THE ROLE OF PLATELETS IN CAPTURING CIRCULATING CELLS FROM FLOW *IN VIVO* AN INTRAVITAL MICROSCOPY STUDY USING A LASER-INDUCED MODEL OF THROMBOSIS IN THE MURINE CREMASTER MUSCLE

By

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ABSTRACT

The role of platelets in capturing circulating cells from flow *in vivo* an intravital microscopy study using laser –induced model of thrombosis in the murine cremaster muscle.

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Literature has increasingly shown how platelets may have roles beyond haemostasis. Platelet related health problems are seen in cancer patients on many occasions. Therefore, there is a possible relationship between platelets and cancer metastasising through the blood stream. Additionally, stem cell homing is an important aspect of stem cell therapy. For tissue regeneration to initiate, a sufficient number of stem cells must adhere to the injured sites. Present literature has shown that the low level of stem cell homing is a problem. Platelets are found at the site of injured tissues, so understanding the interactions between stem cells and platelets will enable research to be carried out to enhance homing mechanisms. In this study microcirculation was examined under an intravital microscope. A laser injury was induced causing damage to the blood vessels within the cremaster, which caused platelets to become adherent to the damaged area and form a thrombus. The labelled stem or tumour cells would then be perfused through in a single dose $1x10^6$ cells when using haematopoietic stem cells or lewis lung carcinoma cells, and $5x10^5$ cells when using mesenchymal stem cells. Results obtained demonstrated low levels of adherence between platelets and both types of stem cells and no adherence was observed between platelets and the tumour cells. Although this investigation has not provided any significant evidence of platelets interacting with stem cell or tumour cells, much literature has demonstrated their interactions and resulting therapeutic effects.

Dedication

I dedicate this to Rozam Ahmed and my family for their love and support throughout my education.

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I would like to take this opportunity to firstly thank my 1st and 2nd project supervisors Dr Neena Kalia and Dr Dean Kavanagh. Their guidance and support through the duration of this project was very valuable for me. I would also like to thank Joseph Robinson and Rebecca White, who are also members of Dr Neena Kalia's research group. Both their technical and emotional support helped me get through these 5 months of hard work. I would also like to thank all my family and friends who have supported me through this whole year, especially Rozam Ahmed. Last but not the least, I would like to thank the University of Birmingham Medical and Dental School; for giving me the opportunity to further develop myself, both as a researcher and as a person by accepting me on to this degree.

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1.0 INTRODUCTION

1.1 Platelets – their role beyond haemostasis

Platelets are recognised as a key component in haemostasis and thrombosis and therefore play a very important role in the prevention of blood loss when tissues are injured. However, the literature has increasingly shown that platelets may have roles beyond those initially recognised. These include a role in promoting inflammatory responses and also in the maintenance of vascular integrity. Furthermore, recent literature has identified that platelets may be involved in various other processes that are not commonly associated with platelets including angiogenesis, tumour progression and metastasis (Jurasz *et al*., 2004; Smyth *et al*., 2009). Of all these additional roles identified for platelets, their ability to physically interact with or modulate the adhesion of other circulating cells has received the most attention. A number of recent studies using mouse models have shown how platelets have the ability to interact with leukocytes and progenitor cells which can be recruited by platelets to sites of inflammation and vascular injury. Results from such studies have provided evidence for the involvement of platelets in many diseases and conditions including acute lung injury, atherosclerosis, hepatitis, sepsis, vascular restenosis and transplant rejection (Smyth *et al*., 2009).

Bioactive mediators that are stored and released by platelets provide them with the ability to affect the functions of various cells. Upon platelet activation the three storage compartments found within platelets, α-granules, dense granules and lysosomes, release their contents. This occurs during exocytosis, which results in proteins being delivered into the blood circulation. The composition of the platelet membrane is also altered during this

process. This further results in P-selectin expression on the cell surface, along with an elevation in the number of integrins $\alpha_{IIb}\beta_3$ [glycoprotein (GP) IIb-IIIa] molecules. One of the main roles of platelets beyond haemostasis is their ability to interact with leukocytes. The exposure of P-selectin is vital for this platelet-leukocyte interaction to occur. This is because the initial interaction between platelets and leukocytes is mediated by the P-selectin receptor. P-selectin expressed on activated platelet cell surfaces also cause leukocytes to roll on the endothelium layer in the blood vessels which also express the P-selectin, the P-selectin glycoprotein ligand 1(PSGL1) is also of importance in this process (Ley *et al*., 2007).

