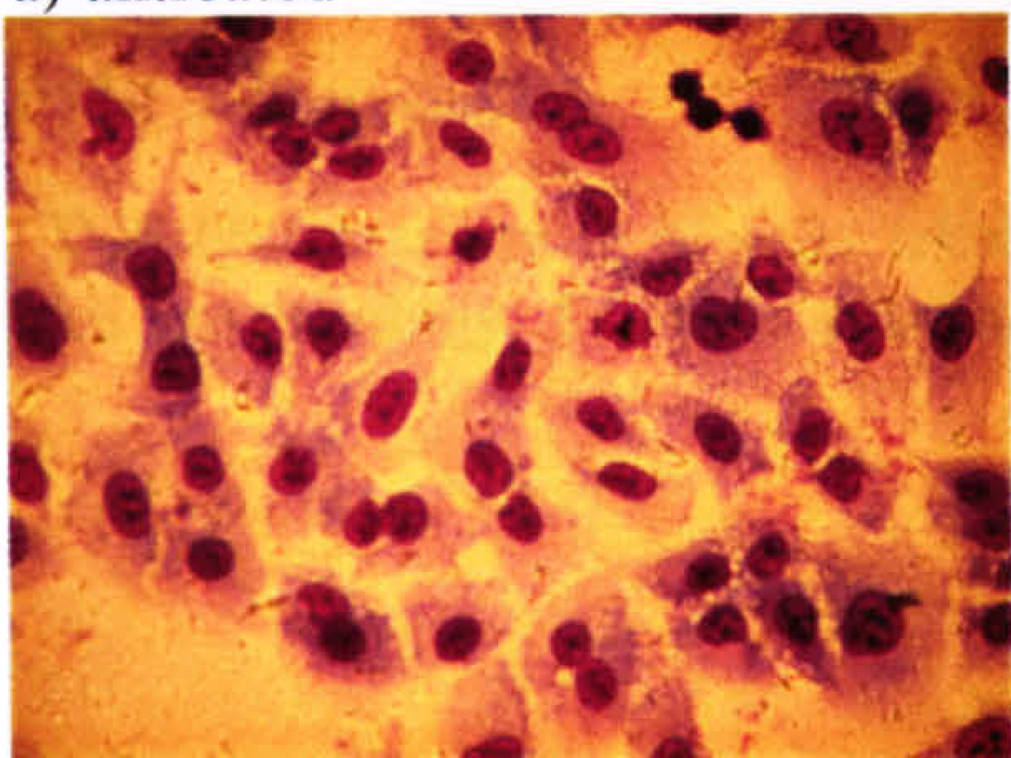
Figure 109: Diff-Quick staining of cells treated with 5μM tBHP for up to 4 hours to show changes in cell morphology. Pictures shown are representative images of repeated experiments (n=3).



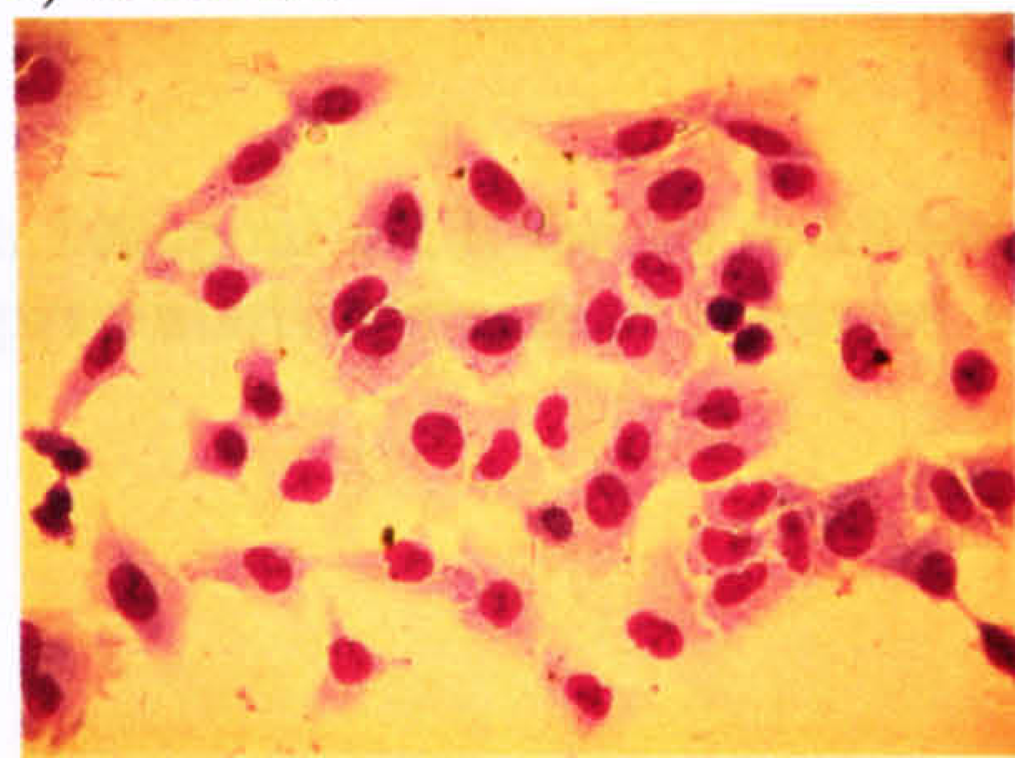
a) untreated



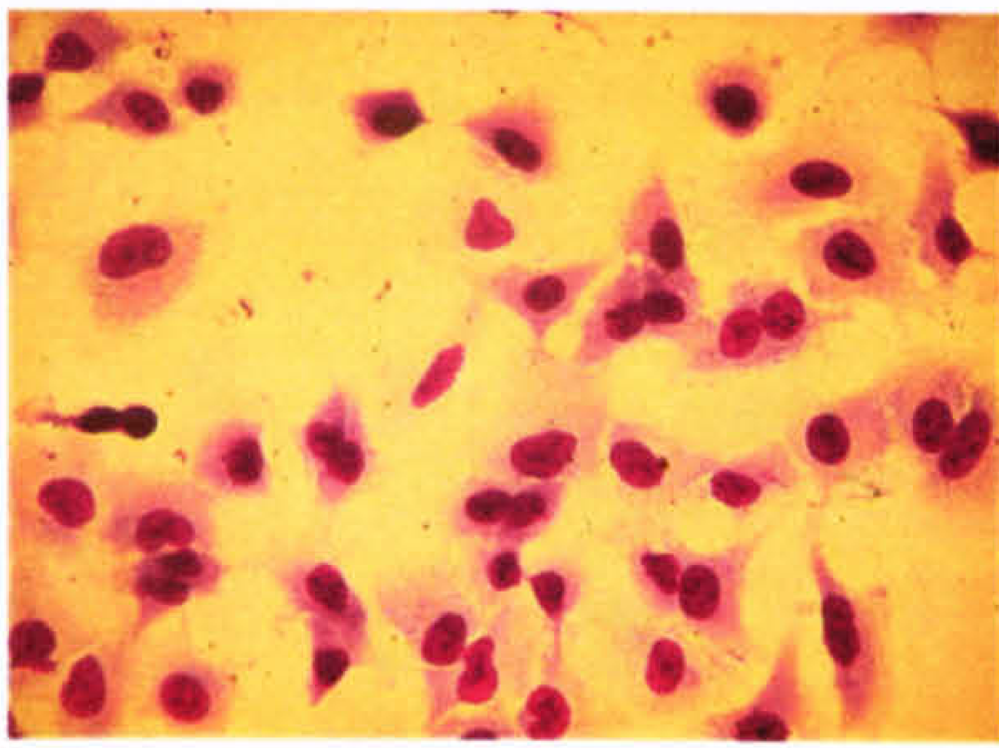
b) ½ treated



c) 1 hour treated



d) 3 hour treated


e) 6 hour treated

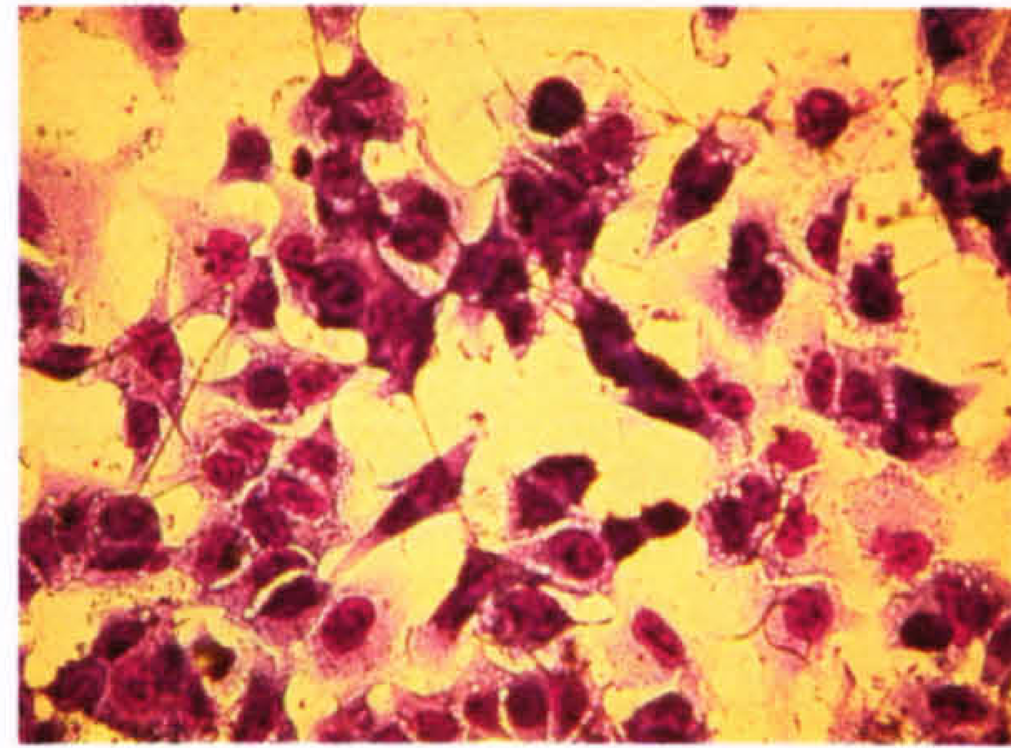
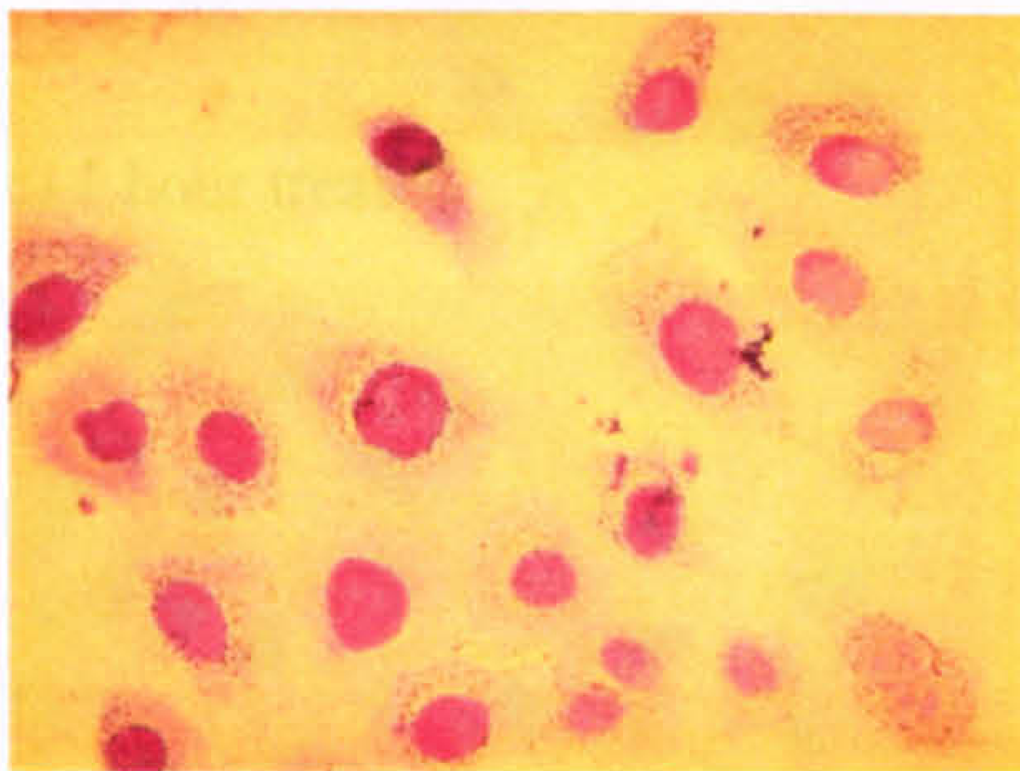
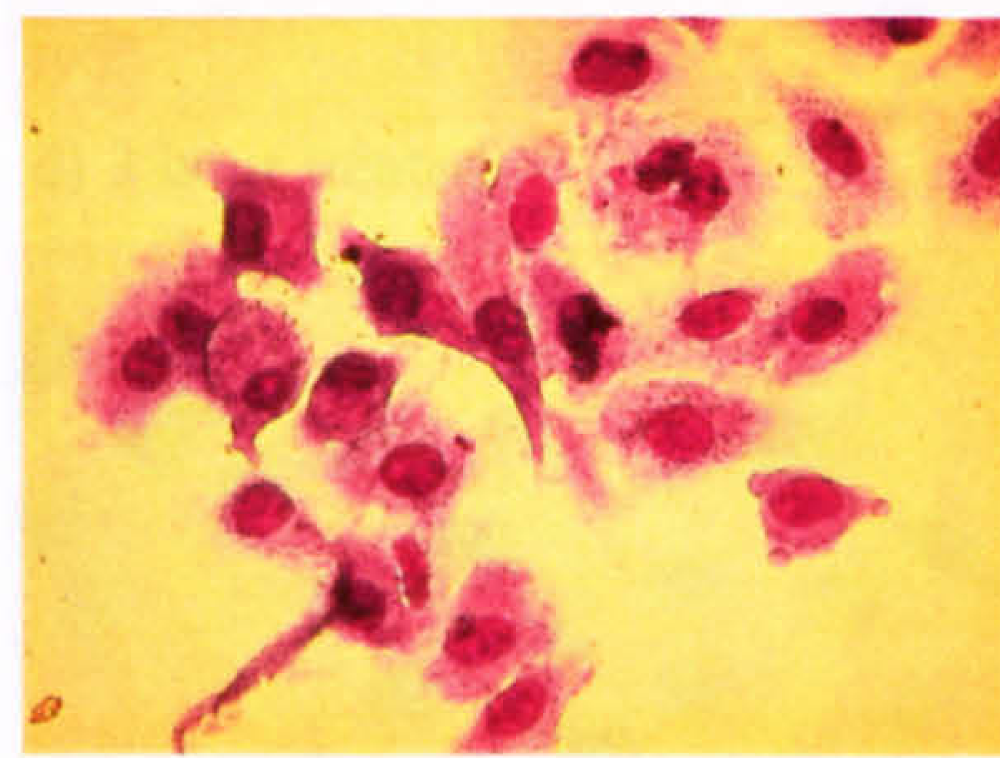
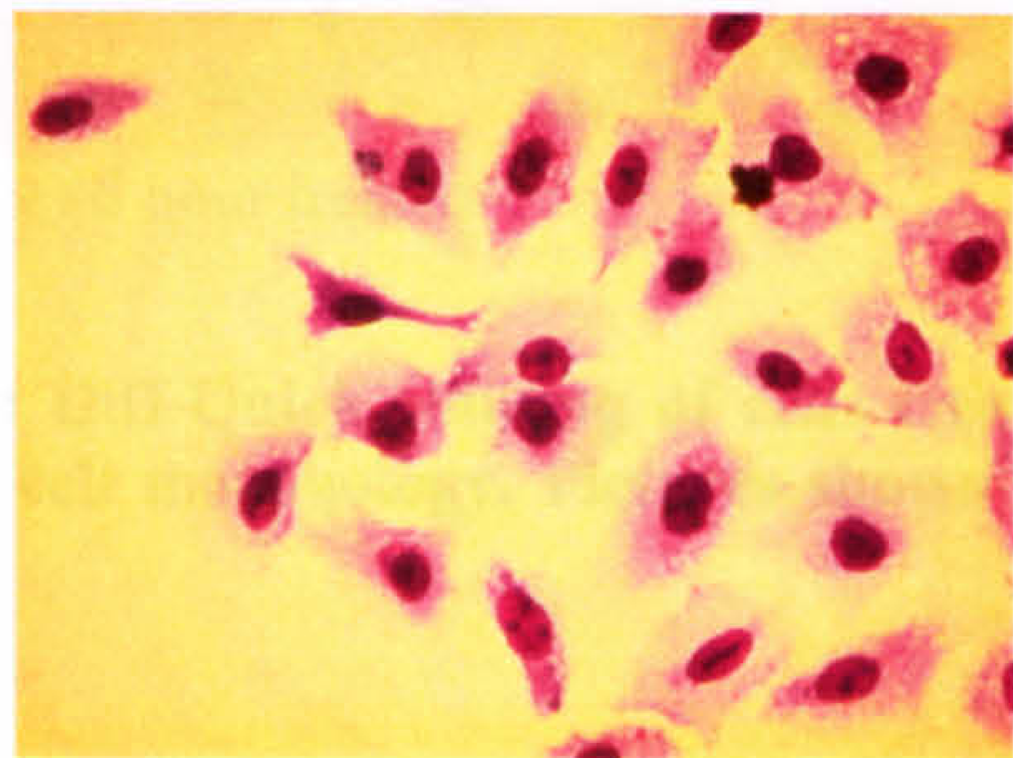
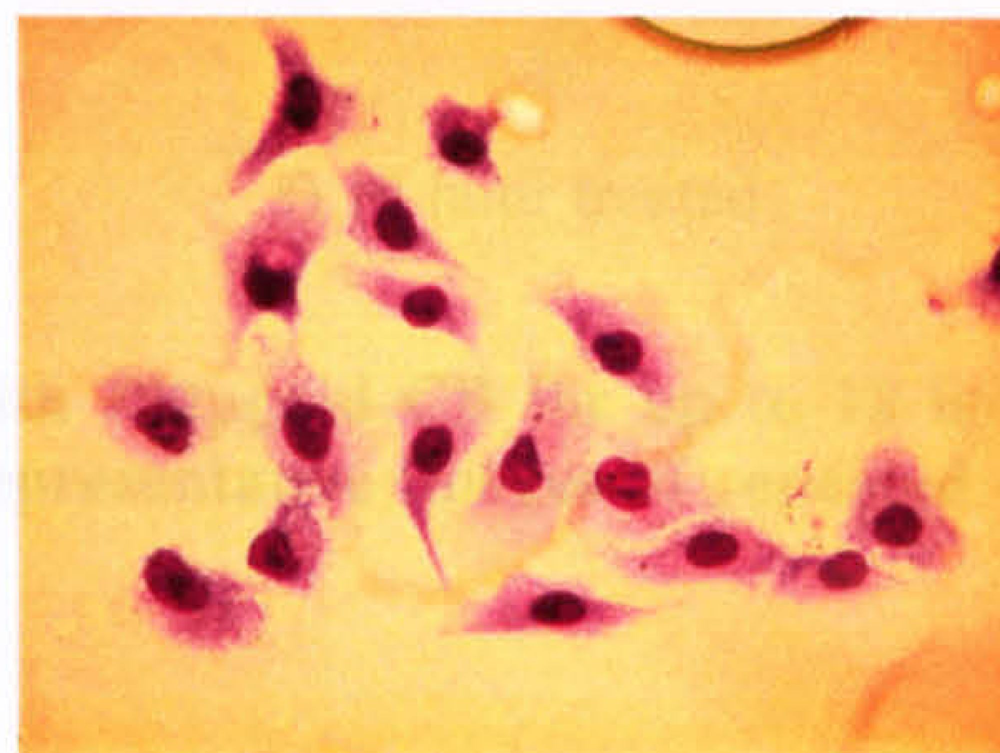

f) 18 hour treated

Figure 110: Diff-Quick staining of cells treated with 80μg/ml, for up to PM10 18 hours to show changes in cell morphology. Pictures shown are representative images of repeated experiments (n=3).