The most abundant immune cell type is the neutrophils. Roughly $5 \times 10^{10} – 10 \times 10^{10}$ new neutrophils are formed in the bone marrow. They spend most of their time in the bone marrow; only 2% of neutrophils are found in the bloodstream with the average life span of 5.4 days, once they migrate to tissues after activation they survive for around 1-2 days. Neutrophils are part of the innate immune response forming the first line of defence against fungal and bacterial pathogens (Christian *et al*., 2011).The recruitment of neutrophils, recirculation of lymphocytes and monocyte trafficking all require adhesion and transmigration through blood-vessel walls. There are 3 steps involved in this; rolling, activation and firm adhesion (Ley *et al*., 2007).Platelets have been identified to interact with neutrophils during inflammation and thrombosis. Interaction between these two cells can be initiated by activation of either the platelet or the neutrophil. This leads to a distinctive pattern of adhesion. The P-selectin on the platelet and the b_2 integrin CD11b/CD18 are responsible for mediating the adhesion between the two cells, when they are activated either primarily or secondarily (Brown *et al*., 1998). The adhesion molecules play an

important role in the interaction of platelets and circulating cells (Li., 2008; Smyth *et al*., 2009; Yang *et al.,* 1999).

Although we know a lot about platelet -leukocyte interactions, less is known about their ability to influence the recruitment of other cells such as therapeutic stem cells or pathogenic tumour cells.

Emerging evidence has shown the potential for platelets to also interact with cancer cells. Platelets from the blood flow have been identified to be involved in aiding cancer metastasis via tumour cell induced platelet aggregation (TCIPA). Metastasis is the process in which cancer cells migrate to areas of the body through blood flow. Cancer cells are more likely to migrate and form a secondary tumour in organs that have a larger blood supply as a higher number of cancer cells would migrate to that area. Tumour cell induced platelet aggregation has many advantages for tumour survival and also for successful metastasis. Platelets coat the tumours and protect it from TNF-α cytotoxicity and also shields the tumours from shear forces that are capable of causing damage (Jurasz *et al*., 2004; Placke *et al.,* 2012; Smyth *et al*., 2009). ADP has been identified to be a (TCIPA) stimulator as it contributes to (TCIPA) induced by many different cancers such as small cell lung cancer, breast cancer, and melanoma. It has been identified that during breast cancer induced (TCIPA) ADP released caused aggregation of platelets via the activation of $P2Y_{12}$ purinergic receptor (Alonso-Escolano *et al*., 2004).

Inhibition of the platelets tumour cell interaction would help cancer therapy along with lowering the levels of metastasis caused by platelets. Additionally, increasing stem cell infusion at this point may play a role in lowering platelet induced metastasis, which could be beneficial in cancer therapy. In relation to cancer therapy, MSCs have been identified to

migrate to areas where damaged cells are in the process of progressing into cancer cells in glioblastoma related studies. Further research would be required to fully understand (TCIPA) and the leading tumour progression in different cancer cell lines. Furthermore, combining the two areas of research, platelet and tumour cell interactions and stem cell and tumour cell interactions, would be valuable (Courtney *et al.,* 2013; Buergy *et al*., 2012; Jurasz *et al*., 2004).

Platelets have been recognised to adhere to damaged sites of endothelium as the sub-endothelial components are exposed, such as von Willebrand factor (vWf) and collagen. Since platelets are often identified to have the ability to adhere to injured tissues, they may also have the ability to specifically guide stem cells for regenerative purposes to injured sites. Currently, no *in vivo* research has been carried out to investigate if circulating cells specifically prefer to bind to activated inflamed endothelium, or to platelets that are already adherent to the blood vessels. Platelets normally adhere to the vessel wall after injury. Injury in the vessel wall causes the tissue collagen and plasma to be exposed, which causes platelets to adhere to initiate the clot formation in order to repair the damage. These blood cells may possibly be involved in the recruitment of MSCs, HSCs and progenitor cells to sites of injury. Additionally, it is not yet apparent if all circulating cells interact with platelets that are already adherent to the blood vessels to the same degree, or if specific cells interact to a greater degree due to the possible presence of specific adhesion molecules at their cell surface (Kavanagh et al., 2011 (a)).