a) untreated


b) ½ treated


c) 1 hour treated


d) 6 hour treated

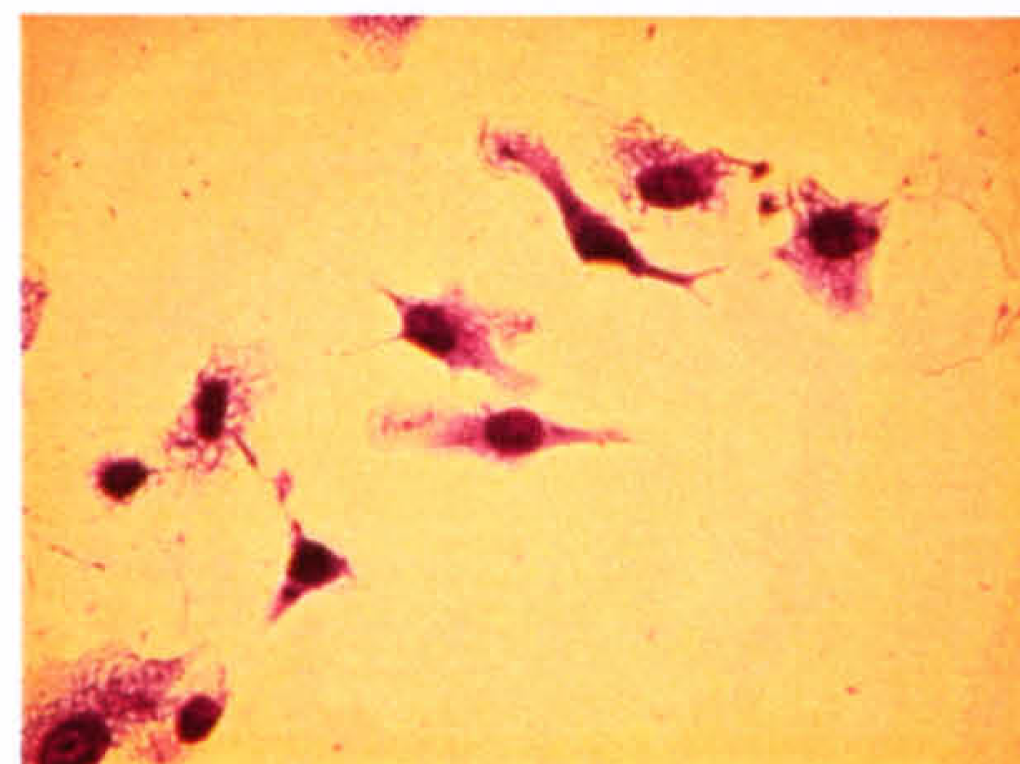
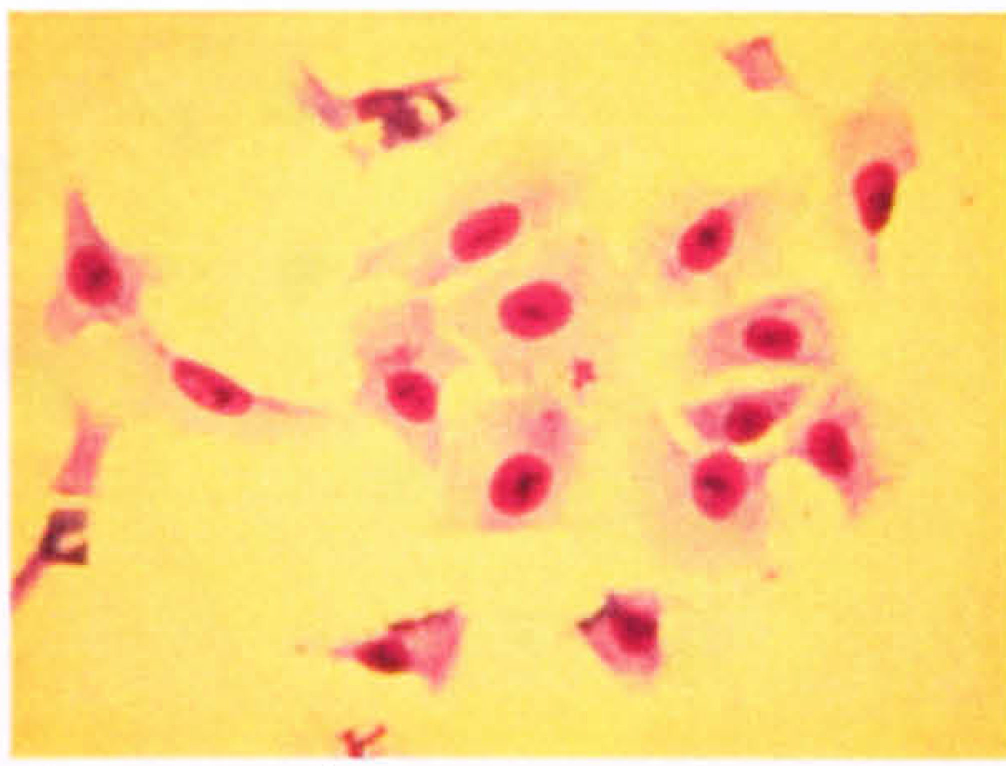
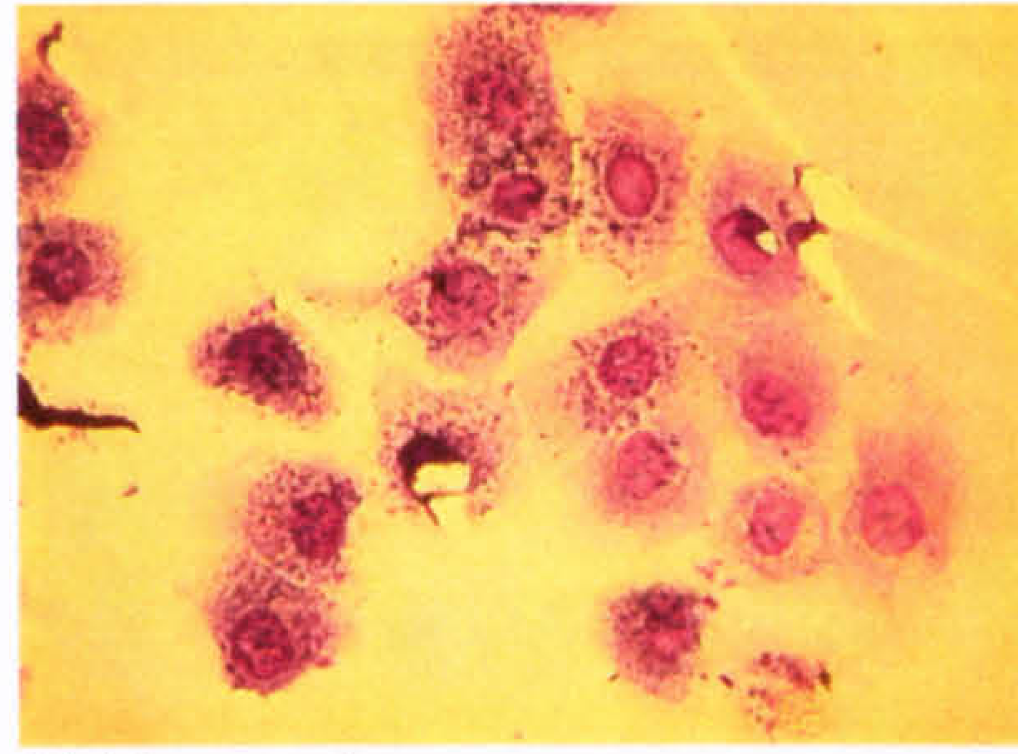

e) 18 hour treated

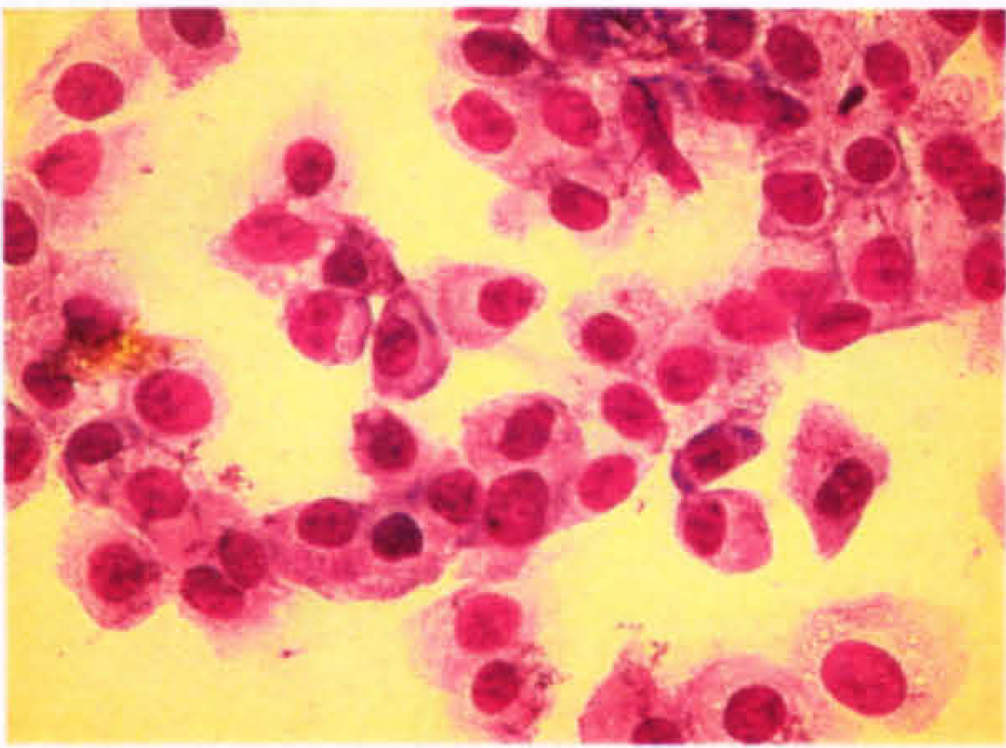
Figure 111: Diff-Quick staining of cells treated with 100μg/ml ufCB for up to 18 hours to show changes in cell morphology. Pictures shown are representative images of repeated experiments (n=3).



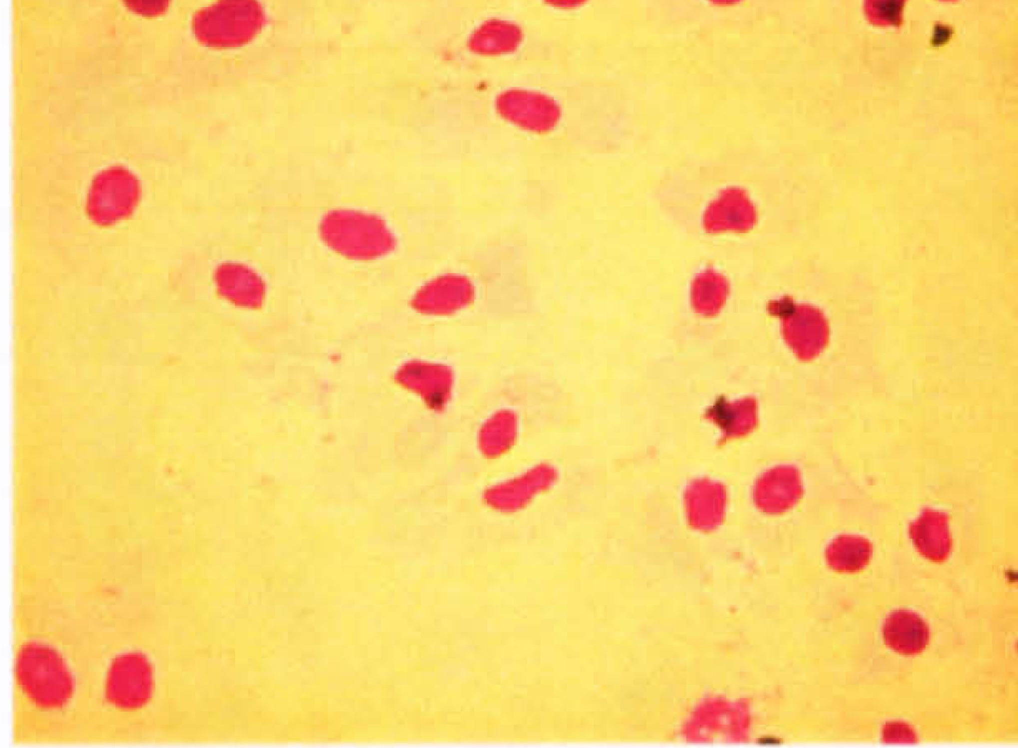
a) untreated



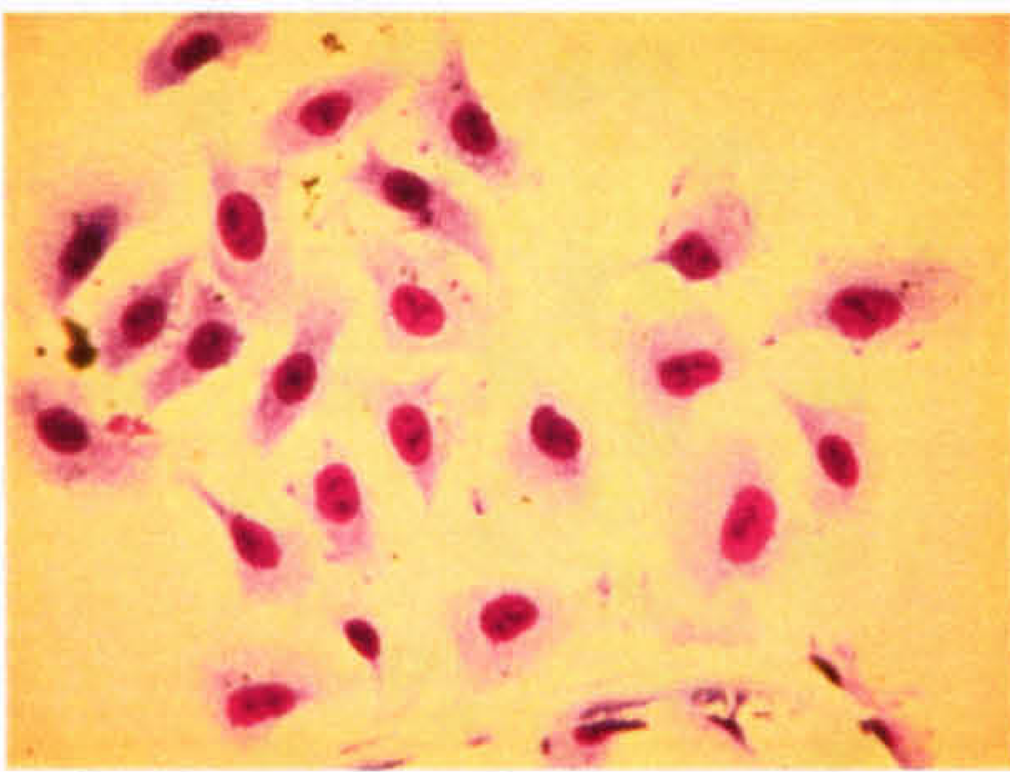
b) ½ treated



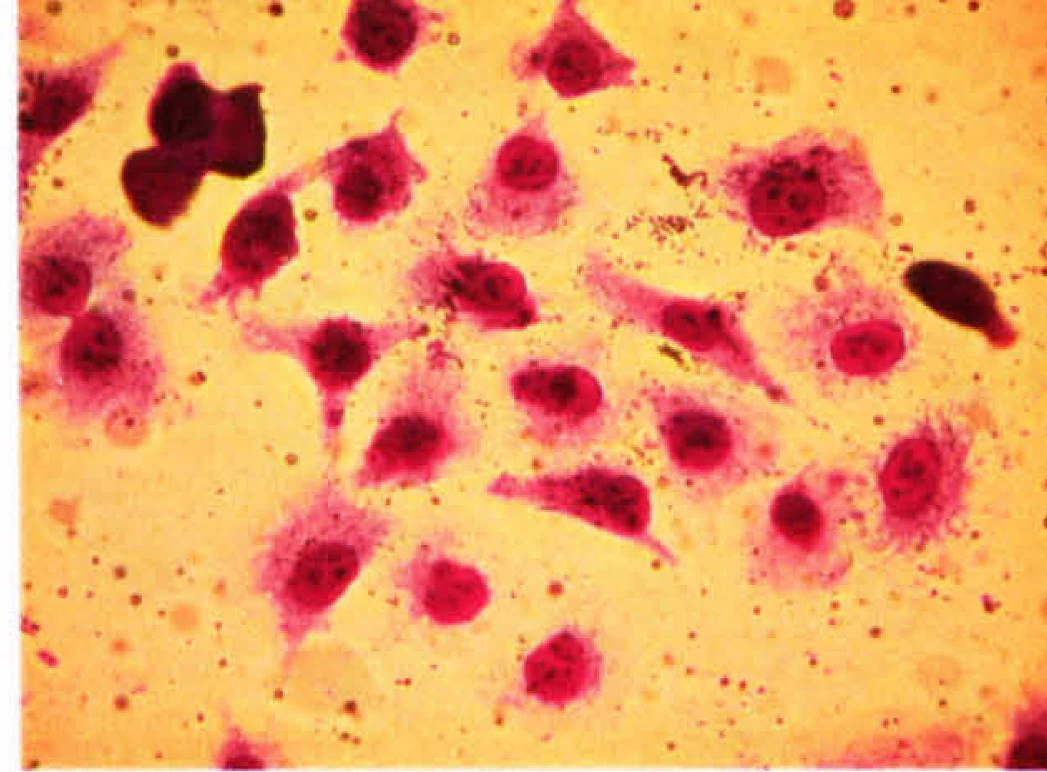
c) 1 hour treated



d) 3 hour treated



e) 6 hour treated



f) 18 hour treated

Figure 112: Diff-Quick staining of cells treated with 100μg/ml CB for up to 18 hours to show changes in cell morphology. Pictures shown are representative images of repeated experiments (n=3).

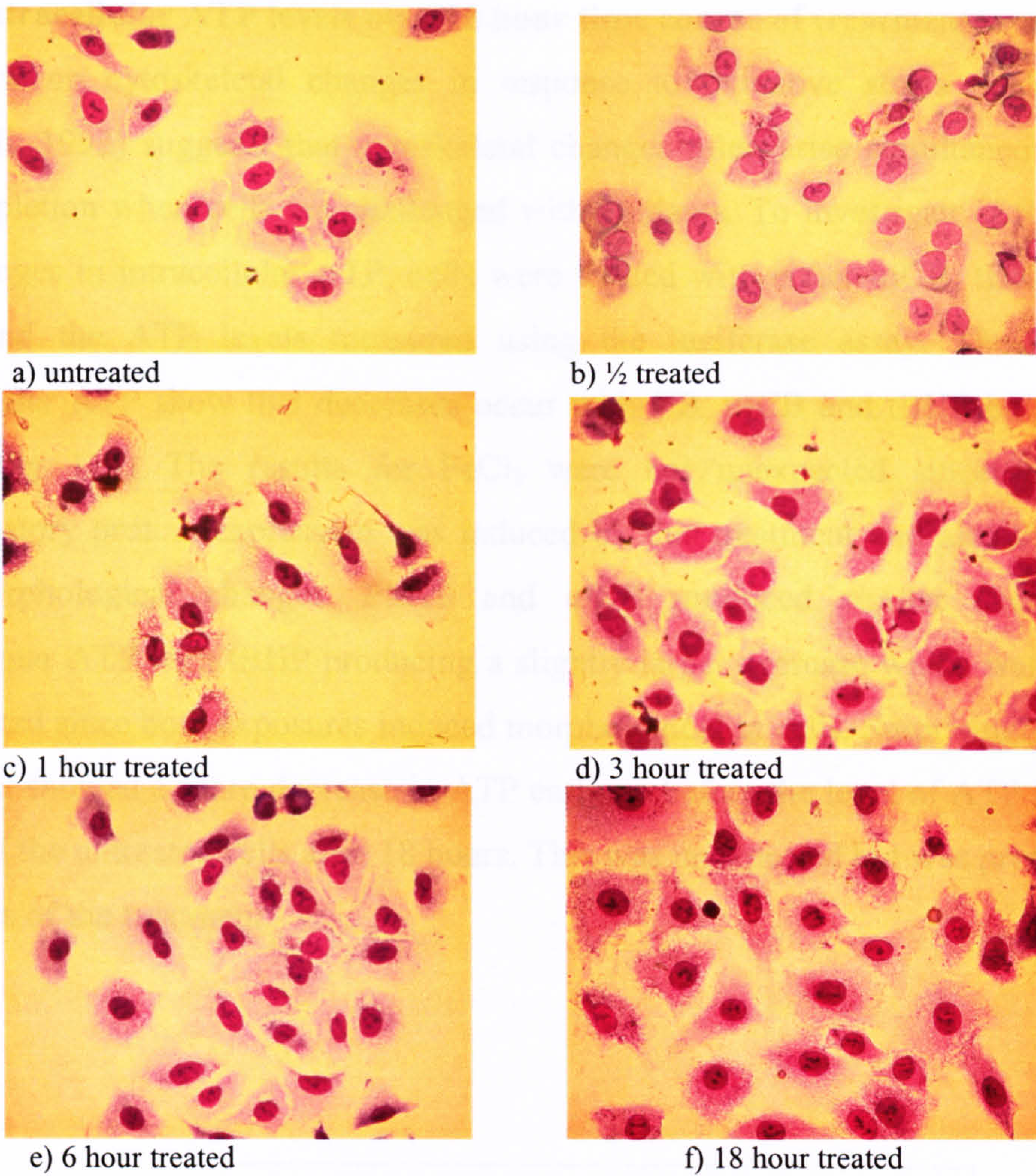


Figure 113: Diff-Quick staining of cells treated with 100 μM $FeCl_3$ for up to 18 hours to show changes in cell morphology. Pictures shown are representative images of repeated experiments (n=3).

5.3.9. Intracellular ATP levels over 18 hour time course of treatments.

A review on cytoskeletal changes in response to oxidative stress (Belomo and Mirabelli, 1992) suggests that cytoskeletal changes often arise simultaneously with ATP depletion when cells are challenged with oxidants. To investigate if there were any changes in intracellular ATP, cells were treated with either ufCB, tBHP, CB or tBHP and the ATP levels measured using the luciferase assay. The levels of intracellular ATP show that decreases occur with CB, ufCB and tBHP but not with $FeCl_3$ (Fig 114). The results for $FeCl_3$ were not unexpected since very little inflammatory protein expression was induced by this treatment and also there were few morphological changes. PM10 and ufCB produced similar decreases in intracellular ATP with tBHP producing a slightly larger decrease which was also not unexpected since both exposures induced morphological in cells. Surprisingly, the CB treatment showed a sharp decrease in ATP early on before the level of ATP increased to that of the untreated cells after 18 hours. This was unexpected but was present in all replicates of the treatment.

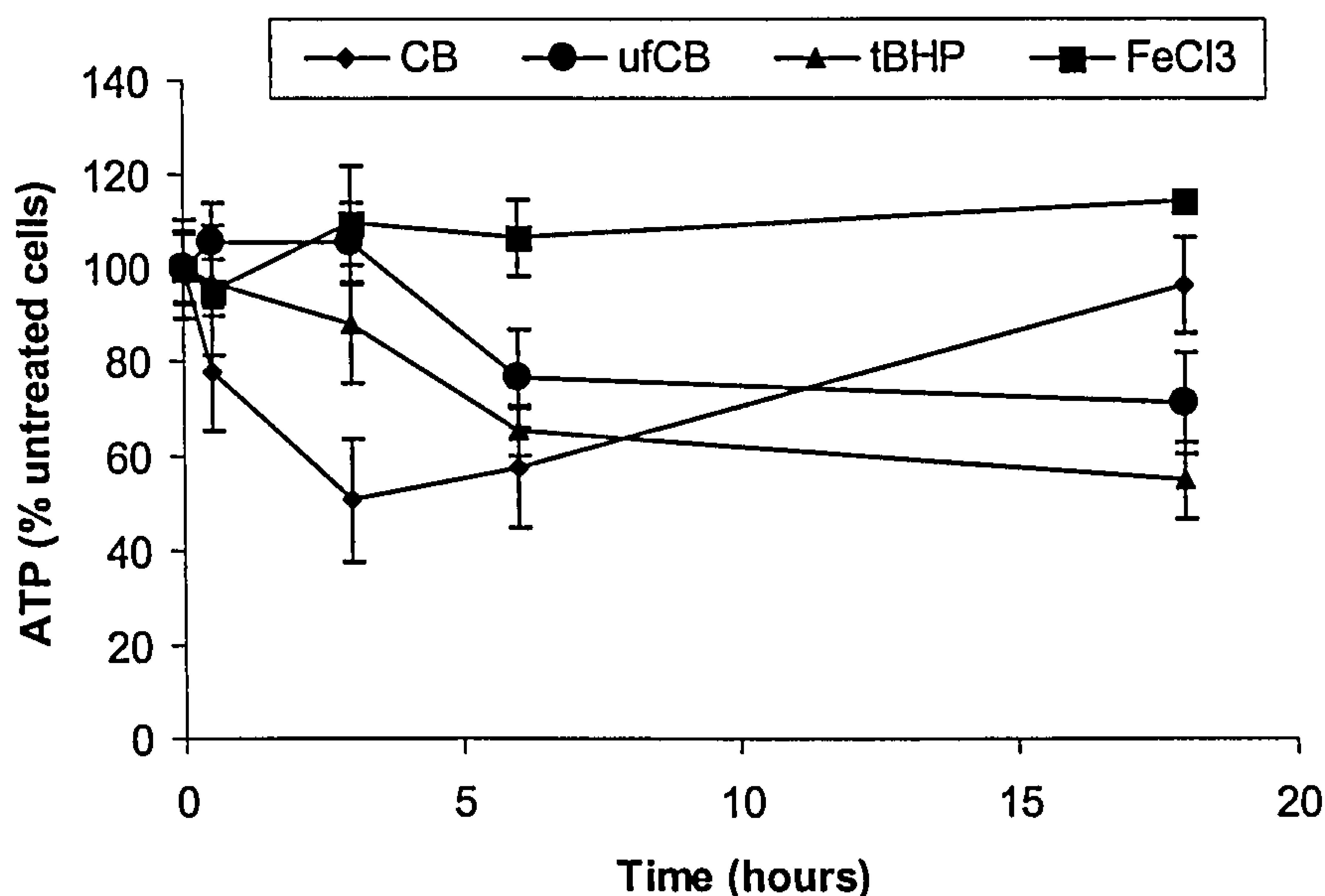


Figure 114: Level of intracellular ATP in A549 cells measured by luciferase assay. Cell treatments are 100mM $FeCl_3$, 100µg/ml CB, 100µg/ml ufCB and 5µM tBHP. The results are the mean of triplicate results from 3 experiments ± SE.