1.2 Stem cell recruitment

Multipotent hematopoietic stem cells (HSCs) are derived from the bone marrow. They have been recognised to maintain haemostasis via continuously replenishing the blood, including leukocytes, lymphocytes, erythrocytes and platelets through self renewal. However, these cells are quite rare, even though these cells have such important roles in the body. Literature has identified that 0.01% present characteristics of HSC in murine BM (Gentile *et al.,* 2013; Kavanagh *et al.,* 2011(b)(c)). The fact that HSC are so rare is a major negative issue in research. The low availability results in limitation when investigating HSCs for their therapeutic abilities *in vivo*. Studies have shown the requirement of 20-30 donor mice to provide approximately $1.5x10^5$ cells, which would only be sufficient for one intravital experiment, which are experiments that are carried out using an intravital microscope to the study microcirculation *in vivo* (Massberg *et al.,*2006).

HPC-7 is an immortalised HSC cell line. This cell line is produced by transfecting murine embryonic SCs with the gene LHχ2 (Pinto *et al*., 1998). HPC-7 display similar characteristics as primary HSC. It has been demonstrated in recent studies that HPC-7 also express adhesion molecules that are present on primary HSCs. These include CD18 and CD49d. Therefore, HPC-7 have been previously used to model hepatic HSC recruitment (Kavanagh *et al.,* 2010).

Recently, literature has also shown that stem cells HPC-7 may be of use in therapy for tissue regeneration after injury. A recent study demonstrated HPC-7 adherence to injured murine intestines *in vivo*. It was identified that after ischemia at 30 minutes and reperfusion at 90

minutes, infusion of $2x10^6$ HPC-7 increased the level of HPC-7 recruitment significantly (Kavanagh *et al*., 2013(a)). Although stem cell therapy has been associated with some clinical success, further research is still required in order to understand how these cells are recruited to sites of injury and if they interact with other circulating cells or the endothelium.

Inflammatory disorder incidences are increasing within the western world. In relation, their treatments are not effective long term (Garcia *et al.,* 2010). Literature has shown HPC-7 have the potential to be clinically beneficial for inflammatory disorders, such as Crohn's patients and colitis (Kavanagh *et al.,* 2011(a)). Anti inflammatory factors and growth factors such as tissue growth factor –β1 (TGF-β1), stem cell factor (SCF), fibroblast growth factor (FGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) are released from HSCs when they are exposed to pro-inflammatory mediators within inflamed tissues. Studies have shown recruitment of HPC-7 to injured liver, kidney and gut, which demonstrated that recruitment of HPC-7 is possible (Kavanagh *et al.,* 2010; Kavanagh *et al.,* 2013(a)).

Cellular interactions mediating recruitment of HSC to injured sites have shown the involvement of CD49d. Recent studies have shown CD49d (α_4 subunit of $\alpha_4\beta_1$ integrin) /VCAM-1 pathway involved in HSC recruitment to murine injured liver and (IR) injured murine small intestines *in vivo* (Kavanagh *et al.,* 2010). Furthermore, literature increasingly demonstrates how platelets may be involved in stem cell (HSC, MSC and progenitor) recruitment to injured sites (Kavanagh *et al.,* 2011(a); Kavanagh *et al.,* 2013(b); Strauer and Kornowski, 2003). Progenitor cells have been recognised not to have the ability to directly bind to the sub-endothelial and therefore, the involvement of platelets has been seen as

vital to mediate their interaction (Massberg *et al.,*2006). Additionally, integrins found on the surface of progenitor cells have been identified to be activated by the ligand CXCL12, secreted by activated platelets (Massberg *et al.,*2006; Peled *et al.,*2009). CXCL12 is the accompanying ligand of G- protein coupled chemokine receptor CXCR4 and this receptor has been identified to play a vital role in the recruitment of HSC to sites of injury (Sipkins *et al.,* 2005).

Literature has shown, that the method of stem cell delivery is also related to the level of engraftment at sites of injury (Strauer and Kornowski, 2003). Stem cell homing is an important aspect of stem cell therapy as a sufficient number of stem cells must adhere to the blood vessels within injured sites for tissue regeneration to occur. Present literature has shown that the low level of stem cell homing is a problem (Kavanagh *et al.,* 2011(a); Kavanagh *et al.,* 2013(a)). Understanding the molecular adhesive factors would help us understand the mechanisms underlying cellular interactions of stem cells (Strauer and Kornowski, 2003). This would provide the basis to allow further work in enhancing stem cell recruitment to injured sites and consequently resulting in possible enhancement of tissue repair.

Mesenchymal stem cells have also been identified to posses the ability to migrate to target areas such as gliomas. MSC can be extracted from adipose tissue, bone marrow, umbilical cord and dental pulp but those used for research purposes and in early clinical studies have been derived primarily from BM and adipose tissue due to the ease of isolation and expansion from these sites. MSCs are also being considered for cancer treatment as they can be used to aid the delivery of drugs used in cancer therapy (Courtney *et al.,* 2013). MSC have been associated with having an anti cancer effect, which has been demonstrated by

MSCs being identified to home at the site of tumour development in many cancers such as; colon, breast, ovarian, melanoma, prostate and gliomas (Courtney *et al.,* 2013). However, further research is required to confirm their anti cancer effect. MSC have also been identified as a potential component in repairing diabetic wounds. Diabetic wounds have impaired blood supply and MSCs have shown potential in enhancing angiogenesis and improving wound healing in comparison to untreated wounds (O'Loughlin *et al.,* 2013).

Literature has also identified that due to the larger size of MSCs, recruitment is more difficult as they become stuck within capillaries and therefore it is more likely that an insufficient number will reach the injured site (Kavanagh *et al*., 2013(b)). In comparison, HSCs are smaller in size and have not been identified to be caught in capillaries and therefore a higher number of these stem cells are more likely to adhere to the injured area. Regardless of the size of these stem cells they have both been identified to be of therapeutic use (Kavanagh *et al*., 2011(a); Kavanagh *et al*., 2013(b)).

However, knowledge of the underlying mechanisms, of the recruitment of stem cells is limited and the involvement of platelets during stem cell recruitment still needs to be better understood and therefore needs to be further investigated using in *vivo* models.

1.3 LLC- Lewis lung cell carcinoma

In USA 20% of deaths caused by cancer are due to lung related cancers. Although, there are many treatments and diagnostic techniques that have been improved over the years the 5 year survival rate in total for all lung cancer patients is a low 15%. Furthermore, the rate lowers to 2% if the patients are suffering from cancer metastasis (Morrison *et al*.,2012).

Literature has demonstrated glycosaminoglycans to be attached to tumour cell surfaces via proteoglycans. These have been identified to be involved in cancer metastasis. The metastasis of lung carcinoma cells was observed to be decreased by preadministration of chondroitin sulphate unit E or by the use of phage display antibody specific for CS-E. However, the underlying mechanism resulting in metastasis needs to be investigated. Literature has discussed the involvement of platelets in cancer metastasis; it is possible that during this process of LLC metastasis, platelets may be involved at some point in enhancing the level of metastasis seen via (TCIPA). However, if the LLC tumour cells do not interact with platelets, it is likely that the metastasis is independent of platelet activity (Mizumoto and Sugahara, 2013).

Therefore, understanding the process of metastasis in different cell lines and what might be involved in the process would lead to potential therapies. Inhibition of metastasis would lead to a better 5 year survival rate for lung cancer patients. Positive findings would be beneficial in forwarding research ideas for many other cancers and their progression (Morrison *et al*.,2012).

1.4 Tumour cell recruitment and metastasis

Patients suffering from cancer commonly display symptoms of thrombotic events which increase as the cancer progresses and metastasises (Gay *et al*., 2011). The specific forms of thrombosis seen in such patients are disseminated intravascular coagulation, pulmonary embolism and migratory thrombophlebitis (Gay *et al*., 2011). In many cases, signs of thrombosis may not always be detected, although an elevation in coagulation factors can

still be identified. Since platelet related health problems are seen in cancer patients on numerous occasions, it is possible that platelets may interact with cancer cells and aid their metastasising through the blood stream and thus contribute to cancer progression. There has been much literature providing evidence of how platelets play an important role in cancer progression (Gay *et al*., 2011; Jurasz *et al*., 2004; Trousseau, 1865).

Literature has shown tumours to have the ability to cause platelets to aggregate. This may explain the high number of thrombotic events seen in cancer patients. Cancer has ten recognised hallmarks; the growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, angiogenesis, invasion, metastasis, deregulated metabolism, unstable DNA, inflammation and evasion of the immune system (Hanahan and Weinberg; 2000). It has been demonstrated in recent studies, that tumours use platelets to evade the immune system in the body (Gay *et al*., 2011; Jurasz *et al*., 2004). Additionally, it is also found that platelets assist the adhesion of tumour cells to the vascular endothelium, providing growth factors which can be used by the tumour cells for growth (Gay *et al*., 2011; Jurasz *et al*., 2004) Understanding this process *in vivo* will aid the development of anti-platelet therapy which could possibly improve and enhance existing therapeutic methods in cancer.