5.4. Is expression of CRP, fibrinogen and Hsp70 a result of newly synthesised protein or the release of stored protein?

5.4.1 RT-PCR for CRP with particle treatments.

To determine if the expression of CRP in cells after particle treatments was a result of *de novo* protein synthesis after transcriptional activation of the CRP gene, RT-PCR was carried out on the cells. CRP is known to be present in the cell cytoplasm bound to the endoplasmic reticulum in an inactive state ready to undergo post transcriptional modifications so that it can be released quickly in response to inflammatory stimuli (Yue *et. al.* 1996). RT-PCR was carried out to examine mechanisms responsible for CRP expression. Cells were exposed for 90 minutes to CB, ufCB, $FeCl_3$, tBHP or TNFα, before RNA extraction. UfCB was found to produce the highest levels of mRNA expression (Fig 115) and this was statistically significant from the untreated cells ($p<0.001$). Small increases in mRNA were also found for tBHP and TNFα and this may be due to the fact that they are both soluble in the culture medium and may induce a faster response than the particle treatments which results in the mRNA being produced before this time point. The increase in mRNA with the treatments indicates that CRP is being produced by the cells in response to a transcription signal being induced by the cell treatments.

5.4.2 RT-PCR for fibrinogen with particle treatments.

To find out if fibrinogen was being produced in the cells as a result of transcriptional activation RT-PCR was carried out. UfCB and tBHP treatments showed the greatest increases in fibrinogen mRNA expression (Fig 116); however, this did not show any statistical significance due to high variation among the replicates although increases were found in each. There were also very small increases with the CB and $FeCl_3$ treatments. There was no increase in the levels of RNA with TNF α treatment which would be expected since TNFα has been shown to reduce or prevent fibrinogen expression (Zhang and Fuller, 2000; Nguyen and Simpson-Haidaris, 2000).

5.4.3 RT-PCR for Hsp70 with particle treatments.

To identify if Hsp70 was being produced in the cells as a result of transcriptional activation RT-PCR was carried out. UfCB, tBHP and $FeCl_3$ treatments all showed an

increase in the levels of Hsp70 mRNA expression (Fig 118). Once again there was no statistical significance of these treatments over the untreated cells due to variation between the replicates. Interestingly the CB treatment showed a decrease in the level of RNA which was unexpected. However, it would appear that Hsp70 was being produced in the cells as a result of transcriptional activation.

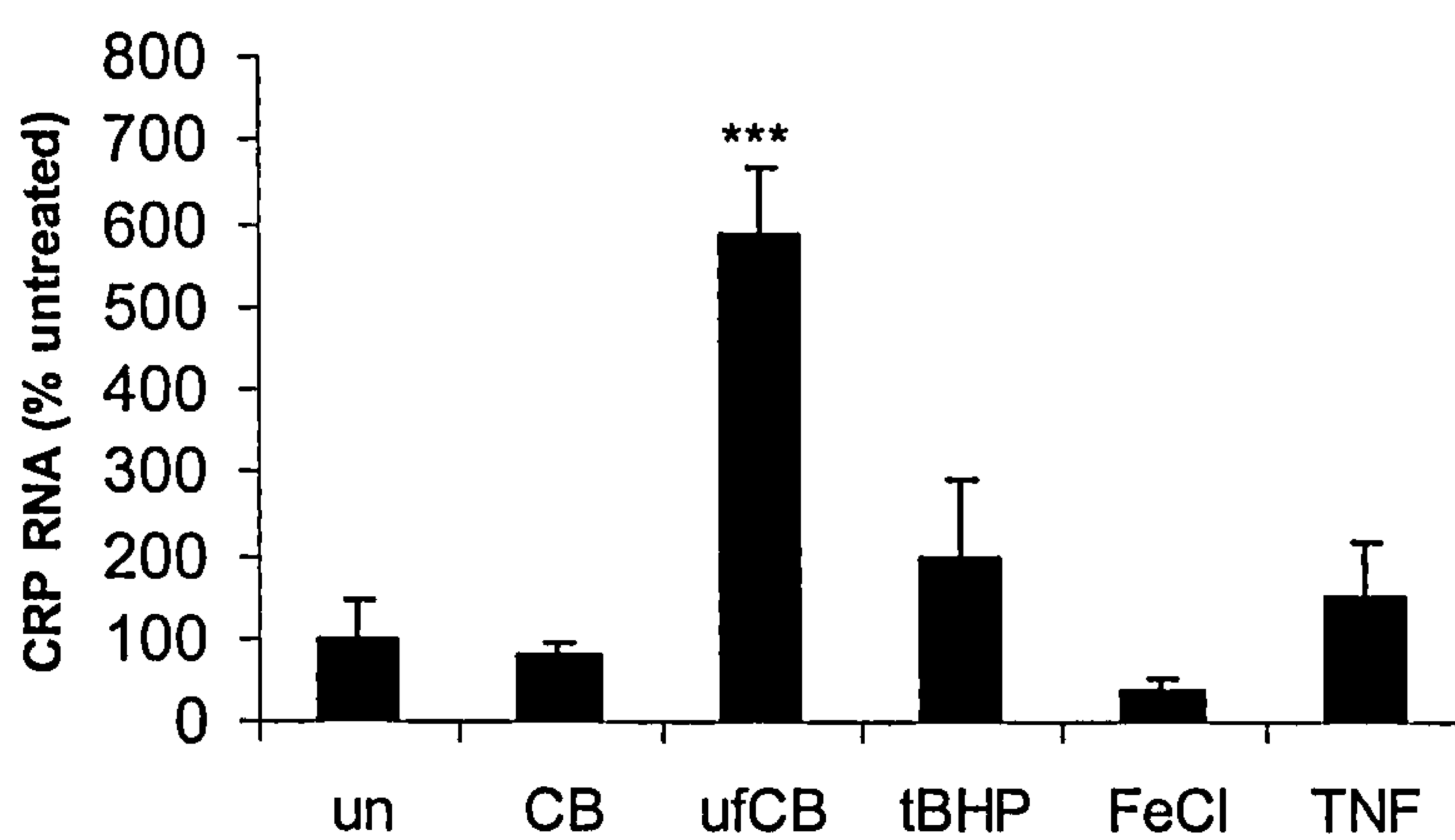


Figure 115: Level of CRP mRNA in A549 cells measured by RT-PCR. Cells were treated with 100μg/ml CB, 100μg/ml ufCB 5μM tBHP, 100mM $FeCl_3$ and 10ng/ml TNFα for 90 minutes. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (***) denote statistical significance from untreated cells $p<0.001$.

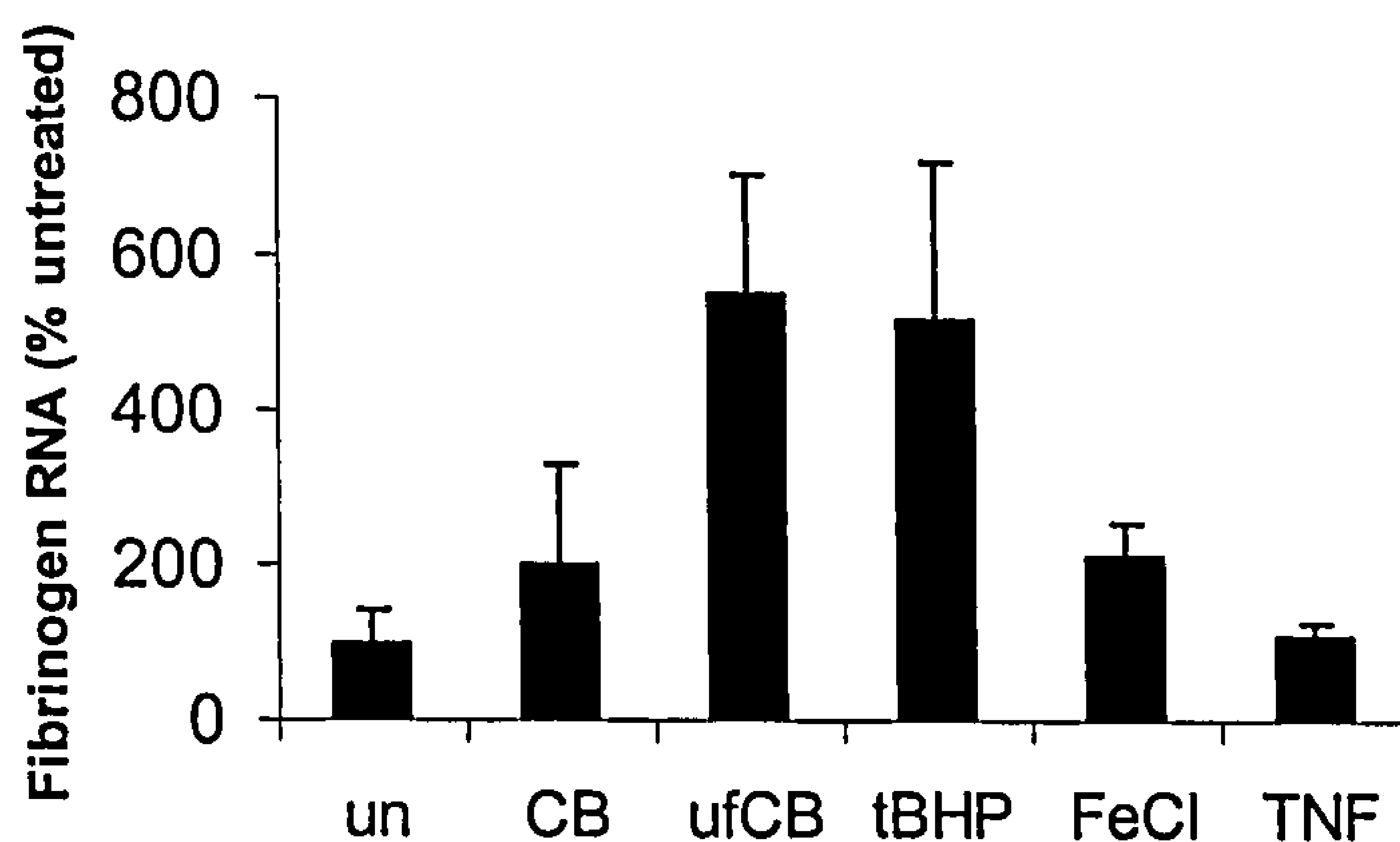


Figure 116: Level of fibrinogen mRNA in A549 cells measured by RT-PCR. Cells were treated with 100μg/ml CB, 100μg/ml ufCB 5μM tBHP, 100mM $FeCl_3$ and 10ng/ml TNFα for 90 minutes. The results are the mean of triplicate results from 3 experiments ± SE.

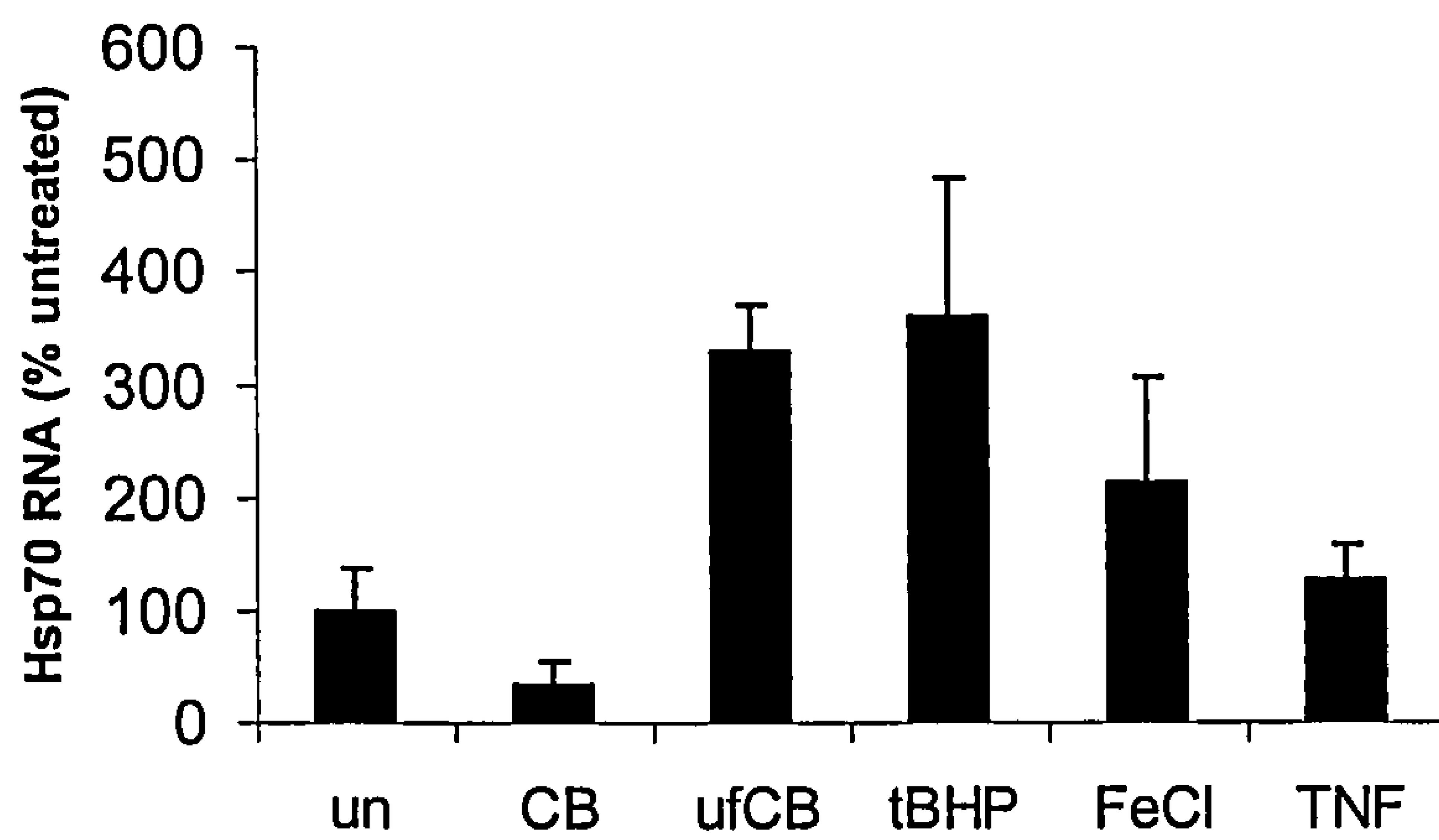


Figure 117: Level of Hsp70 mRNA in A549 cells measured by RT-PCR. Cells were treated with 100µg/ml CB, 100µg/ml ufCB 5µM tBHP, 100mM $FeCl_3$ and 10ng/ml TNFα for 90 minutes. The results are the mean of triplicate results from 3 experiments ± SE.

Chapter 6

Investigation into functions of secreted pro-inflammatory proteins.

6.1.0 Effect of CRP and Fibrinogen treatment on Monomac 6 (MM6) cells.

6.1.1 Effect of CRP treatment on MM6 cell line

To determine the effect of secreted CRP on other cells, monocyte-like MM6 cells were treated with recombinant CRP. The concentration of CRP used was determined by the amount of CRP present in the cell supernatants of particle treated cells. MM6 cells were treated with 4μg/ml of recombinant CRP for up to 4 hours. Cells were then used for indirect immunofluoresence and examined for CRP, fibrinogen, Hsp70 and NFκB expression. This showed that CRP treatment induced the expression of CRP and NFκB within the cells but very little fibrinogen and Hsp70 expression was induced (Figure 118). In the NFκB stained cells there was some nuclear staining present which may be responsible for CRP expression. However, there is no increase in fibrinogen expression which indicates that the presence of CRP may arise due to activation of stored CRP and not NFκB induced transcription of CRP. Fibrinogen expression may arise in response to conditioned media (CM) treatments at a later time point.

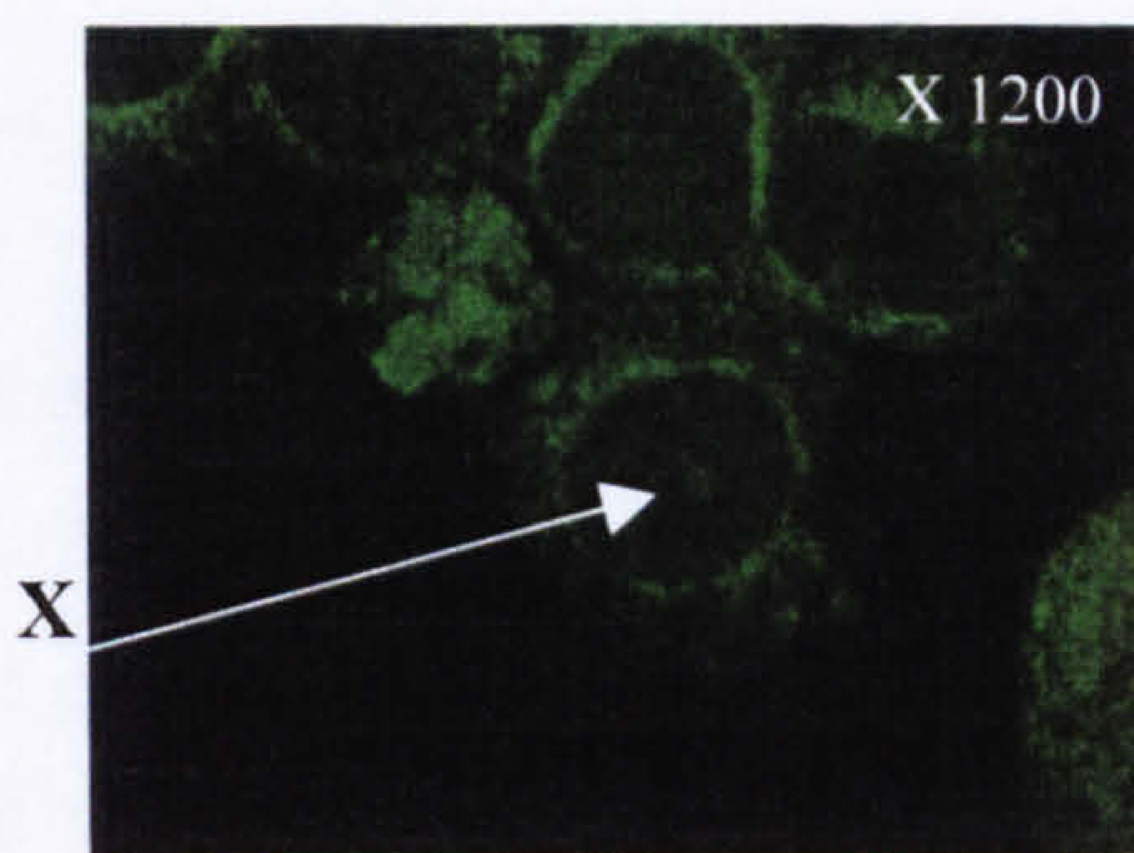
6.1.2 Effect of fibrinogen treatment on MM6 cell line

To determine the effect of secreted fibrinogen on MM6 cells, the cells were treated with recombinant human fibrinogen. The concentration used was similar to the amount of fibrinogen secreted from the cells treated with particles. MM6 cells were treated with 2μg/ml fibrinogen for 4 hours. Cells were then examined using indirect immunofluoresence for CRP, fibrinogen, Hsp70 and NFκB expression. This shows that fibrinogen treatments induced the expression of all proteins except CRP after 4 hours of treatment (Fig 119). Once again, as for the CRP treatment, there is some nuclear staining of NFκB with fibrinogen treatment. The expression of Hsp70 may suggest that the cells are stressed by the fibrinogen treatment.

a) CRP

b) Fibrinogen

c) Hsp70



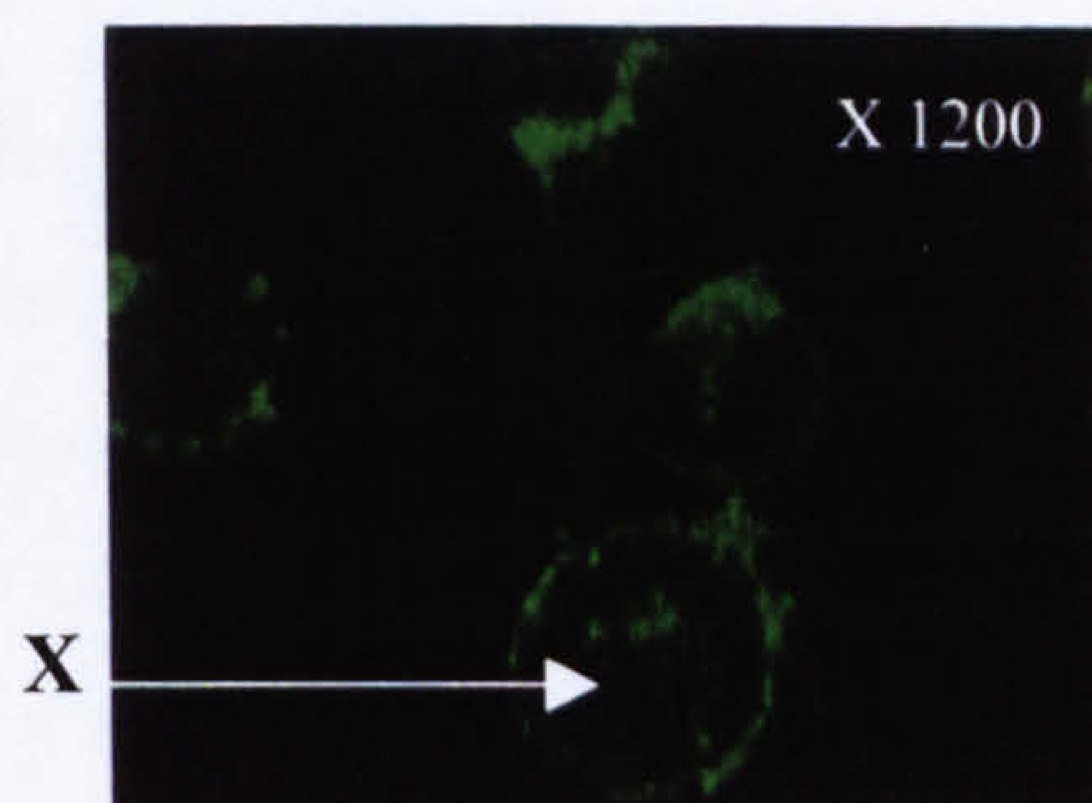
d) NFκB

Figure 118: Expression of inflammatory protein expression in MM6 cells treated with, 4mg/ml recombinant human CRP after 4 hours of treatment. Negative controls show little background staining which is at a similar level to the cells stained for Hsp70. X indicates nuclear localisation of NFκB.

a) CRP

b) Fibrinogen

c) Hsp70



d) NFκB

Figure 119: Expression of inflammatory protein expression in MM6 cells treated with 2mg/ml recombinant human fibrinogen after 4 hours of treatment. Negative controls show little background staining which is at a similar level to the cells stained for CRP. X indicates nuclear localisation of NFκB.