In order for cancer cells to metastasise they need to interact within the host microenvironment. This involves the circulation, lymphatic vessels and target tissues. The specific cells that are involved in the process of metastasis include endothelial cells, lymphocytes, macrophages, mast cells, fibroblasts, bone marrow derived progenitor cells and platelets. Currently to understand aspects of metastasis the most extensively studied cells are platelets (Gay *et al*., 2011; Joyce and Pollard, 2009).

Trousseau was the first to identify the link between platelets and cancer in the middle of the nineteenth century. His finding led to the belief that tumours have the ability to activate platelets leading to the likelihood of spreading platelets from clots while they are forming (Gay *et al*., 2011; Trousseau, 1865).

Platelet counts during different phases of cancer development are useful in the prediction of clinical outcomes. Thrombocytosis is linked with poor prognosis in many cancers, including colon, breast, lung, gastric, renal cervical endothelium, brain, pancreatic and ovarian cancer. Additionally, during chemotherapy the event of thrombocytopenia has been recognised to occur due to suppression of platelets being produced in the bone marrow, which could possibly be contributing to the anti-cancer effects of chemotherapy, as lower levels of platelets would cause less incidents of metastasis, as a result of less platelet-tumour interactions (Gay *et al*., 2011; Kaushansky, 2008).

Much research is required to gain a better understanding of tumour progression stages affected by platelet function, and at what point are the platelets adhesive functions involved in enhancement of tumour progression. Anti-platelet therapy may be of use in many treatments for many other diseases and therefore, its development through research and clinical trials should be focused on.

1.5 Further research

Looking at previous literature around this area it is obvious that there are many unanswered questions which require further research. It is important to understand the underlying mechanism of the interaction between platelets and circulating cells. Most of the studies

that have been carried out are in vitro rather than in vivo. The techniques used within the current studies to investigate cellular interactions and interactions between circulating cells and damaged tissues, have included cannulation of a close artery to investigate the organ of interest. This is done by using the cannulated area for infusion of cells or other substances; investigating cellular interactions in a specific area of the mouse. This has been proven an efficient technique of investigation (Kavanagh *et al.,* 2011(c)(d)). The cremaster has been used quite a lot through this sort of research mainly due to the fact that it is a good preparation to study microcirculation. Although the cremaster itself is rarely a target of injury or disease, but it is a good model of the vasculature of muscle. Therefore, it is an efficient model to use to investigate cellular interactions (Bagher and Segal, 2011).

In this study platelets and their ability to capture circulating cells from the blood flow, via intravital observations of the cremaster muscle in a mouse model will be investigated. It will be very useful to gain knowledge on how platelets capture circulating cells from the blood flow using this technique as it is more direct, it would show platelets and the cells of interest at the same time physically interacting with each other. This has not been shown previously in an in vivo study. This study will allow comparisons to be made in regards to the effectiveness of platelets in recruiting or interacting with different circulating cell populations. Additionally, it will provide an insight on the behaviour of platelets in recruiting different cells, and if they are selective with regards to which cell they interact with. Investigating the cellular interactions between platelets and tumours cells could possibly lead to a better understanding of how and why these cells interact and further lead to the development of methods to inhibit any interactions. Investigating the cellular interactions between stem cells and platelets may possibly lead to methods of enhancing

their interactions to benefit the process of tissue regeneration possibly for many different diseases.

1.6 Aims and Hypotheses

- Platelets and HSCs will interact with each other following the development of laser-induced thrombi within the mouse cremaster muscle microcirculation.

- Platelets and MSC will interact in the cremaster after thrombi formation.

- Platelets and mouse lung cancer cells will interact after thrombi formation.

- HSCs will interact at a higher level in comparison to MSC.

The route of infusion will influence the interactions of platelets and circulating cells.