6.2.0 Effect of A549 conditioned media on pro-inflammatory protein expression and secretion MM6 cells.

The effect of CM from A549 cells, treated with 100μg/ml ufCB for 18 hours, was examined using MM6 cells. There is likely to be high concentrations of many proteins in the CM including cytokines, CRP, fibrinogen and Hsp70, which may have various effects on the MM6 cells. MM6 cells were treated with the CM for 4 hours and indirect immunofluoresence was carried on the cells (Fig 120). This showed an increase in CRP, fibrinogen, Hsp70 and nuclear and cytoplasmic expression of NFκB.

ELISAs were than carried out to find out what proteins were present in the medium and to see if MM6 were producing pro-inflammatory proteins in response to this treatment. This shows that the acute phase protein fibrinogen was increased after CM treatment suggesting that fibrinogen was being secreted from the MM6 cells (Fig 121). However there was little secretion of CRP by MM6 after CM treatment although there was CRP present in the A549 CM.

ELISAs for the cytokines IL6, IL8 and TNFα were carried out (Fig 122). This showed that there was an increase in production of IL6 and TNFα by MM6 treatment after CM treatment. There was however a large increase in the amount of IL8, present in the supernatant after treatment with the A549 CM which is statistically significant ($p<0.001$) from the untreated MM6 cells and the A549 CM. This may be the result of cellular damage since IL8 is known to be involved in the recruitment of circulating neutrophils to tissue damage

6.3.0 Does A549 CM have damaging effects on MM6 cells?

The production of the pro-inflammatory protein expression suggested that MM6 cells may be maintaining an inflammatory response and this may be associated with cell damage. Therefore the cells were examined for morphological changes associated with cellular stresses. This was carried out as before by staining the treated cells with Diff-Quick staining solutions (Fig 123). This shows that there is a dramatic response of the cells at ½ hour of CM treatment shown by nuclear condensation of the cells with some signs of necrosis shown by loss of normal cellular morphology and cellular disintegration. By 1 hour of treatment the cells still show nuclear condensation but

there are far fewer cells present and many more necrotic cells. At 1 hour there also appears to be some apoptotic cells displaying nuclear fragmentation. By 2 hours of treatment most of the cells appear to be recovering with some necrotic cells present and also some phagocytic activity by some of the cells. At 3 hours of treatment the cells appear to be more normal in shape and by 4 hours of treatment the cells look as healthy as the untreated cells. This suggests that the CM is inducing a huge inflammatory/stress response in these cells.

A LDH assay was than carried out to find if the cells were indeed becoming damaged by the treatment as would appear by looking at the morphological changes. LDH is released by the cells during membrane damage which can occur as cells become necrotic. The assay (Fig 124) showed that there was an increase in the amount of LDH release by the cells with CM treatment which is statistically significant ($p<0.05$). There was little production by the untreated cells and the A549 cells which were treated by the ufCB particles. This also suggests that the ufCB treated A549 cells were producing something which was causing a huge stress response in MM6 cells.

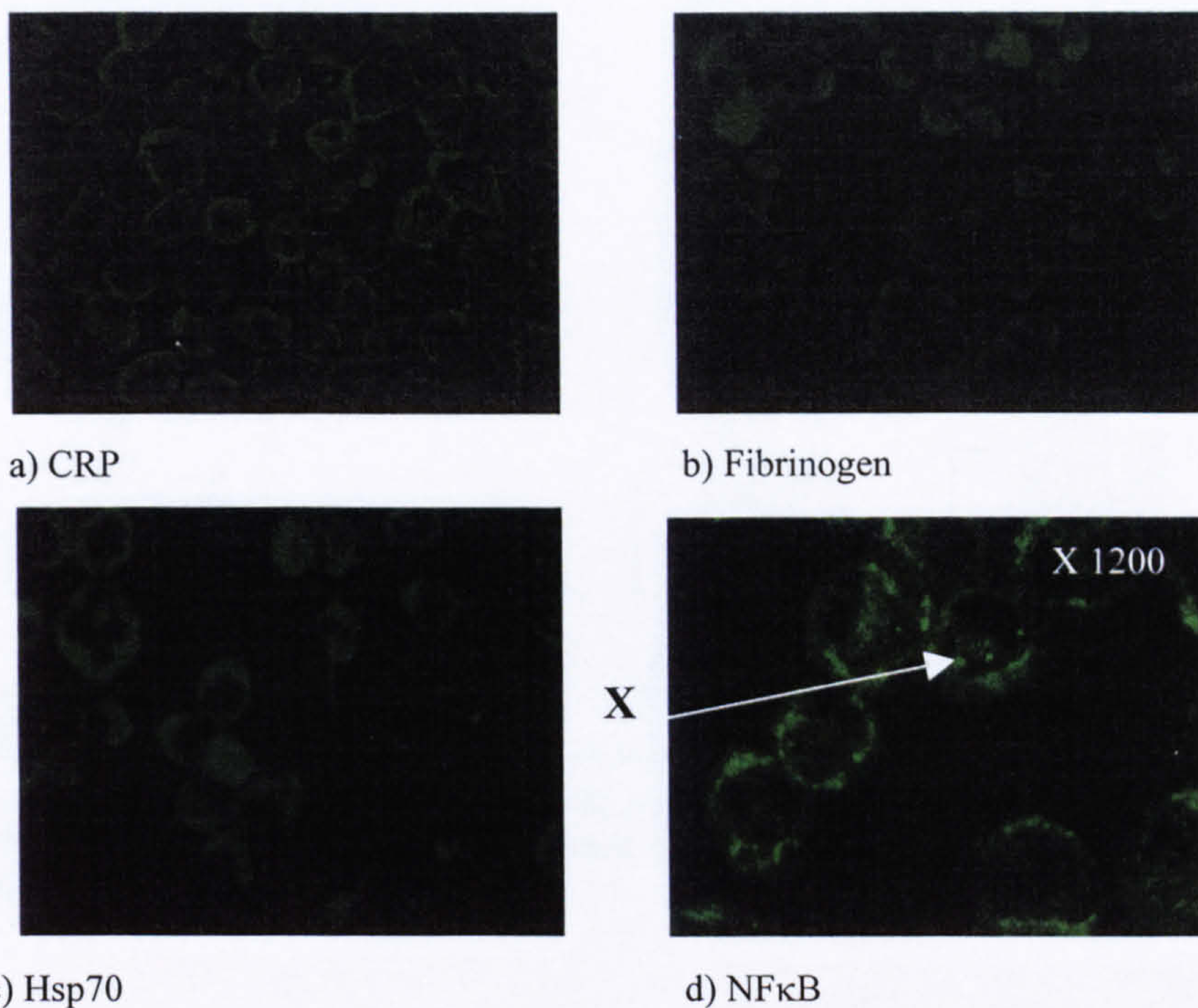


Figure 120: Expression of inflammatory protein expression in MM6 cells treated with ufCB treated A549 conditioned medium for 4 hours. Negative controls show little background staining. X indicates nuclear localisation of NFκB.

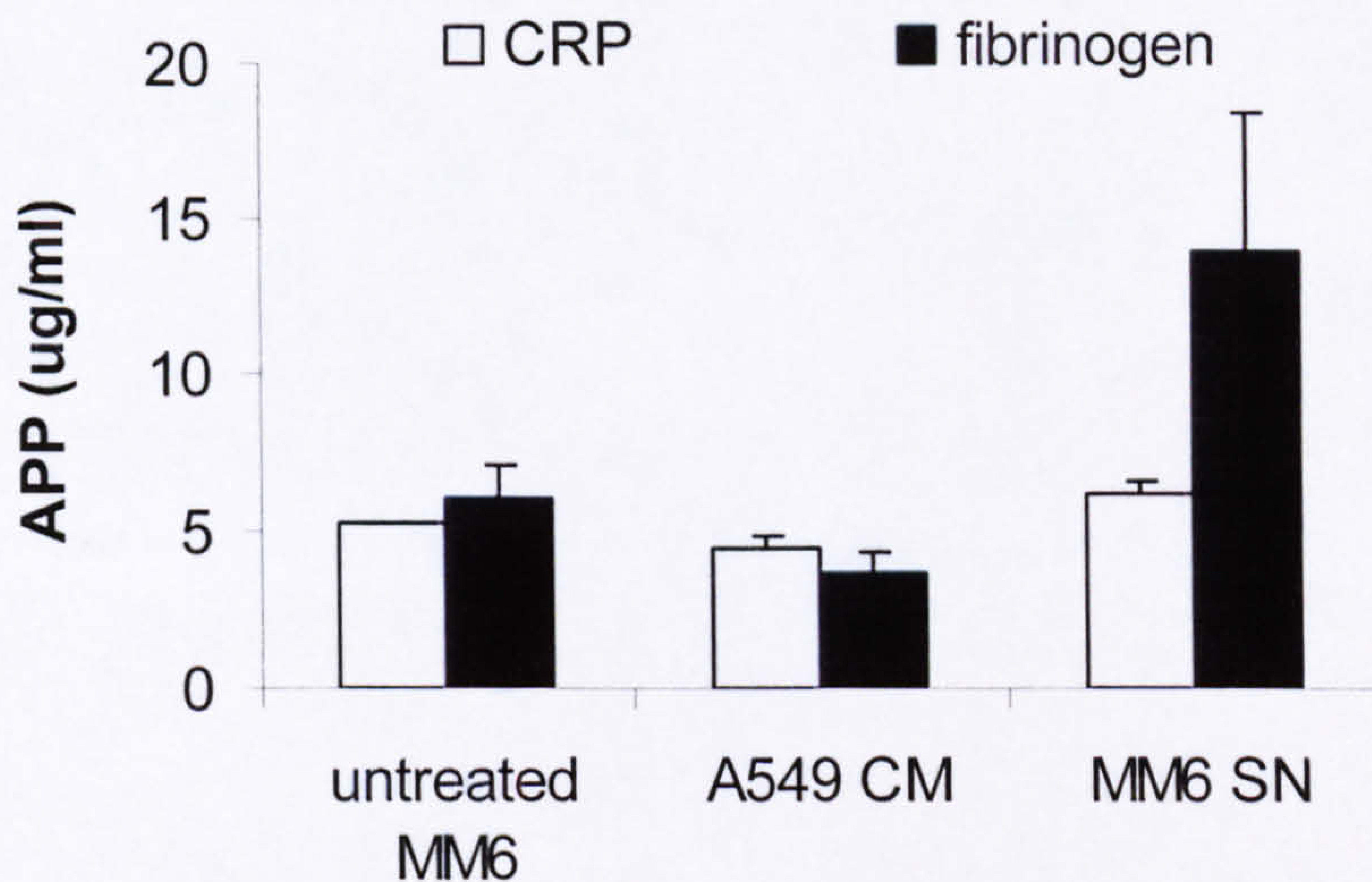


Figure 121: Acute phase protein concentration in cell supernatants with A549 CM. The results are the mean of triplicate results from 3 experiments ± SE.

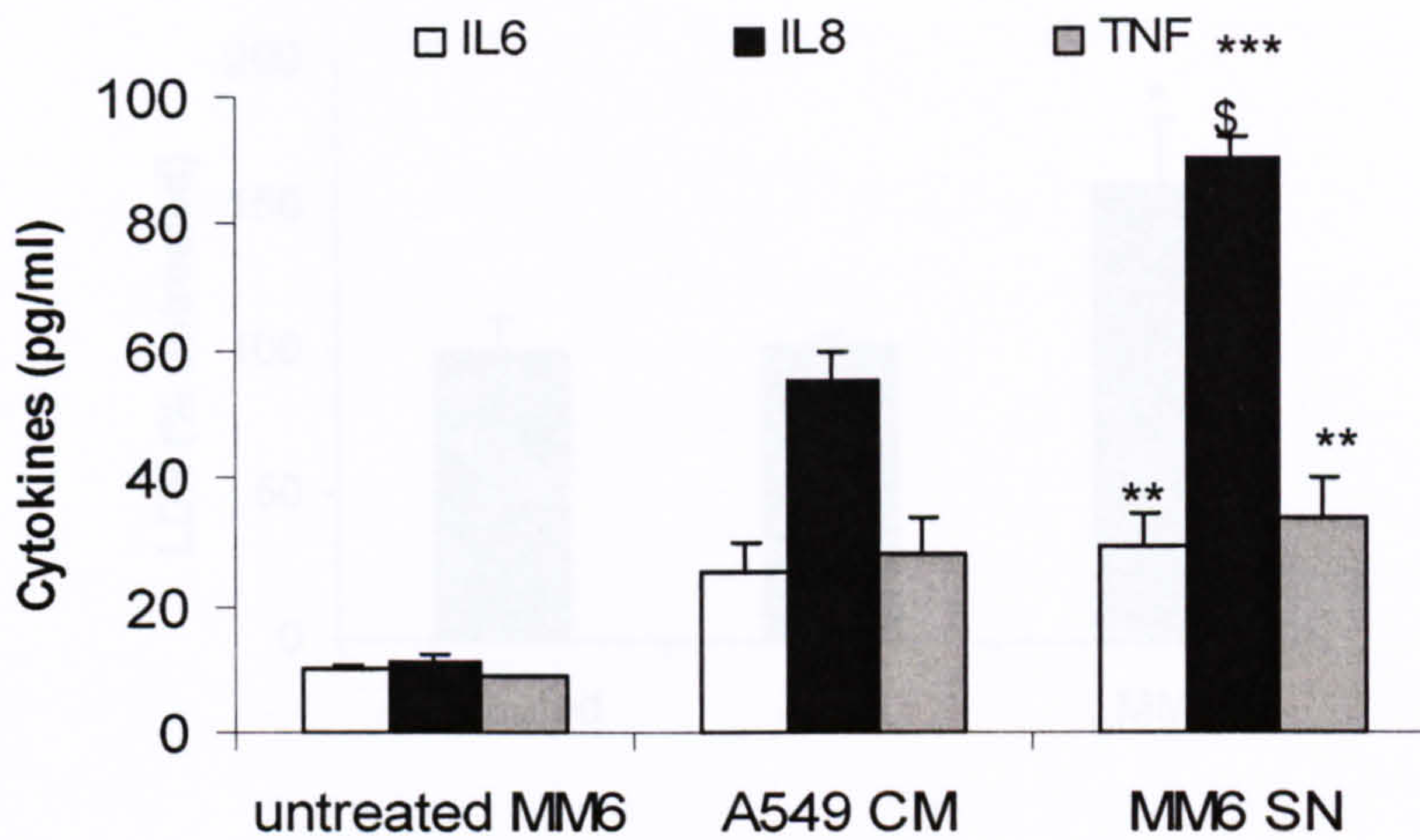


Figure 122: Cytokine concentration in cell supernatants with A549 CM. The results are the mean of triplicate results from 3 experiments ± SE. Asterisks (*, **) denote significant changes from untreated MM6 cells and $ denotes significant change in IL8 present in MM6 supernatant over A549 supernatant.**

a) untreated

b) ½ hour

X

Y

Z

c) 1 hour

d) 2 hours

e) 3 hours

f) 4 hours

Figure 123: Diff-Quick staining of MM6 cells treated with A549 CM. X- necrotic cells, Y- apoptotic cells, Z- phagocytosis.

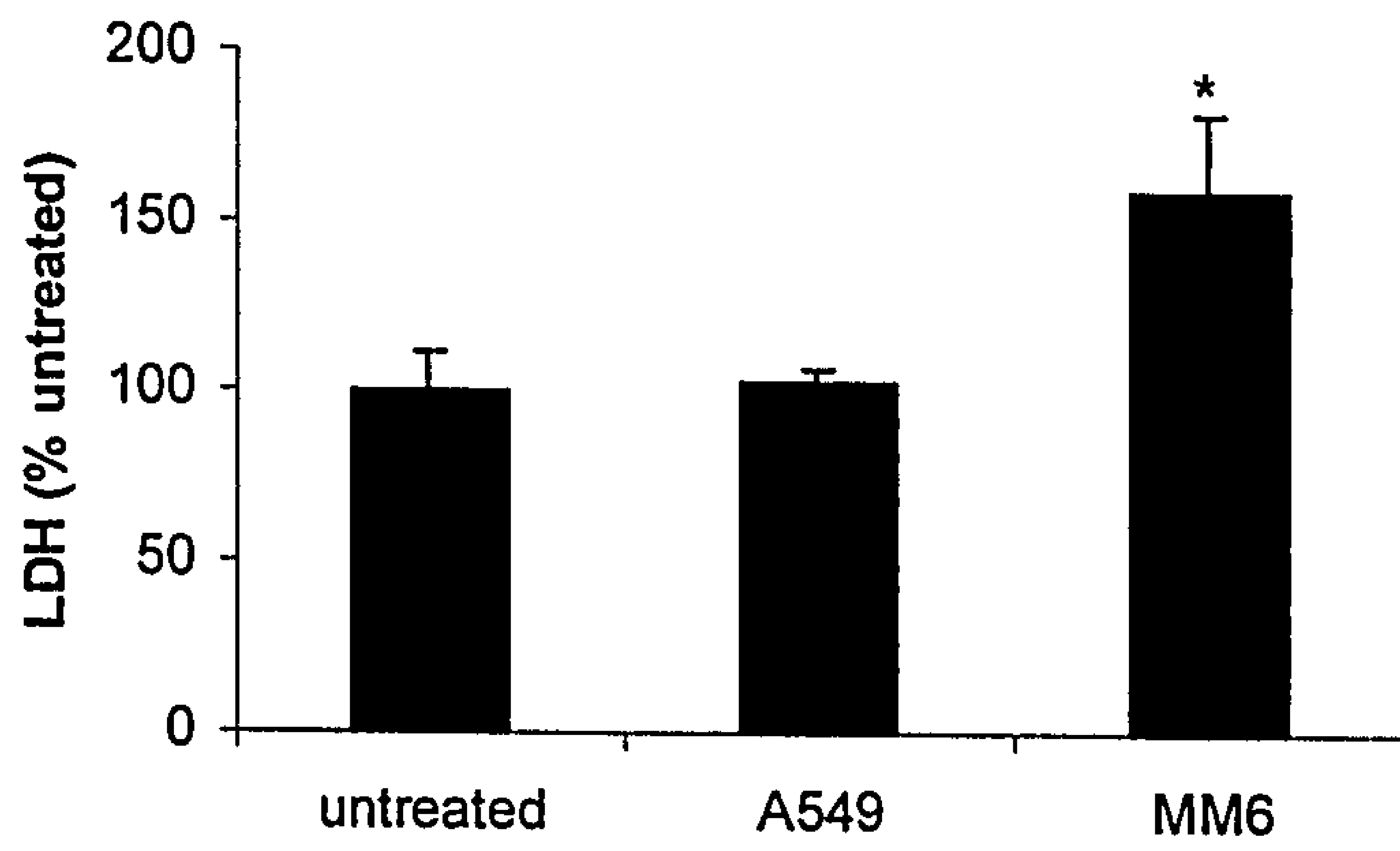


Figure 124: LDH assay for CM treated MM6 cells. The results are the mean of triplicate results from 3 experiments ± SE. Asterisk (*) denotes significant changes from untreated MM6 cells and A549 supernatant.

Chapter 7

Discussion and Conclusions.

7.1.0 Expression of acute phase proteins in A549 cells after cytokine treatment.

7.1.1 Fibrinogen expression after cytokine treatment.

Fibrinogen has been shown to be produced in the lung during inflammatory events. It has been shown that fibrinogen can be produced in the lung epithelial cells in response to IL6 (Simpson-Haidaris, 1997), parasitic infections (Simpson-Haidaris *et. al.*, 1998), and PM10 exposure (Anderson *et. al.*, 2002). Hepatic expression of fibrinogen is known to arise from stimulation by IL6. It has also been shown that IL6 induced fibrinogen production can be inhibited by the presence of IL1β (Zhang and Fuller, 2000). Therefore the effect of cytokines on fibrinogen expression in the lung epithelium was investigated in A549 cells.

In agreement with studies mentioned above, fibrinogen expression was found to be stimulated in A549 cells with IL6 treatment. Treatment with TNFα and IFNγ produced little increase in fibrinogen expression as shown by indirect immunofluoresence and ELISAs. When the effect of simultaneous cytokine treatment was tested it was found that IL6 and TNFα together could stimulate the production of fibrinogen above the level of TNFα alone as shown by immunofluoresence. The decrease in fibrinogen production with simultaneous treatment with IL6 and TNFα has been found previously in human hepatoma cell lines (Mackiewicz *et. al.*, 1991) and in A549 cells in serum free condition (Anderson, 2003). However, analysis of the cell supernatant showed that the simultaneous treatment with IL6 and TNFα induced the greatest amount of fibrinogen secreted from the cells. This indicates that IL6 and TNFα induce the production of fibrinogen by different mechanisms and that in combination they can induce optimal secretion of fibrinogen from the cells in the presence of fetal calf serum. The lower intracellular levels of fibrinogen in the simultaneous treatment may be the result of faster secretion than is seen with IL6 treatment alone.

Simultaneous treatment was also carried out with IL6 and IL1β. This showed that fibrinogen could be found expressed in the cytoplasm of the cytokine treated cells and simultaneous treatment of IL1β with IL6 had no evident additional effect from IL6 only-treated cells. A study by Zhang and Fuller (2000) found a large decrease in

fibrinogen expression with IL6 and IL1β co-treatments which was not found in this study. This may indicate that fibrinogen expression in human lung epithelial cells may occur in a different manner to that in the rat liver. Activation of fibrinogen by IL6 can occur by signalling via two transcription pathways (Fuller and Zhang, 2001; Siebenlist *et. al.*, 1994). The first of these is by CCAAT enhancer binding proteins (C/EBP) and the other is by the STAT pathway, whereas IL1β activates NFκB. In rat liver, NFκB associates with the STAT3 binding site preventing IL6 activating the fibrinogen gene but in the human lung there may be a greater distance between the binding sites of these transcription factors.

Simultaneous treatment was also carried out with IL6 and IL8. IL8 is produced during inflammation as a result of signalling from TNFα and IL1β. It was found that IL8 induced a small increase in fibrinogen but IL6, and the simultaneous treatment of IL6 with IL8, produced a greater response. This indicates that fibrinogen can be produced in the lung epithelial cells in response to several cytokine stimuli and most of these can increase the level of expression over and above the effect produced by IL6 alone (results summarised in Table 1).

7.1.2 CRP expression after cytokine treatment.

The results from this study indicate that CRP can be produced in the human alveolar epithelial cell line, A549. This occurred when the cells were exposed to various cytokines on their own or in combination with other cytokines. Of particular interest is the finding that TNFα induced the greatest amount of secreted CRP whereas there were relatively low levels of CRP expressed in the cytoplasm of the cells. This suggests that TNFα induced CRP in a different way to that of the other cytokines resulting in the early secretion of CRP. In hepatocytes, CRP is synthesised at a low rate in normal cells and is found bound to the endoplasmic reticulum by two carboxylesterases (gp60a and gp60b, Yue *et. al.*, 1996). This allows CRP to be quickly released from the cells during an acute phase response, which may occur with TNFα treatment. However, treatment with the other cytokines may be having a transcriptional effect on CRP expression which takes longer for the production of CRP. IFNγ was shown to stimulate the production of IL6 in A549 cells after 24 hours (Crestani *et. al.*, 1994). The IL6 produced by the IFNγ may then be able to induce the

expression of CRP intracellularly at this time point but not the secretion of CRP (results summarised in Table1).

The induction of high levels of cytoplasmic CRP and nuclear localisation in the combined treatment of the cytokines IL6 and IL1β suggests that within the lung there is the potential for high levels of expression of CRP in epithelial cells and that CRP may have a cytoprotective role. Other proteins with nuclear localisation sequences include transcription factors, heat shock proteins, and nucleoplasmin all of which are involved in protecting the cell from cellular stress (Malek *et. al.*, 1998; Benjamin *et. al.*, 1998; Santoro, 1999). Transcription factors enter the nucleus during inflammation to signal the production of important inflammatory mediators. HSPs are known to enter the nucleus to protect nuclear material from various stresses including thermal, oxidative and radiation effects. Studies by Du Clos (1989, 1990, 1991) have found CRP associated with histones, snRNPs (Jewell *et. al.*, 1993) and chromatin which suggests that CRP may be involved in protecting the nucleus during an inflammatory response.

Table 1: Summary of the effect of cytokine treatments on CRP and fibrinogen expression.

	CRP	Fibrinogen
IL6	Highly increased expression.	Highly increased expression.
TNFα	Increased level of secretion from cells	Little effect on expression or secretion.
IL6 + TNFα	Expression and secretion increased. Expression is similar to that of IL6 treatments alone, while secretion is similar to treatment with TNFα alone.	Expression and secretion increased. Expression is similar to that of IL6 treatment alone but secretion is increased over the levels of either IL6 or TNFα alone.
IFNγ	Increased expression and secretion.	Little effect on expression or secretion.
IL1β	Increased expression and secretion. With nuclear localisation.	Increased expression and secretion.
IL6 + IL1β	Highly increased expression and secretion, above that of either IL6 or IL1β alone. With nuclear localisation.	Slightly decreased expression and secretion when compared to effect of IL6 but above effect of IL1β or control cells.
IL8	Little effect on expression or secretion.	Little effect on expression or secretion.

IL6 + IL8	Highly increased expression over either IL6 or IL8 treatment alone. Little effect on secretion.	Highly increased expression over either IL6 or IL8 treatment alone. Little effect on secretion.

7.1.3 Effects of acute phase proteins (APP) in the lung.

The present studies show the ability of lung epithelial cells to mount and acute phase response. Another APP known as haptoglobin (Hp) has also been found to be expressed in alveolar macrophages and airway epithelial cells (Yang *et. al.*, 2000). This suggests that this family of inflammatory proteins may have important consequences on pulmonary inflammatory events. All three APP mentioned are not present in the normal lung yet appear to be present in high quantities during infection and injury.

Both CRP and Hp have been shown to reduce the movement of lymphocytes and neutrophils towards stimuli (Oh *et. al.*, 1990; Kew *et. al.*, 1990; Shepherd *et. al.*, 1992; Heuertz *et. al.*, 1993; Heuertz *et. al.*, 1994; Heuertz *et. al.*, 1996; Zhong *et. al.*, 1998). Fibrinogen degradation products (FDP) have been associated with increased chemotaxis of lymphocytes and neutrophils (Altier *et. al.*, 1993; Leavell *et. al.*, 1996; Gross *et. al.*, 1997; Forsythe *et. al.*, 2001; Rubel *et. al.*, 2001). This suggests that each APP may have specific roles in the recruitment of lymphocytes and neutrophils to sites of inflammation and therefore this may be the reason for differing effects in response to specific cytokines.

Other effects of APP proteins in the lung during inflammation can involve interaction between exogenous APP produced locally or systemically from the circulation and leucocytes and neutrophils. CRP has been shown to induce the expression and production of the cytokines IL1α, IL1β and TNFα from alveolar macrophages (Galve-de Rochemonteix *et. al.*, 1993) and monocyte chemotatic protein-1 (MCP-1) from human monocytes (Zhou *et. al.*, 1995). Monocytes, macrophages and neutrophils express surface receptors for both CRP and fibrinogen suggesting that these proteins can be bound to the cell surfaces. CRP is often found associated with neutrophils at sites of inflammation (Du Clos, 1981). CRP acts as an opsonin and there are receptors on monocytes and leucocytes for CRP to assist in phagocytosis of pathogens and damaged cells (James *et. al.*, 1981a, b; Mold *et. al.*, 1982; Bharadwaj

et. al., 1999; Mold *et. al.*, 2001). Fibrinogen also has the ability to bind to macrophages and monocytes, stimulating NFκB activation and chemokine secretion (Sitrin *et. al.*, 1998; Smiley *et. al.*, 2001). Both CRP and fibrinogen may also have the ability to exert effects on the lung epithelium. One study has found that fibrinogen can be phagocytosed by A549 cells (Ordjilin *et. al.*, 2001). CRP has been found to have direct pro-inflammatory effect on vascular endothelial cells (Pasceri *et. al.*, 2000). These all suggest that the expression and secretion of CRP and fibrinogen during inflammatory stimuli from epithelial cells may have important effects on the progression of lung inflammation.

7.2.0 Effect of air pollution particles on pro-inflammatory protein expression.

In chapter 4 the effect of inflammogenic air pollution particles (PM10) on lung epithelial cells was investigated. This was carried out using roadside PM10, a heterogenous air pollution particle, which is composed of a mixture of organic and elemental carbon, transition metals, acid salts, organic pollutants (polyaromatic hydrocarbons, nitroaromatic hydrocarbons, etc.) and biological components such as bacterial products and pollen spores. The cells were also treated with fine and ultrafine carbon black (CB and ufCB) and $FeCl_3$, a transition metal salt. This allowed a comparison between the inflammatory potential of PM10 and some of the components which make up PM10.

7.2.1 Effect of air pollution particles on CRP expression.

When the cells were treated with PM10, CB or ufCB there was an increase in the levels of CRP expression within the cells. This was determined using indirect immunofluoresence of the treated cells and by ELISA. It was also determined by ELISA that CRP could be secreted from the cells in response to these three particle treatments. The treatments were carried out over an eighteen hour time course and the production of CRP was found to be time dependent. However the treatment with the iron salt produced no increase in intracellular or secreted CRP.

Air pollution particles are known to be associated with oxidative stress in the lung. It is thought that this is a result of high surface area of the smaller (ultrafine) particles

where chemical reactions can occur. This surface chemistry occurs between species on the surface such as the transition metals (Li *et. al.*, 1996; Dellinger *et. al.*, 2001; and reviewed in Donaldson *et. al.*, 1998; MacNee and Donaldson, 1999). There is also evidence that free radical production can occur on the surface of ufCB particles (Stone *et. al.*, 1998). This oxidative stress is known to be involved in the progression of inflammatory events and has been shown to be associated with cytokine production on alveolar macrophages and epithelial cells (Becker *et. al.*, 1996; Carter *et. al.*, 1997). Therefore, it would be expected that PM10 and ufCB treatments would produce more CRP due to their greater inflammatory potential.

When examined it was found that there was little difference between the intracellular levels of CRP with particle treatments. The intracellular levels of CRP were increased by around 1mg/ml as shown by ELISA. The main difference between the treatments was in the ability to induce secretion of CRP. Using ELISAs, CB induced a maximal concentration of 1.5 mg/ml (+/-0.1), ufCB a concentration of 1mg/ml (+/- 0.1) and PM10 with a concentration of 8mg/ml (+/- 1). This suggests that adsorption of the protein onto the particle surface was occurring and this was found to be the case. Since ufCB has a larger surface area than CB more protein adsorption is able to occur therefore underestimating the amount of secreted protein.

When examining the effects of the particle treatments on the intracellular CRP by indirect immunofluoresence other differences in the effects of the treatments become apparent. All three treatments are able to induce the nuclear localisation of CRP. As discussed above this is important in establishing that the particles are stimulating an inflammatory response and that this may be harmful to the cells. Both carbon particles induced nuclear localisation only after 18 hours of treatment while nuclear localisation occurs after 3 hours with PM10 treatment. This indicates that the PM10 is able to induce a greater response in the cells than the carbon particles alone and this would not be unexpected. This is due to the fact that the components of PM10 are known to interact with each other and this can cause synergism of the biological effects (Wilson *et. al.*, 2002). Cells were also treated with $FeCl_3$ which produced no effect on CRP expression and secretion however metals contribute to induction CRP expression but only when present in PM10.

Also shown in the ufCB treatment is an alteration in the cell morphology after 18 hours of treatment. This gives the impression of a polarised staining pattern. This may be the result of oxidative stress. Free radicals produced can cause depletion of intracellular ATP in cells. This can result in the depolymerisation of actin and breakdown of the acto-myosin network which are responsible for maintaining cell shape (Bellomo and Mirabelli, 1992). Interestingly there does not appear to be any changes in cell morphology in the PM10 treatment after 18 hours, although there is some evidence to suggest that minor alterations may be occurring at the 3 and 6 hour time points.

Table 2: Summary of the effects of air pollution particles on CRP expression

	Effect on CRP
CB	Increased expression and secretion in a time-dependent manner. Nuclear localisation at 18 hours.
ufCB	Increased expression and secretion in a time-dependent manner. Nuclear localisation and polarisation of cytoplasmic staining at 18 hours.
PM10	Increased expression and secretion in a time-dependent manner. Nuclear localisation at 3, 6 and18 hours.
$FeCl_3$	No effect on either expression or secretion.

7.2.2 Effect of air pollution particles on fibrinogen expression.

The effect of environmental air pollution was investigated for fibrinogen as for CRP. However unlike CRP, fibrinogen expression was stimulated by all treatments including the $FeCl_3$ treatment. The treatment of the cells was carried out over an 18 hour time course and the cells analysed by indirect immunofluorescence and ELISA. The expression of fibrinogen was found to be highest for the PM10 treatment with an increase of 3 mg/ml (+/- 0.5), followed by ufCB with an increase of 1mg/ml (+/- 0.1), CB with an increase of 0.5 mg/ml (+/- 0.1) and $FeCl_3$ with an increase of 0.2mg/ml (+/- 0.1) over untreated cells. This suggests that oxidative stress may have a role in the effects on intracellular levels of fibrinogen.

Analysis of the cell supernatants for fibrinogen secretion shows that all four treatments were capable of stimulating the secretion of fibrinogen. Treatment of the cells with CB and ufCB appeared to show an increase in secreted fibrinogen in the first few hours of treatment but after 3 hours the levels decrease. This may be due to

absorption of fibrinogen onto the particle surface. However the treatment with PM10 does not appear to show this phenomenon. This may be due to the fact that there is a relatively low level of fibrinogen present during the first 6 hours of treatment and this may be due to absorption of the protein onto the particle surface (Kim *et. al.*, 2003); this may only be overcome when the levels of fibrinogen saturate the particle surface at 18 hours and this may explain the high degree of deviation among the three experiments. Another explanation for these results may be due to endocytosis of fibrinogen (Odrljin *et. al.*, 2001) by binding to the integrin receptor $\alpha v\beta 3$. This would result in the degradation of fibrinogen within the cells and it has been hypothesised that this may be involved in the process of alveolar tissue repair and matrix remodelling. Fibrinogen may also be degraded by the cells or proteins in the cell supernatant giving arise to fibrinogen degradation products (FDP) which are not detectable in the ELISA assay (degradation of fibrinogen reviewed by Herrick *et. al.*, 1999).

When examining the intracellular localisation of fibrinogen it can be seen that fibrinogen is localised in the cell cytoplasm in the peri-nucelar region. This is the same for treated and untreated cells. The only difference between the treatments is seen in the ufCB treated cells. The ufCB treated cells show some of the morphological changes shown in the cells stained for CRP: there is elongation and in the majority of the cells fibrinogen is localised densely at only one end of the cell. This may have consequences on fibrinogen secretion which occurs basolateraly due to the arrangement of the cell microtubules. However, disruption of the microtubules results in the inhibition of fibrinogen secretion (Guadiz *et. al.*, 1997). This may also contribute to the variable results for fibrinogen secretion shown by ELISA.

Table 3: Summary of the effects of air pollution particles on fibrinogen expression.

	Effect on fibrinogen
CB	Highly increased expression and secretion in a time-dependent manner.
ufCB	Highly increased expression and secretion in a time-dependent manner. Polarisation of cytoplasmic staining at 6 and 18 hours.
PM10	Highly increased expression and secretion in a time-dependent manner.
$FeCl_3$	Increased expression and secretion in a time-dependent manner

7.2.3 Effect of air pollution particles on Hsp70 expression.

The effect of PM10, CB, ufCB and $FeCl_3$ on Hsp70 expression in A549 cells was investigated. All four treatments stimulated an increase in the expression of Hsp70. The particle treatments all were able to stimulate Hsp70 expression in a time dependent manner but the $FeCl_3$ treatment produced a different response. Indirect immuofluorescence showed that the particle treatments not only generated an increase in intracellular Hsp70 but also produced nuclear localisation of Hsp70 after around 6 hours of treatment. Nuclear localisation of Hsp70 is known to result from exposure to stresses including oxidative stress. This is thought to be cytoprotective since Hsp70 bound to the DNA can protect against radical-induced genotoxicity (reviewed by Jacquier-Sarlin *et. al.*, 1994). Another function of Hsp70 in the nucleus during stress is to associate with the peri-ribosomal granular region of the nucleolus and assist in maintaining ribosome assembly and translocation (Welch and Suhan, 1986). In the nucleus Hsp70 can also bind to the DNA at HSF1 sites and inhibit the HSP gene transcription when high levels of HSP are present in the cells to control the stress response (Shi *et. al.*, 1998).

Indirect immunofluorescence of the $FeCl_3$ treated cells shows a different Hsp70 response from the particle treatments. It can be seen that there is a fast response to the iron treatment and this is resolved quickly leaving the cells with a lower level of Hsp70 than was present in the untreated cells. This may be due to stressing of the cells prior to treatment, since there is more Hsp70 present in the cells than was shown for the particle treatments. Another explanation could be that a quick response to the treatment occurred, since the iron is soluble in the culture medium, and that this was quickly resolved, therefore explaining the lack of CRP production to this treatment. Hsp70 has been shown to inhibit inflammatory responses by inhibiting the activation of the transcription factor, NFκB (Guzhova *et. al.*, 1997; Ayad *et. al.*, 1998; Yoo, *et. al.*, 2000; Stuhlheimer, 2000; reviewed by Santoro, 2000; Malhotra and Wong, 2002). Other studies have examined the effects of other transition metals on Hsp70 production. It has been found that Hsp70 can be induced in various cell types in response to treatments with zinc and cadmium salts (Timblin *et. al.*, 1998; Wagner *et. al.*1999; Gaubin *et. al.*, 2000; Croute *et. al.*, 2000). HSPs have also been found to be stimulated in alveolar macrophages by quartz coated with cadmium or vanadium

(Radloff *et. al.*, 1998). Other metals have been shown to induce HSP production in cells including copper, cobalt, nickel, mercury and arsenic (reviewed by Hall, 1994).

Analysis of the cell supernatants showed that there was some secretion of Hsp70 from the cells. There is little in the literature on secretion of Hsp70 from cells suggesting this may be one of the first studies to report this. Hsp70 is known to be present in the circulation during infection and inflammation (reviewed by Wallin *et. al.*, 2002). The presence of Hsp70 has normally been attributed to release from apoptotic and necrotic cells and from bacterial sources. Hsp70 has been found to be released after coronary bypass surgery (Dybdahl *et. al.*, 2002) although the cells responsible for producing Hsp70 were not identified. The presence of extracellular Hsp70 was presumed to be a result of necrosis. Although most of the Hsp70 present would appear to be the result of secretion some may also arise from apoptosis or necrosis from the treatment however concentrations used were subtoxic. Extracellular Hsp70 has been associated with the stimulation of an immune response through the generation of fusion proteins. These HSP fusion proteins can induce cytotoxic T-lymphocyte-mediated immune responses, protective anti-viral immunity, and stimulate classical and/or alternative MHC class I pathways (reviewed by Wallin *et. al.*, 2002). Extracellular Hsp70 has been associated with the generation of an inflammatory response through the production of cytokines in blood monocytes, peritoneal macrophages and various monocyte-like cell lines through the Toll like receptors TLR2 and TLR4 (Asea *et.* al., 2002; Dybdahl *et. al.*, 2002).

Table 4: Summary of the effect of air pollution particles on Hsp70 expression.

	Effect on Hsp70
CB	Highly increased expression and secretion in a time-dependent manner. Nuclear and peri-nuclear localisation at 6 and 18 hours.
ufCB	Highly increased expression and secretion in a time-dependent manner. Nuclear and peri-nuclear localisation at 6 and 18 hours.
PM10	Highly increased expression and secretion in a time-dependent manner. Some morphological changes.
$FeCl_3$	Rapid increase in expression and secretion which is reduced to control levels after 3 hours of treatment

7.2.4 Effect of air pollution particles on NFκB and IκB expression.

Treatment of A549 cells with CB, and ufCB produced the degradation of IκB followed by the activation of NFκB as shown by immunofluoresence. The PM10 treatment induced NFκB activation without any apparent IκB degradation, while $FeCl_3$ treatment failed to stimulate the degradation of IκB or the activation of NFκB in an IκB-independent manner (Jimenez *et. al.*, 2000). IκB degradation in the CB and ufCB treatments occurred within 30 minutes of particle treatment. The expression of IκB was increased between 3 and 6 hours for CB and after 1 hour of ufCB treatment. This was followed by nuclear localisation of IκB. During this time NFκB was found to have entered the nucleus of the cells at around 3 hours for CB treatment and ½ an hour for ufCB treatment. This suggests that the degradation of IκB led to the nuclear localisation of NFκB which in turn bound to the DNA to induce the expression of pro-inflammatory proteins including IκB (Le Bail *et. al.*, 1993; Cheng *et. al.*, 1994; reviewed by Baeuerle and Henkel, 1994; Baldwin *et. al.*, 1996; Christman, *et. al.*, 2000; Karin and Ben-Neriah, 2000). Once the newly formed IκB was present in the cell it was able to translocate to the nucleus and bind to the NFκB and remove the NFκB-IκB complex back into the cytoplasm of the cells where it is retained (Beg *et. al.*, 1992; Arenzana-Sesdedos *et. al.*, 1997; reviewed by Bachelerie *et. al.*, 1998), as shown by immunofluoresence at the 6 and 18 hour time points. The PM10 treatment did not appear to induce the degradation of IκB although NFκB was found in the nucleus of the cells at the ½ hours and 1 hour time points. This has been found previously in this cell line and the explanation for this phenomenon was attributed to the effects of transition metals, notably iron (Jiminez *et. al.*, 2000). It was suggested that oxidant-induced activation of NFκB occurred via a proteosome-independent pathway.

The treatment of cells with $FeCl_3$ appeared to generate no activation of NFκB or have any effect on the expression of IκB. This may have been expected since this treatment had little effect on the expression of any of the other proteins studied. This indicates that iron has no effect on the lung on its own but on the surface of PM10 it can interact with the other components and create redox cycling resulting in the generation of free radicals and oxidative stress (Wilson *et. al.*, 2002).

Table 5: Summary of the effect of air pollution particles on NFκB and IκB expression.

	Effect on NFκB	Effect on IκB
CB	Slightly increased expression during time course.	Nuclear localisation at 6 hours. Little difference from untreated cells at other time points.
ufCB	Highly increased expression nuclear localisation at ½ - 1 hour. Decrease in levels at 4 hours followed by increase at 6 hours with more nuclear localisation.	Increased expression after 1 hour with nuclear localisation at 1 and 3 hours.
PM10	Increased expression with nuclear localisation at ½ hour.	Little effect.
$FeCl_3$	Little effect.	Slight increase in cytoplasmic levels in treated cells.

7.2.5 Effect of air pollution particles on inflammatory response in lung epithelial cells.

The results from this chapter indicate that CB, ufCB and PM10 have the potential to elicit an inflammatory response in this cell line. Many of the results would indicate that oxidative stress may be generated, particularly in the ufCB and PM10 treated cells, and this is what is driving the inflammatory response. Oxidative stress and free radicals have been attributed to the activation of transcription factors including NFκB (Schreck *et. al.*, 1991; Barchowsky *et. al.*, 1995; reviewed by Baeza-Squiban *et. al.*, 1999). The oxidative stress and free radicals would also result in the production of Hsp70. The response time for the induction of the proteins would appear to occur at around the same time with NFκB apparently being activated just before Hsp70. The production of Hsp70 after NFκB would explain why an inflammatory response to the particle occurs in the cells when Hsp70 has been shown to inhibit NFκB activation (Guzhova *et. al.*, 1997; Ayad *et. al.*, 1998; Yoo, *et. al.*, 2000; Stuhlheimer, 2000; reviewed by Santoro, 2000; Malhotra and Wong, 2002).

The activation and nuclear localisation of NFκB would be expected to induce the expression of important pro-inflammatory proteins. These proteins would include IκB, CRP and fibrinogen as well as cytokines. The results indicate that all three of these proteins were produced after the nuclear localisation of NFκB suggesting that

this may be the case. Indeed a recent study has indicated that the over expression of NFκB can result in the production of CRP (Agrawal *et. al.*, 2003).

7.3.0 Investigation into mechanisms of pro-inflammatory protein production.

In chapter 5 the possible mechanisms of pro-inflammatory protein expression and secretion are discussed. Firstly the secretion of CRP, fibrinogen and Hsp70 was investigated to ensure that the proteins were indeed being secreted from the cells and not present in the cell supernatant as a result of cellular damage caused by the cell treatments. Then the effect of transcription inhibitors used in the presence of ufCB treatment was investigated to identify the transcription pathways being stimulated by the particle treatments which were responsible for the pro-inflammatory protein production. This chapter also discusses the effect of oxidative stress on the cells using tBHP and antioxidant treatments to investigate if the particles were able to stimulate the production of the pro-inflammatory proteins via oxidative stress. After establishing that oxidative stress would appear to induce pro-inflammatory protein production the effects of oxidative stress on cell was investigated looking at cell morphology and intracellular ATP levels. Finally this chapter identifies that these treatments are stimulating *de novo* synthesis of these inflammatory proteins and not the release of stored inactive protein subunits.

7.3.1 Is presence of CRP, fibrinogen and Hsp70 in supernatant a result of active secretion from the cell?

Firstly the cells were stained with propidium iodide after ufCB treatment to determine if the cell membrane was intact. If the treatment was damaging to the cell the membrane would be disrupted and the dye would enter the nucleus and would be visible under the UV microscope. The cells were counted after treatment with ufCB for various time points and it was found that there was a small decrease in cell viability over the time course but this is unlikely to be responsible for the amounts of each protein found in the supernatant.

Secretion of CRP and fibrinogen occurs through the conventional secretory pathway (Kushner and Feldmann, 1978; Hartwig and Danishefsky, 1991; reviewed by Redman

and Xia, 2001) via the rough endoplasmic reticulum, smooth endoplasmic reticulum and the Golgi apparatus before packaging into secretory vesicles to exit the cell by exocytosis (Lodish *et. al.*, 1995). The cells were treated with ufCB in the presence of Brefeldin A which has been shown to block the intracellular transport of secretory proteins. Brefeldin A impedes the transport of proteins from the endoplasmic reticulum to the Golgi complex therefore preventing the secretion of the proteins (Misumi *et. al.*, 1986). Treatment of ufCB simultaneously with Brefeldin A was shown to greatly reduce the levels of CRP, fibrinogen and Hsp70 in the cell supernatants. This indicates that these proteins are being actively secreted from the cells.

The actively secreted pro-inflammatory proteins could have consequences on the lung as a whole as discussed previously. After particle inhalation or during lung inflammation the released proteins may also enter the circulation. The presence of cytokines produced during inflammation such as TNFα, IL1β, and IFNγ have been shown to increase vascular permeability which allows the infiltration of proteins and cells across the lung-blood barrier (Sedgewick *et. al.*, 2002).

7.3.2. Effect of transcription complexes on particle induced pro-inflammatory protein production.

To investigate the effects of transcriptional events on the expression of CRP, fibrinogen and Hsp70 inhibitors of transcription complexes were used in combination with ufCB treatment. Previous studies suggest that NFκB is involved in the signalling of CRP and fibrinogen expression in cells during inflammatory stimuli (Agrawal *et. al.*, 2003; Siebenlist *et. al.*, 1994). Hsp70 has been shown to be under the transcriptional control of the JAK/STAT complex during oxidative stress (Stephanou *et. al.*, 1999; Madamanchi *et. al.*, 2001) while STAT3 (also known as APRF, acute phase response factor) has been associated with the induction of fibrinogen and CRP expression (Siebenlist *et. al.*, 1994; Zhang *et. al.*, 1996; Agrawal *et. al.*, 2001; Agrawal *et. al.*, 2003).

These transcription factors can be activated by various stimuli including stress (oxidative, chemical, UV), pro-inflammatory cytokines, and pathogens including

bacterial LPS via Toll-like receptors. NFκB as mentioned previously is particularly redox sensitive and is readily activated by free radicals (Schreck *et. al.*, 1991; Meyer, *et al.*, 1994; Barchowosky *et. al.*, 1995; and reviewed by Haddad, 2002; Rahman, 2002). The JAK/STAT pathway can also be activated in response to intracellular ROS (Simon *et. al.*, 1998) and endogenous hydrogen peroxide (Carabello *et. al.*, 1999). These two transcription factors can also be activated by a wide variety of cytokines including TNFα, IL1β for NFκB, and IL2, IL3, IL4, IL5, IL6, IL11, IL12, IFNγ, and GM-CSF for JAK/STAT (reviewed by Hanada and Yoshimura, 2002; Heim, 1996; Boscoe *et. al.*, 1996).

Inhibitors of these transcription complexes were found and treated with ufCB to determine if the production of CRP, fibrinogen and Hsp70 were dependent on the activation of these transcription complexes. The inhibitor for NFκB was a cell permeable peptide known as sn50. Sn50 contains the NFκB nuclear translocation signal sequence allowing it to enter the nucleus and bind to the DNA mimicking and preventing the binding of NFκB (Lin *et. al.*, 1995). The inhibitor used for the JAK/STAT activation was a tyrosine kinase blocker known as Ag490. Ag490 is a member of tyrosine kinase inhibitors known as tyrophostins (Gazit *et. al.*, 1991). Ag490 works by blocking the constitutive activation of STAT3 (Nielsen *et. al.*, 1997).

Treatment with ufCB and these inhibitors indicated that intracellular levels of CRP were dependent on NFκB and not by JAK/STAT and that secretion of CRP required both transcription complexes. The dependence of CRP expression and secretion on NFκB activation is not surprising since CRP is known to be activated by NFκB during inflammatory events. JAK/STAT inhibition however shows only reduced levels of CRP in the cytoplasm compared to ufCB treated cells but there does appear to be the requirement for the JAK/STAT pathway in the secretion of CRP. The lack of CRP inhibition with Ag490 treatment indicates that either the treatment does not activate the JAK/STAT pathway or that the use of this inhibitor was not able to inhibit the pathway efficiently. However, the inhibition of secreted CRP indicates that the inhibitor may be having a small effect on the production of CRP which is more evident at a later stage in the production and secretion of CRP. The use of this inhibitor may be having a small effect on the synthesis of CRP which may have an

effect on the normal synthesis and secretion pathway for example the rate limiting step in CRP secretion is the time of exit from the RER, so if the inhibitor had an effect on the RER this would result in reduced secretion of CRP from the cells. CRP secretion efficiency has also been shown to be related to the intracellular concentration of CRP so if there was a slight decrease in this concentration it may have a larger consequence on the amount of secreted CRP (Hu *et. al.*, 1988).

The effect of transcription inhibitors of fibrinogen expression is very similar to the effects on CRP expression with NFκB apparently being responsible for the expression and secretion of fibrinogen from the cells in response to ufCB treatment. A small decrease was also shown for treatment with Ag490 indicating that the JAK/STAT pathway may have a small effect but not as much as NFκB. The effect on Hsp70 expression indicated that NFκB had little effect on Hsp70 expression but was capable of inhibiting the secretion of Hsp70. The treatments with Ag490 indicated that Hsp70 expression and secretion was dependent on the JAK/STAT pathway.

7.3.3. Effect of oxidative stress on particle induced pro-inflammatory protein production.

The results of the particle treatments and the use of transcription inhibitors suggested that oxidative stress may play a role in the expression of these pro-inflammatory proteins. Therefore the effect of oxidative stress on the expression of these proteins was carried out using treatment with tBHP. Treatment with tBHP showed similar responses to the particle treatments on CRP, fibrinogen and Hsp70 expression and secretion. Since tBHP was soluble in the culture medium a shorter time series was used but a time dependent increase in expression occurred for all three proteins. Nuclear localisation of CRP and Hsp70 occurred at the later time points and there was some evidence of changes in cell morphology as was found in the ufCB and PM10 treatments.

7.3.4. Effect of antioxidant treatment on particle induced pro-inflammatory protein production.

In the lung environment epithelial cells cope with oxidative stress by producing antioxidants which scavenge free radicals. The epithelium is covered by lining fluid

which contains many antioxidants produced by the cells including mucin, uric acid, protein (albumin), ascorbic acid (Vitamin C) and reduced glutathione (Cross *et. al.*, 1994). Several studies have found that synthetic lung lining fluid can reduce oxidative damage in cells treated with particles (Sun *et. al.*, 2001; Greenwell *et. al.*, 2002).

Many studies have also treated cells with PM10 in the presence of other antioxidants and found that these antioxidants scavenged the free radicals preventing NFκB activation. Many studies have used N-acetyl cysteine (NAC) which has been shown to increase intracellular levels of the antioxidant glutathione and deplete the oxidative effects associated with PM10 and DEP (Quay *et. al.*, 1998; Kennedy *et. al.*, 1998; Stringer *et. al.*, 1998; Gaubin *et. al.*, 2000; Omara *et. al.*, 2000; Harper *et. al.*, 2001; Whitekus *et. al.*, 2002). Studies carried out using Vitamin E analogues such as Trolox and α-tocopherol have shown that these compounds can reduce oxidative effects in cells. Trolox is a water soluble derivative of vitamin E and can penetrate biomembranes and protect the cells. Trolox has been shown to prevent hydroxyl radical formation, H_2O_2 formation, lipid peroxidation, and intracellular plasmid degradation in cultured epithelial cells (Menzel, 1992; Luo, X *et. al.*, 1999; Luo, X. *et. al.*, 2003).

To further investigate the effects of oxidative stress on the expression and secretion of CRP, fibrinogen and Hsp70, treatment of the cells with ufCB was carried out in the presence of antioxidants. The antioxidants used were either a N-acetylcysteine analogue, Nacystalin (NAL) or Trolox. Firstly, the antioxidant and ufCB treated cells were examined using indirect immunofluoresence for NFκB to show that the concentration of antioxidants were capable of mopping-up any free radicals, thereby preventing the activation and nuclear transclocation of NFκB. This showed that both antioxidants prevented the nuclear localisation of NFκB. Similar results have also been found in human blood monocytes using ufCB and these antioxidants (Brown *et. al.*, 2003). As for the use of the transcription inhibitors there was a reduction in the levels of intracellular expression of CRP, fibrinogen and Hsp70 although this was not statistically significant for any of these proteins. Similarly to the transcription factor inhibitors there was a huge reduction in the levels of secreted proteins from the cells for CRP and fibrinogen showing statistically significant reductions for both

antioxidants compared to the ufCB treated cells. This indicated that oxidative stress was capable of inducing the transcription factors responsible for the production of these proteins. Interestingly both antioxidants were able to produce similar effects on protein expression although these antioxidants work by different mechanisms. Many antioxidants have been shown to reduce lipid and protein peroxidation during oxidative stress however thiol antioxidants such as NAL are the only antioxidants which decrease the intracellular GSH:GSSH ratio during oxidative conditions (Whitekus *et. al.*, 2002). Trolox works by preventing increased hydroxyl radical and hydrogen peroxide formation. This reduction in hydrogen peroxide in culture medium can prevent programmed cell death by apoptosis which occurs when the levels of intracellular thiols are depleted (Luo *et. al.*, 1999). Therefore the protection of intracellular thiols by different means (NAL by maintaining intracellular GSH:GSSH ratio and Trolox by mopping-up radicals in culture medium) may help explain the similar results for both antioxidants.

7.3.5. Morphological changes in A549 cells with particle treatments.

To investigate further the apparent morphological changes occurring with particle treatment the cells were treated and Diff-Quick staining of the cells was carried out. This enabled identification of any changes in morphology and revealed any indications of cell apoptosis or necrosis associated with the treatments. Treatment with $FeCl_3$ showed little morphological changes over the time course which was not unexpected since there was no previous evidence to indicate free radicals and oxidative stress were having a role in the effects of this treatment. However, tBHP and all the particle treatments (PM10, ufCB, CB) induced huge morphological changes occurring during the first few hours of treatment which increased with time. This suggests that oxidative stress may be causing these changes in the cells which if this occurred *in vivo* would result in increased epithelial permeability and movement of cells and proteins across the epithelial blood barrier with resulting inflammation (Bellomo and Miarbelli, 1992; Morrison *et. al.*, 1999, reviewed by Rahman and MacNee, 2000).

Morphological changes are thought to occur during oxidative stress as a result of ATP depletion. The depletion of intracellular ATP causes depolymerisation of actin resulting in an elongated cell shape which has been shown by immunofluoresence and

the Diff-Quick staining. Intracellular levels of ATP were measured using a luciferase assay after CB, ufCB, tBHP and $FeCl_3$ treatments. It would be expected that tBHP would induce oxidative stress which would induce a large reduction in ATP levels (Nowak *et. al.*, 1998; Min *et. al.*, 2000) and the $FeCl_3$ treatment would have little effect. When carried out this was indeed the case. The ufCB treatment showed a very similar response to the tBHP treatment indicating that free radical production is likely to contribute to the effects of ufCB on the activation of NFκB and subsequent pro-inflammatory protein production. Surprisingly, the CB treatment showed a sharp decrease in the levels of ATP in the first few hours of treatment and the levels recovered between 3 and 18 hours. This was unexpected and was reproducible when repeated on a number of occasions. This may indicate that free radical production is associated with this treatment and is responsible for the effects found for CB treatment.

7.3.6. RT-PCR for particle treated cells

To ensure that the CRP, fibrinogen and Hsp70 levels detected in the cell cytoplasm and supernatant are the result of protein synthesis arising from the cell treatments RT-PCR was carried out. One time point was chosen for CB, ufCB, tBHP, $FeCl_3$ and TNFα treatments. However, not all these treatments are likely to induce optimal RNA synthesis at the time point since CB and ufCB are particulate and tBHP, $FeCl_3$, and TNF are soluble in the culture medium. However, the results suggest that all these treatments are having an effect in the first few hours. After 90 minutes of treatment increased mRNA expression was found after ufCB, and tBHP exposures indicating that the treatments were indeed inducing the synthesis of these proteins presumably as a result of transcriptional activation by free radicals.

7.4.0 Effect of A549 cell supernatants on the monocyte-like cell line MM6.

After establishing that CRP and fibrinogen can be secreted from A549 cells the effect of these proteins on surrounding cell populations which occur in the lung was investigated. Plasma CRP and fibrinogen are both known to interact with various cell types including blood monocytes and tissue macrophages during an inflammatory response (Galve-de Rochmontiex *et. al.*, 1993; Pue *et. al.*, 1996; Sitrin *et. al.*, 1998; Smiley *et. al.*, 2001). Several studies have focused on the interactions among lung

cell populations using cell lines. One study examined the cross talk of cytokines during inflammatory stimuli between pulmonary epithelial cells and peripheral blood mononuclear cells (Krakauer, 2002). Another study examined the effect of PM10-exposed macrophages on lung epithelial cells (Jimenez *et. al.*, 2002). Both these studies indicate the communication between cells by secreted proteins, namely cytokines, during inflammatory stimuli.

7.4.1 Effect of CRP and fibrinogen on pro-inflammatory protein expression in MM6 cells.

Treatment was firstly carried out using exposure of MM6 cells to recombinant CRP or fibrinogen. Immunofluoresence showed very low levels of CRP, fibrinogen, NFκB and Hsp70 in untreated cells. However CRP treatment induced CRP and NFκB expression while fibrinogen induced Hsp70, fibrinogen and NFκB expression. This indicates that CRP and fibrinogen both can stimulate a response in MM6 cell but each protein would appear to induce different responses. The expression of CRP in MM6 cells with CRP treatment could arise from phagocytosis or uptake of CRP molecules by the cells (Mold *et. al.*, 2001) or by the release of stored CRP subunits (Yue *et. al.*, 1996). While fibrinogen treatment induced the expression of fibrinogen in the MM6 cells which may arise from the phagocytosis of fibrinogen molecules, it is more likely to be the result of NFκB dependent production. Fibrinogen treatment was also able to induce the expression of Hsp70 in the MM6 cells and this may occur through Toll-like receptor 4 (TLR4). Fibrinogen has been shown to activate chemokine expression in macrophages through TLR4, and Hsp70 expression can be activated by binding to TLR4 on the surface of monocytes (Smiley *et. al.*, 2001; Dybdahl *et. al.*, 2002). The synthesis of Hsp70 by macrophages has been shown in response to TNFα (Heimbach *et. al.*, 2001; Wantanabe *et. al.*, 1997; Wantanabe *et. al.*, 1998) which could result from TLR4 binding or by activation of NFκB. Interestingly, both CRP and fibrinogen treatments were able to induce the expression and nuclear localisation of NFκB. NFκB activation in monocytes by fibrinogen has been shown previously (Sitrin *et. al.*, 1998) but the same has not been found for CRP.

7.4.2 Effect of A549 CM on protein expression in MM6 cells

The effect of CM from A549 cells treated with ufCB (100μg/ml for 18 hours) on protein expression in MM6 cells was investigated. This indicated that CM exposure induced the expression of CRP, fibrinogen, Hsp70 with activation and nuclear localisation of NFκB. To identify if there was also secretion of CRP and fibrinogen from the cells ELISAs were carried out. This showed that MM6 cells were capable of secreting CRP and fibrinogen constitutively without treatment. ELISAs for CRP and fibrinogen in the supernatants of the A549 treated cells were carried out to confirm the levels present in the CM used to treat the MM6 cells with. This showed that there was constitutive secretion of both proteins in untreated MM6 cells and there was CRP and fibrinogen present in the CM prior to treatment. That there was less CRP present after MM6 treatment than expected may be due to phagocytosis of CRP or degradation of CRP by MM6 cells (Robey *et. al.*, 1997; Mortensen and Zhong 2000). The secretion of fibrinogen from MM6 cells was higher than either the untreated MM6 cells or the A549 conditioned media treated MM6 cells as expected and indicates the secretion of fibrinogen.

The cell supernatants were also analysed for cytokine production by MM6 which may be a result of the NFκB activation induced by the CM treatment. This indicated that IL8 could be produced by MM6 cells in response to the CM treatment. There was also a small increase in the levels of TNFα and IL6. The high levels of IL8 indicate that there is an inflammatory response occurring which may be associated with cell damage. IL8 is often produced during tissue injury and is involved in the recruitment and activation of circulating and tissue neutrophils (Luscinskas *et. al.*, 1992). Oxidative stress has been shown to induce NOS production in many cell types including epithelial cells and NOS production is often associated with concomitant formation of superoxide and hydrogen peroxide (Adcock *et. al.*, 1994). The presence of these species in the CM as well as the presence of pro-inflammatory proteins and cytokines provides a further mechanism for the activation of NFκB in MM6 cells. Other mechanisms which may trigger the secretion of cytokines from MM6 cells with A549 CM treatment could include ICAM-1 expression in response to Hsp70 secreted from the A549 cells (Galdiero *et. al.*, 1997). Increased ICAM 1 expression can lead to

cytokine production (in particular IL8), reactive oxygen species production and cell proliferation (Hubbard and Rothlein, 2000)

7.4.3 Investigation into damaging effects of A549 CM on MM6 cells

The high levels of IL8 produced by MM6 cells after treatment with ufCB treated A549 culture medium indicated cell damage. The increased expression of NFκB and Hsp70 in the cells also indicated that the treatment was inducing stress (presumably oxidative stress) in the MM6 cells. Diff-Quick staining of the cells at various time points of treatment shows that a huge stress response occurred in the first 30 minutes of treatment which induced cell apoptosis and necrosis. The remaining cells were shown to phagocytose the damaged cells and after 3 hours the cells remaining viable appear to be of normal cell morphology. This indicated that the A549 cells are producing species associated with oxidative stress for example NOS and/or ROS (Adcock *et. al.*, 1994) and this is then having and effect on the MM6 cells.

To further investigate the damaging effects of the A549 CM on MM6 cells a lactate dehydrogenase (LDH) assay was carried out. LDH is released from cells during membrane damage and is a good indicator of toxicity. This showed that there was little LDH present in the untreated MM6 cell and the A549 CM. However, after treatment of MM6 with CM for 4 hours there were increased levels of LDH present in the cell supernatant which is likely to be produced by the necrotic cells found after 1 hour of treatment with the CM.

7.5.0 Conclusions.

The results discussed above indicate the importance of CRP, fibrinogen and Hsp70 expression and secretion in the progression and pathology of inflammatory lung conditions In this thesis the main focus of the work was on the expression of these proteins in A549 lung epithelial cells in response to inflammatory stimuli such as cytokines and particulate air pollution (see overview in Figure 125). The production of these proteins in the lung could provide insight into inflammatory effects associated with infection (microbial, viral and parasitic) in the lung and also in other organs. Both CRP and fibrinogen production have been associated with infection locally and systemically (Simpson-Haidaris *et. al.*, 1998; Szalai *et. al.*, 1999; Gould and Weiser, 2001; Horan *et. al.*, 2001; Du Clos and Mold, 2001; O'Grady *et. al.*, 2001).

The expression of all the proteins studied with respect to environmental air pollution particle exposure also indicates the powerful effects of oxidative stress on the progression of lung inflammation. PM10 exposure has been associated with adverse health effects (Donaldson and MacNee, 1998; Salvi *et. al,*. 1999; MacNee *et. al.*, 2000). Many of these effects include morbidity and mortality due to not only respiratory but also cardiovascular diseases (Dockery *et. al.*, 1993; Seaton *et. al.*, 1995; Schwarz, 1999; Donaldson *et. al.*, 2001). The secretion of CRP, fibrinogen and Hsp70 from the lung epithelium would appear to have important consequences on the localised inflammatory response. This study indicates that the secretions from epithelial A549 cells in response to particulates is capable of having a huge response on monocytic cells. Several studies have shown inability of alveolar macrophages (AM) to cope with the clearance of inhaled particulates (discussed by Renwick *et. al.*, 2001) and this may arise due to impaired function of the AM arising from epithelial cell secretory products as well as the association with particle overload.

Other studies have indicated that CRP, fibrinogen and Hsp70 have effects on the cells present in the lung during inflammation. For example CRP is involved in the influx and impairment of neutrophils which are recruited to the lung during inflammation (Buchta *et. al.*, 1988; Shepherd *et. al.*, 1992; Heuertz *et. al*, 1993, 1994, 1996, 1999; Kew *et. al.*, 1990; Heurtz and Webster 1997; Zouki *et. al.*, 1997; Zhong *et. al.*, 1998).

In the rabbit lung the effects of CRP in the circulation has been associated with decreased vascular permeability (Abernathy *et. al.*, 1996). Fibrinogen has also been shown to have effects on the function of neutrophils and monocytes. Fibrinogen unlike CRP, has been shown to promote neutrophil activation (Altier, *et. al.*, 1993; Walzog *et. al.*, 1999; Rubel, *et. al.*, 2001; Forsyth *et. al.*, 2001) and activate monocytes (Sitrin *et. al.*, 1998) all via signalling through integrin (CD11b/CD18) binding. The effects of Hsp70 during inflammation generally occur intracellularly. However, there has been evidence recently that Hsp70 can also be secreted from cells and extracellular Hsp70 can be involved in the signalling of pro-inflammatory cytokines by monocytes (Asea *et. al.*, 2002). Until this time the circulatory pool of Hsp70 was thought to arise from necrotic cells or was assumed to be bacterial in origin (Hall, 1994). Hsp70 also has been associated with endothelial cells, particularly in and around the heart where it is associated with increased risk of myocardial ischemia (Yellon *et. al.*, 1994).

The results shown here may be useful in understanding the signalling events arising in response to oxidative stress and the production of CRP, fibrinogen and Hsp70 during inflammation. The expression of CRP and fibrinogen has been shown to be similar to that in hepatocytes by being inducible by cytokines which stimulate the activation and nuclear localisation of NFκB. The expression and secretion of Hsp70 in response to oxidative stress via the JAK/STAT pathway in the lung epithelium also provides another mechanism by which inhaled particles can mediate their effects.

The secretion of CRP, fibrinogen and Hsp70 from the cells also provides a mechanism by which particles can exert extrapulmonary effects. Evidence here indicates that particle-induced oxidative effects can cause morphological changes in epithelial cells which, if this occurs *in vivo*, may contribute to the increased permeability of the lung during inflammation. The permeabilised lung then could allow not only the extravasion of inflammatory cells and proteins from the circulation but allow the movement of secreted proteins from the lung, including CRP, fibrinogen and Hsp70, contributing to the systemic inflammatory response. Increased levels of environmental particles have been associated with increased cardiovascular disease and morbidity (Costa *et. al.*, 2000; Donaldon *et. al.*, 2001; Donaldson and MacNee,

2001). Since CRP, fibrinogen and Hsp70 are all well recognised risk factors for cardiovascular disease there may be a link between the secretion of these proteins from the lung epithelium and the increased risk of cardiovascular events.

Many studies on the effect of PM10 on systemic immune responses have found increased plasma levels of CRP and fibrinogen. Increased levels of plasma CRP have been found with exposure to PM10 in several studies (Seaton *et. al.*, 1999; Peters *et. al.*, 2001). Increased plasma levels of fibrinogen have also been found in response to PM10 and this has been associated with increased plasma viscosity (Pekkanen *et. al.*, 2000; Gardner *et. al.*, 2000; reviewed by Donaldson *et. al.*, 2001). While Hsp70 has not previously been found to be present in the circulation during particulate air pollution episodes these results indicate that it should be investigated further in exposure studies.

These results may provide useful information for further studies on inflammation in the lung in particular particle-induced inflammation. This study indicates the potential for further investigation into cellular mechanisms of pro-inflammatory protein production and signalling through the JAK/STAT pathway. Further investigation into the secretion of proteins into the lung alveolus and fate of secreted proteins would also provide insight into the proposed mechanism by which cardiovascular disease is associated with inhalation of environmental particles.

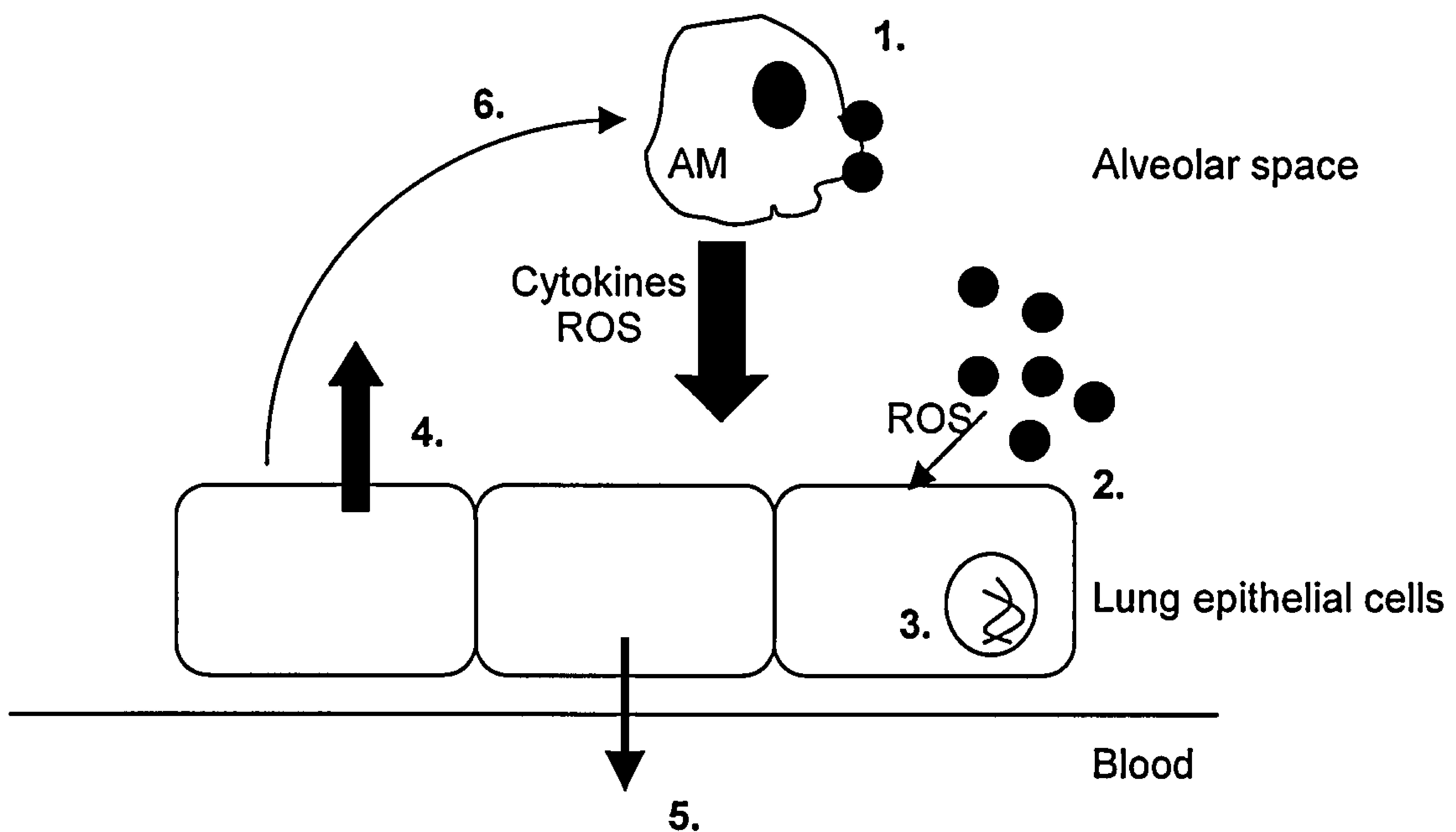


Figure 125: Overview of potential mechanisms by which inflammatory proteins are produced in lung in response to particle exposure. 1. Particles enter lung and come into contact with AM. AM phagocytose particles and generate cytokines and ROS. 2. Direct contact of particles and ROS with epithelial cells. 3. Activation of transcription and production inflammatory proteins. 4. Secretion of proteins into alveolus. 5. Possible secretion of proteins into circulation during inflammation/membrane permeability. 6. Interaction between secreted inflammatory proteins and AM and potential exacerbation of inflammatory response.

7.6.0 Ideas for future work

Further work based on this project could focus on the *in vivo* production of CRP, fibrinogen and Hsp70. Studies on human volunteer and animal exposure to PM10 inhalation could be carried out to assess whether the production of these proteins still occurs *in vivo.* Analysis of human lung secretory products could also be carried out to analyse the secretion of these proteins during inflammatory conditions. To examine if these proteins can enter the circulation from the lung, labelled proteins could be administered in to the lungs of animals and the blood analysed for the labelled proteins.

Futher *in vitro* experiments could also be carried out on primary epithelial cells to determine if they behave similarly to that of the A549 cell line. Analysis using

confocal microscopy could help determine the exact location of the proteins within the cells and help to study the nuclear translocation of proteins. Multiple staining of the cells could also be carried out to find if the proteins are localised in the same areas of the cells at the same time e.g. the acute phase proteins CRP and fibrinogen or NFκB and IκB. Further analysis of the effects of particles on NFκB activation could be carried out. This could include detailed examination of cytosolic and nuclear fractions of the cells to determine when NFκB was in each fraction and this could be measured by a more sensitive technique than the western blot such as EMSA or ELISA.

Chapter 8

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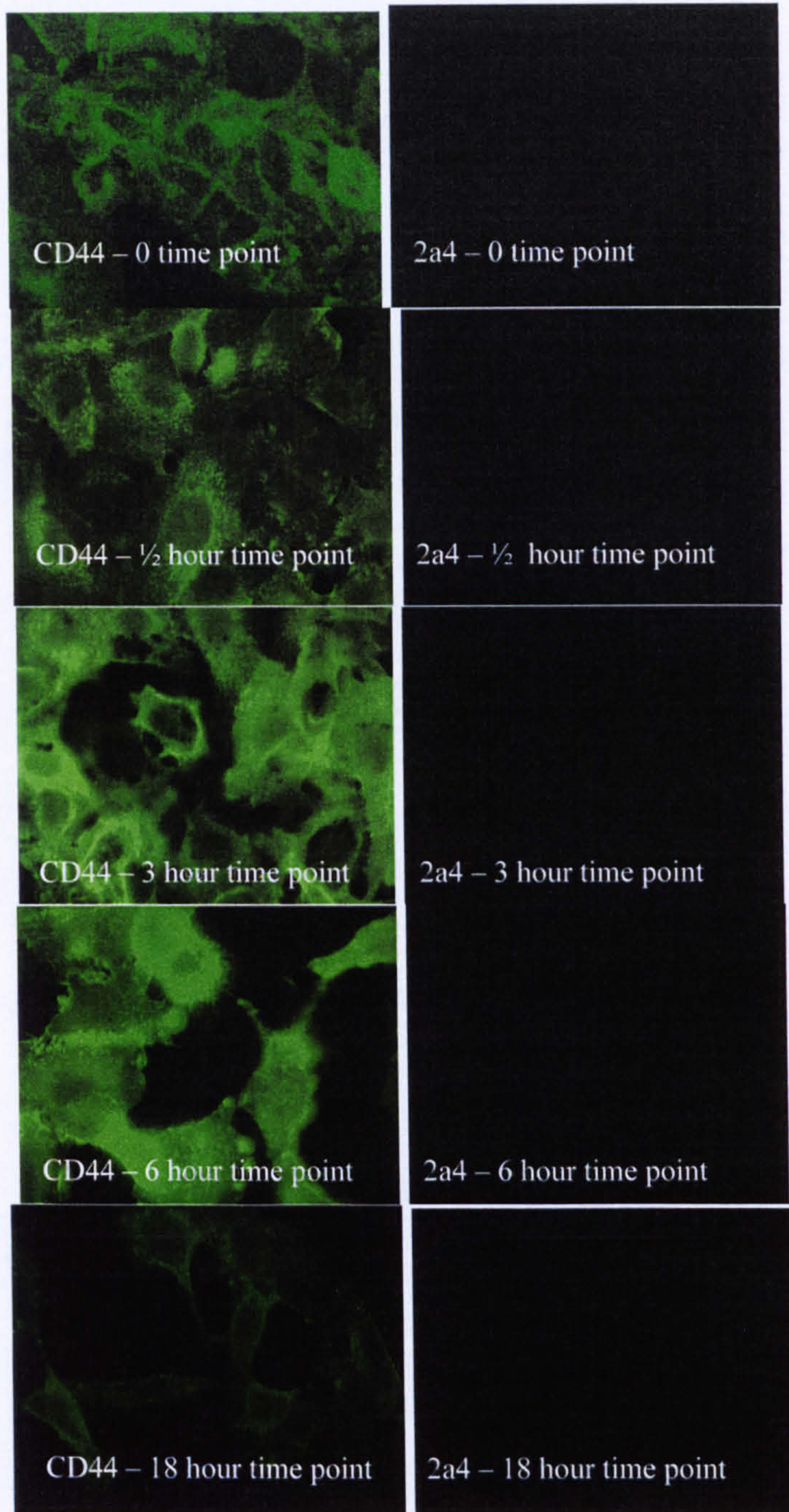
Appendix I: Controls for immunofluorescence of A459 cells shown in Chapter 4.



Appendix II: Comparison between effects of CB and ufCB on pro-inflammatory protein production.

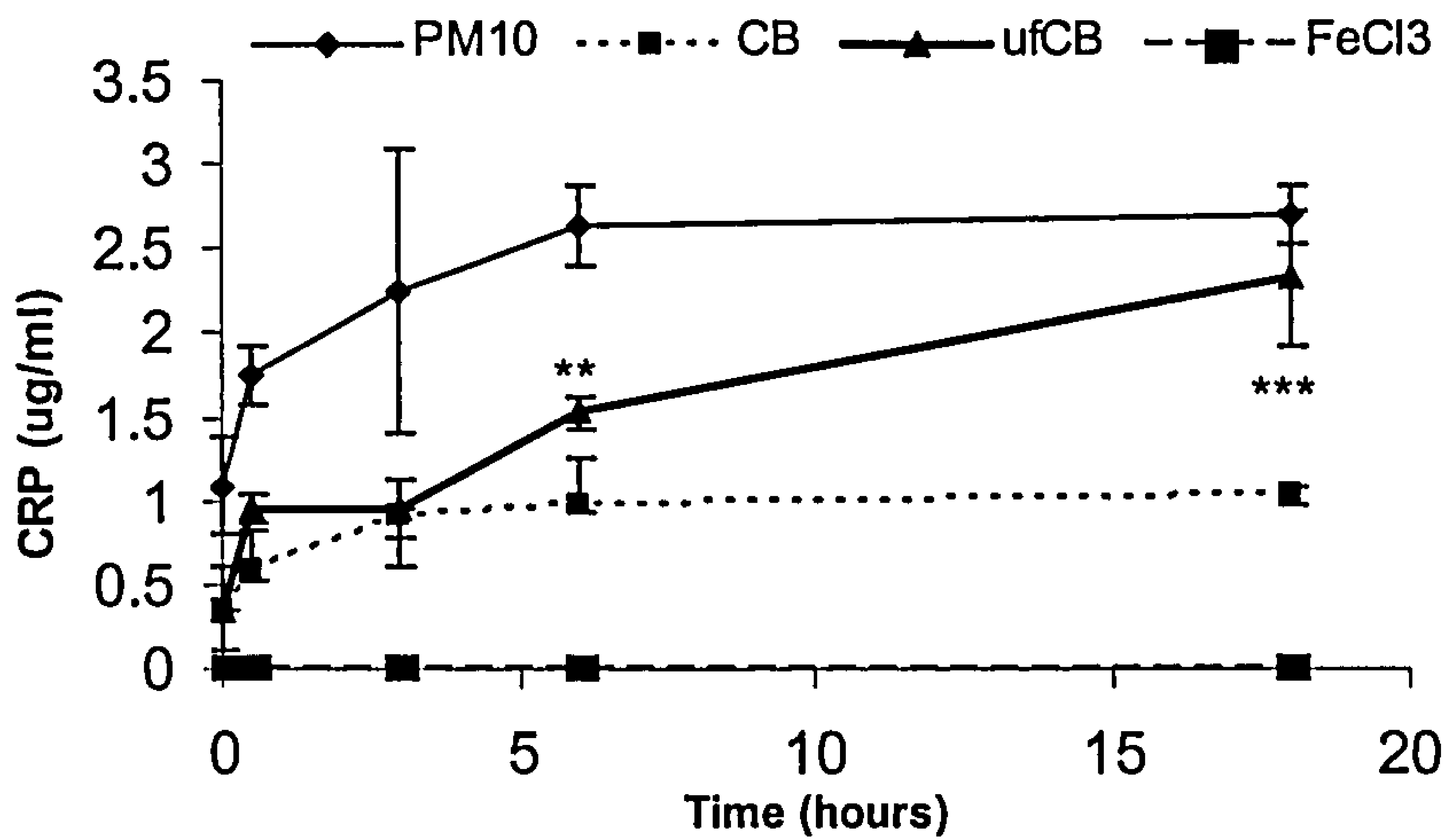


Fig a) Concentration of CRP in cell lysate, measured by ELISA.

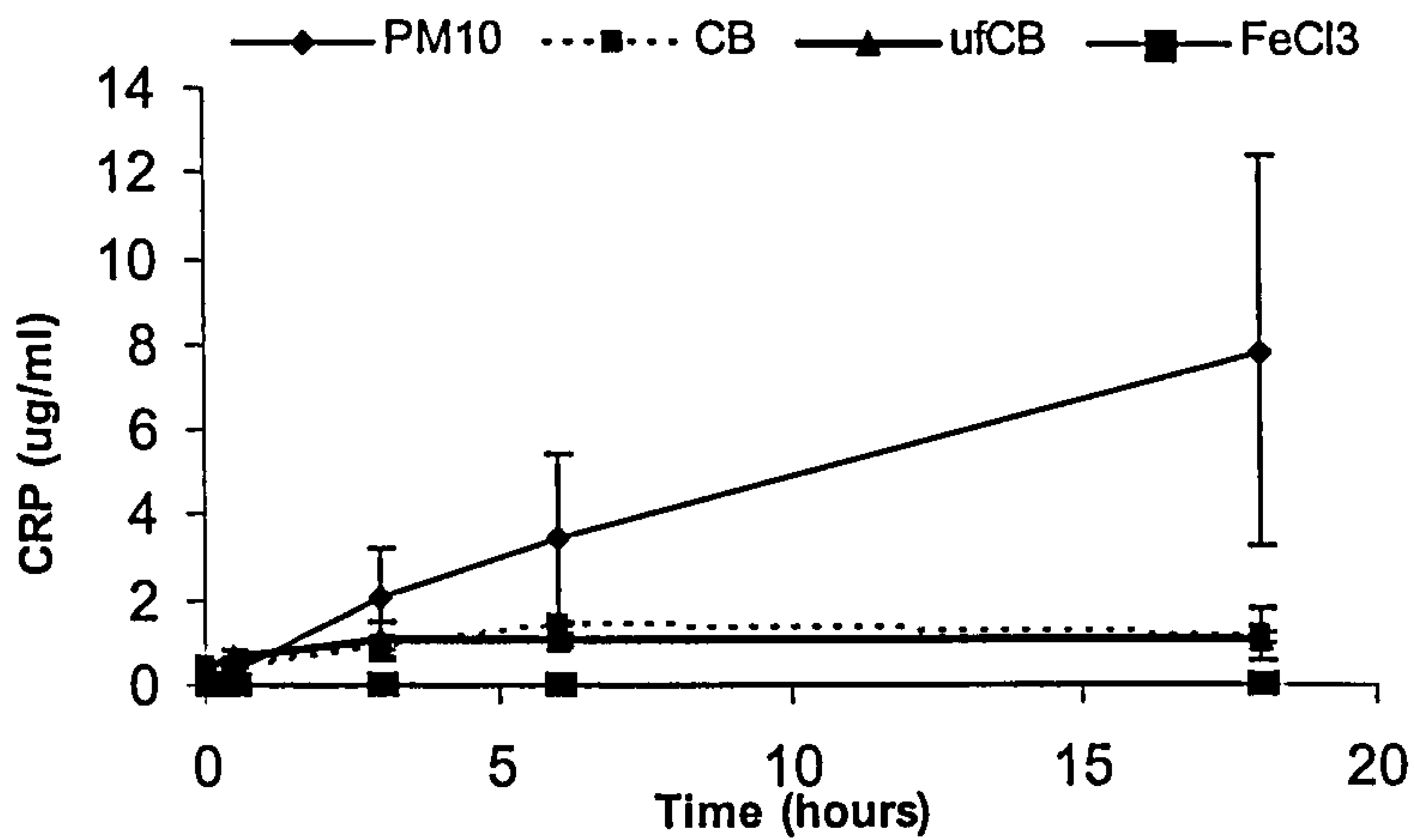


Fig b) Concentration of CRP in cell supernatant, measured by ELISA.

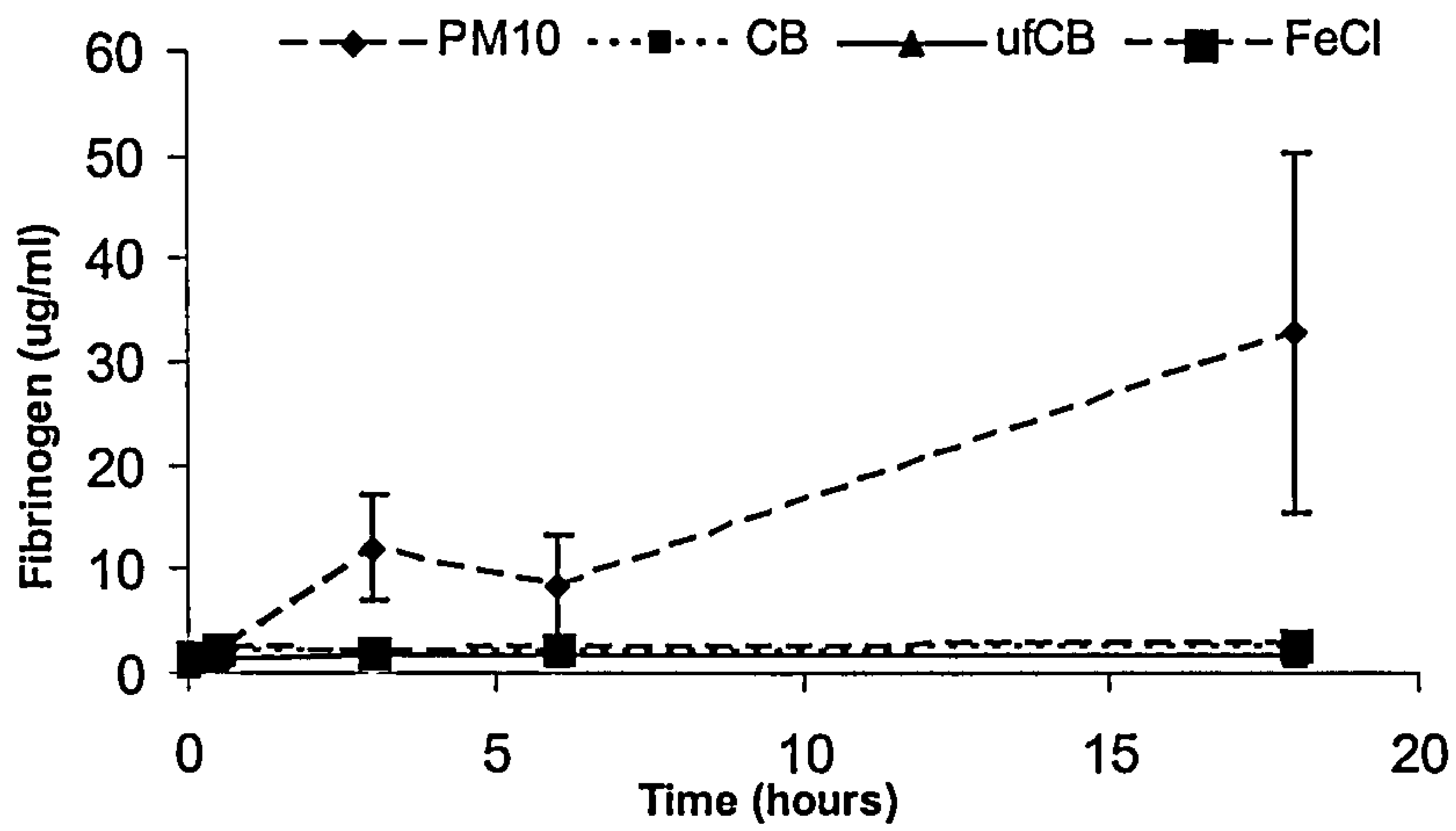


Fig c) Concentration of fibrinogen in cell lysate, measured by ELISA.

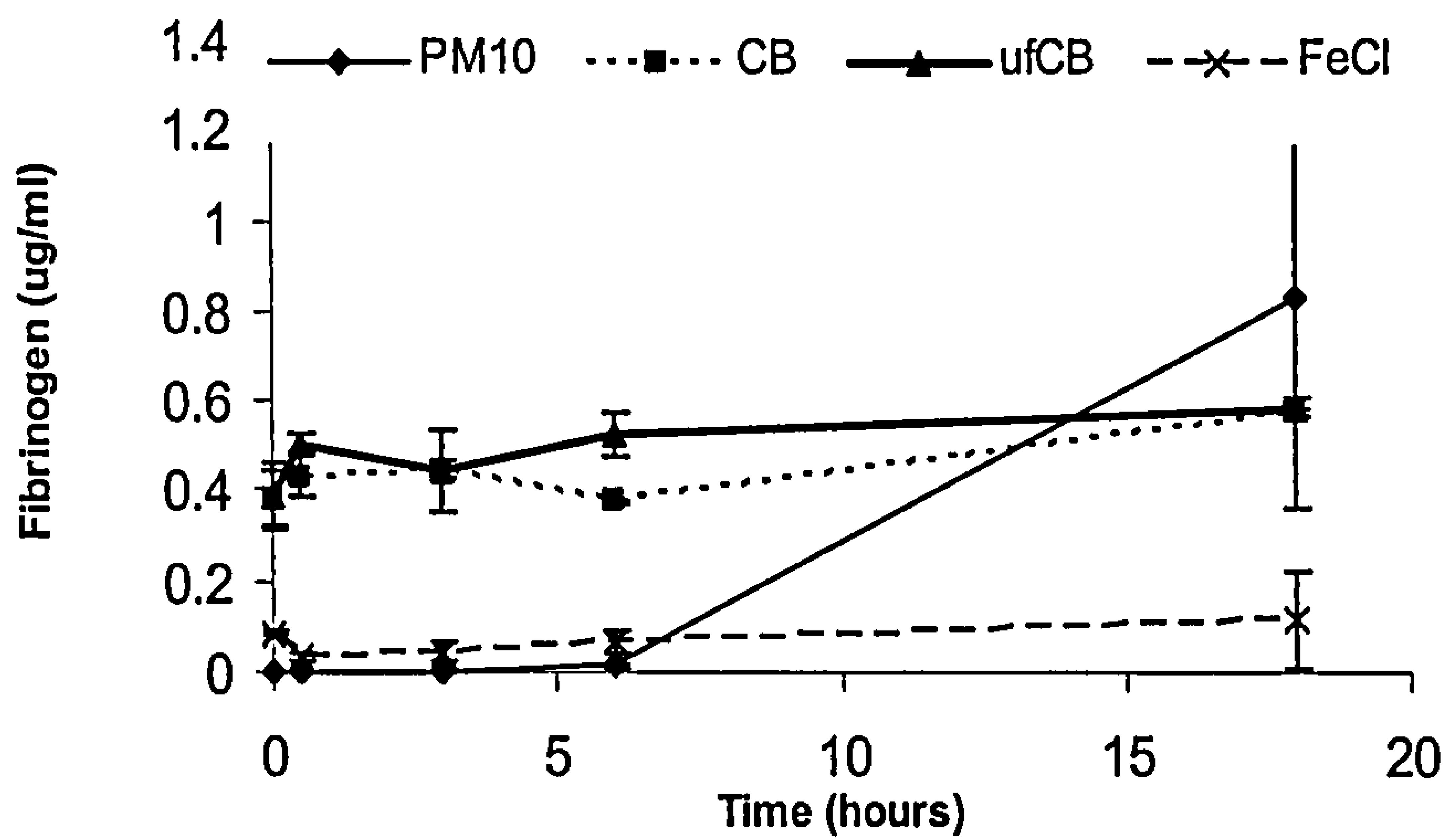
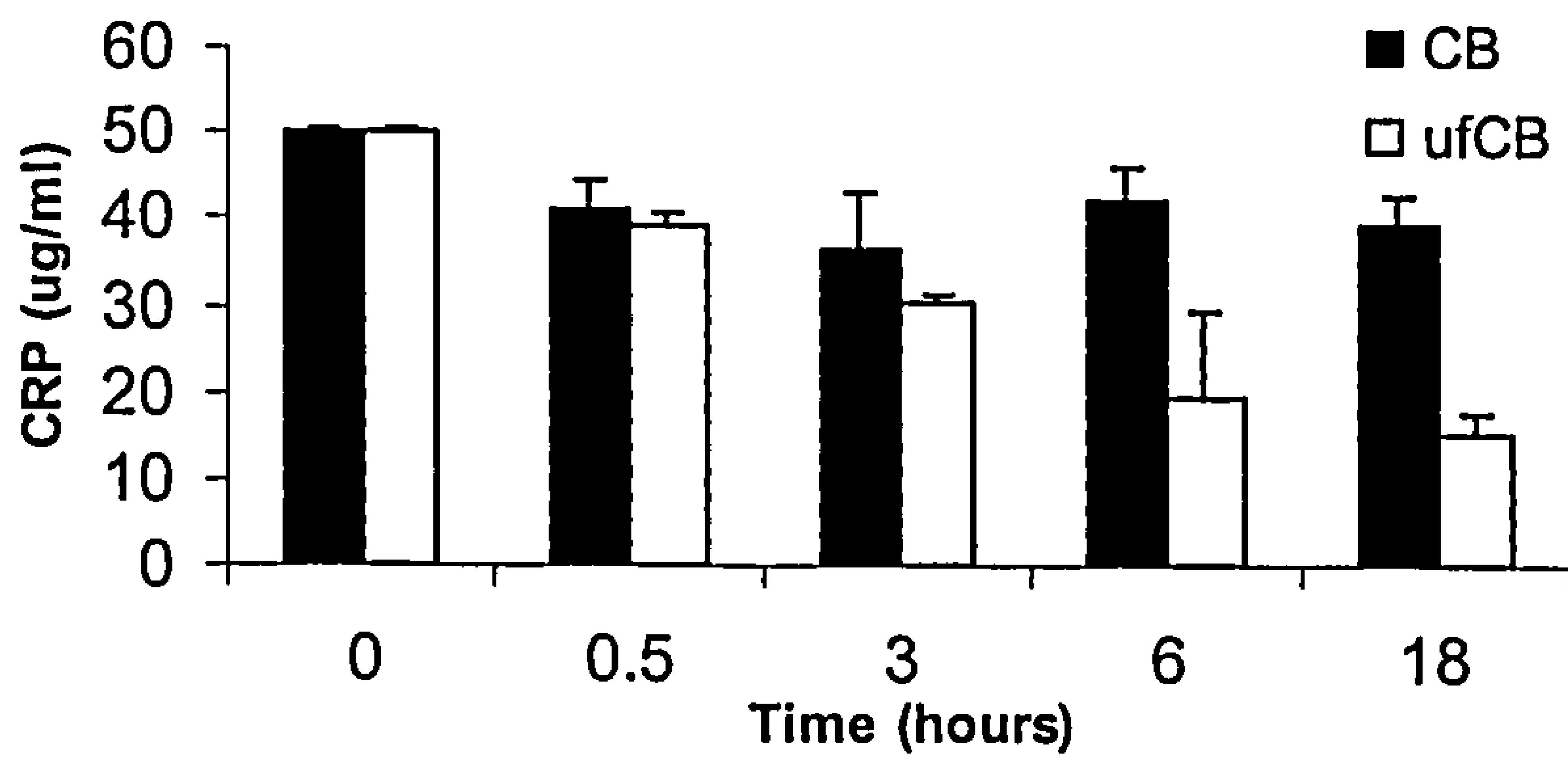
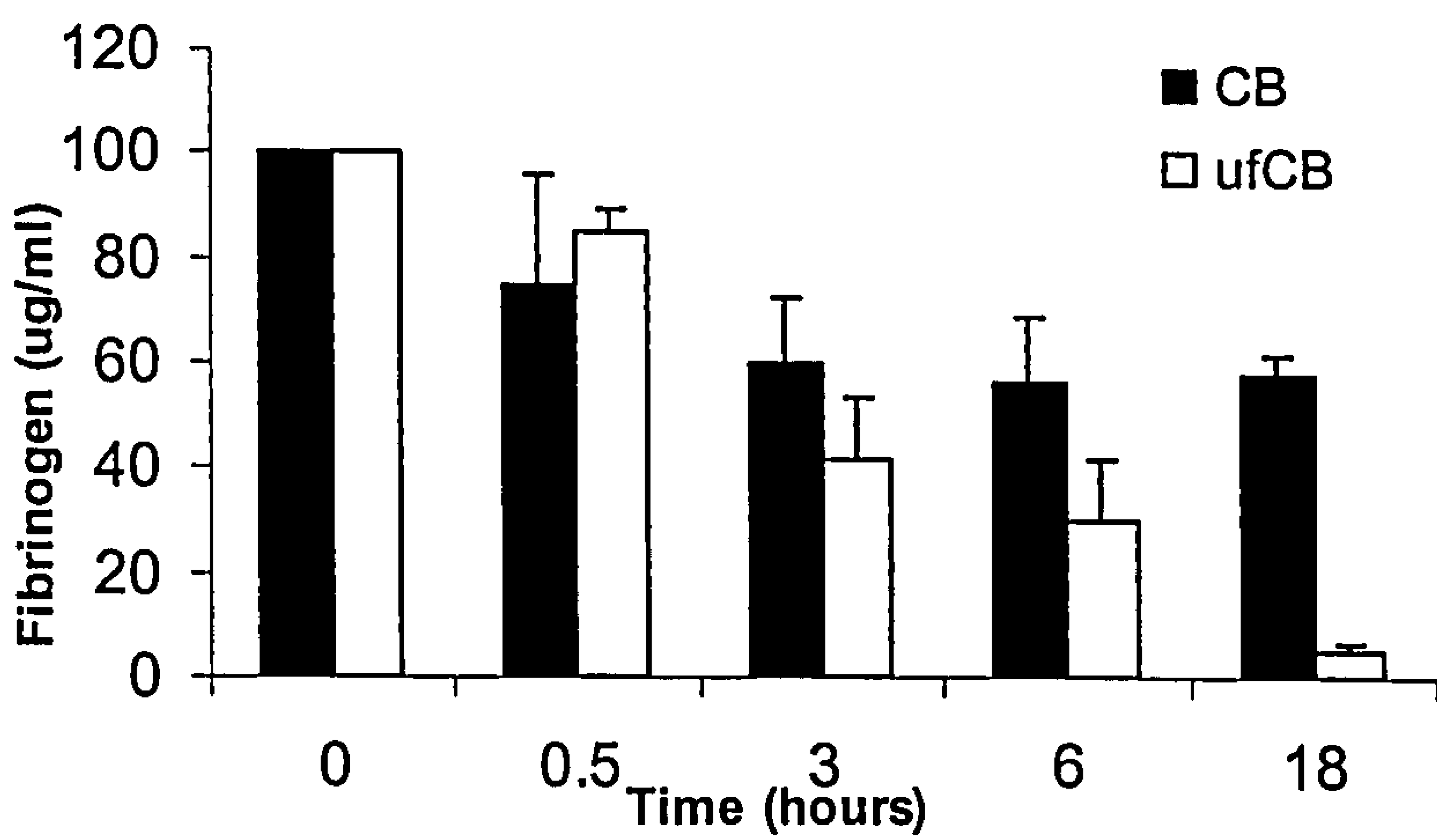


Fig d) Concentration of fibrinogen in cell supernatant, measured by ELISA.

Appendix III: Adsorption of protein on to particle surface.

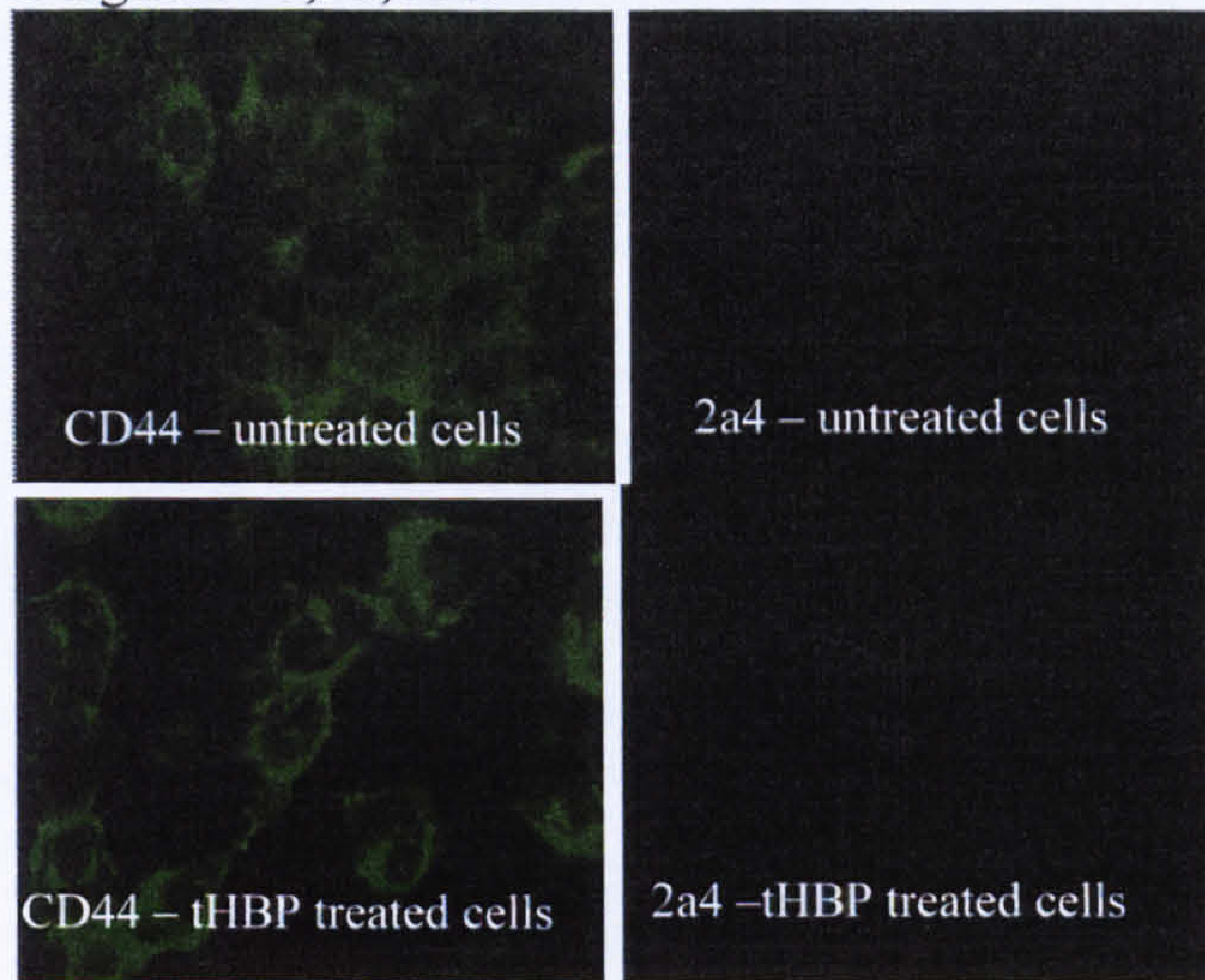
A known concentration of either CRP or fibrinogen was incubated with CB or ufCB for various time points. The particles were spun out of the suspension and the concentration of CRP or fibrinogen was determined by ELISA.



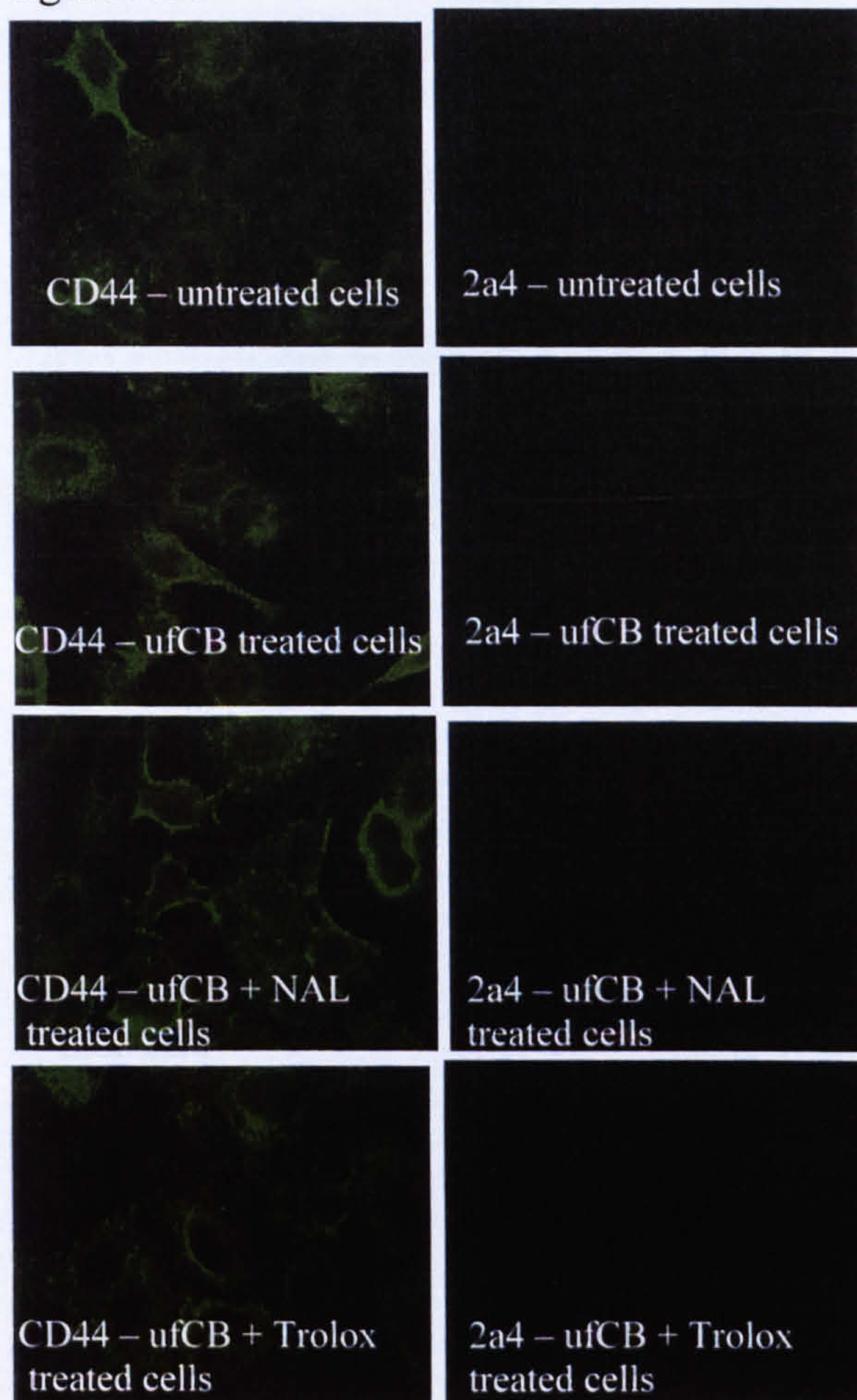


Appendix IV: Controls for immunofluorescence of A549 cells shown in Chapter 5.

i. Controls for figures 96,98,100.



ii. Controls for figure102.



Appendix V: Controls for immunofluorescence of MM6 cells shown in Chapter 6