2.0 METHODS

2.1 Cell culture: Hematopoietic Stem Cells

A stock solution of HSC specific culture media was made using 45ml of stem Pro 34 SFM, 1.3ml stem cell supplement, 20µl(100ng per ml) stem cell factor (Life Technologies), 0.5ml penicillin/streptomycin and 0.5ml L-glutamine (P.A.A Laboratories). This culture media was changed every day by discarding the old media and adding fresh media to maintain a cell concentration of $1x10^6$ cells/ml. After fresh media was added the flask was left in an incubator at 37˚C and 5% CO_2. New media was added to the cells after being pelleted via centrifugation at 300g for 5 minutes and counted. The cells could then be distributed to a new flask once they reached confluence. In this study HPC-7 were used instead of primary HSCs. Prof. Leif Carlsson (University of Umeå, Sweden) gifted the HPC-7 cells used in this study.

2.2 Cell culture: Mesenchymal Stem Cells

The MSCs used were derived from the bone marrow. A stock solution of MSC specific culture media was made using 47ml Modified Eagles medium alpha- modification (α-MEM) (Sigma Aldrich) and 0.5ml foetal bovine serum , 0.5ml penicillin/streptomycin and 0.5ml L-glutamine (P.A.A Laboratories) and 10µl (10ng per ml)transforming growth factor β (New England Biosciences). The culture media was changed 2-3 times per week when culturing these cells. Cells were passaged after being trypsinised. New media was added to the cells after being pelleted via centrifugation at 300g for 10 minutes and counted. The cells could

then be distributed to a new flask once they reached confluence. The flask was incubated at 37˚C and 5% CO_2. The MSC can only be used up until passage 9.

2.3 Cell culture: Lewis lung carcinoma cell line

The LLC cell line is a well established lung carcinoma derived from the lungs of C57BL/6 mice. These cells are reported to be highly tumorigenic. Although, some studies suggest they are only weakly metastatic in mice. A stock solution of LLC specific culture media was made using 500ml Dulbecco's modified eagles medium (DMEM), (Sigma Aldrich), 50ml foetal bovine serum, 5ml penicillin/streptomycin and 5ml L-glutamine (P.A.A Laboratories). The culture media was changed was changed 2-3 times per week when culturing these cells. The cells were trypsinised before passaging as they were adherent. New media was added to the cells after being pelleted via centrifugation at 300g for 10 minutes and counted. The cells could then be distributed to a new flask once they reached confluence. The flask is then incubated at 37˚C and 5% CO_2.

2.4 Reagents

Modified eagles medium alpha-modification (α-MEM), Dulbecco's modified eagles medium (DMEM), Dulbecco's phosphate buffered saline were all purchased from Sigma Aldrich. Carboxyfluorescein diacetete succinimidyl ester (CFSE), 0.25% Trypsin-EDTA, Stem Pro 34 SFM, stem cell supplement and stem cell factor were purchased from Life Technologies. Foetal bovine serum, penicillin/streptomycin and L-glutamine were purchased from P.A.A Laboratories. Transforming growth factor β was purchased from New England Biolabs.

2.5 Sterile conditions

Keeping sterile conditions were important in order to avoid infecting the cells. Sterile conditions were kept by using 70% ethanol. Ethanol was sprayed on everything that was placed inside the Class-II culture cabinet. The cabinet created a sterile environment to work in. In order for the Class-II cabinet to be at a sterile state it must be switched on and left for at least 10-15 minutes prior to use.

2.6 Cell counting

A haemocytometer was used to count the cells (Figure 2.1), in order for the cells to be fed or split so they can divide efficiently. For this the media was discarded and the cells were washed using 5ml of PBS. The cells needed to be washed twice to make sure all the media was removed. Then 7ml of trypsin- EDTA solution (purchased from Life Technologies) was added to the flask of cells. After the trypsin was added, the flask needed to be returned to the incubator for around 10 minutes. This whole procedure was carried out so that the adherent cells can be detached from the bottom of the flask. The cells adhere to the bottom due to protein attachments. Trypsin is used as it is an enzyme that digests these proteins and releases the cells from the bottom of the flask. After 10 minutes the flask needs to be examined under a light microscope to confirm that the adherent cells have completely been detached from the bottom of the flask. If they have been released, on examination they will appear to be floating, if they are not, the flask needs to be returned to the incubator for a few more minutes until the adherent cells appear to be detached completely.

Once the cells are detached completely 7 ml of full medium needs to be added to the flask which results in neutralisation of the trypsin activity. Before the media is added it needs to be warmed to 37˚C in a water bath for around 20 minutes.

To count the cells a cover slip was placed on the haemocytometer 10 µl of cells was then added to 90 µl of trypan blue from which 10 µl of cells were pipetted into the haemocytometer. The haemocytometer was placed under a microscope and the cells could be counted. The trypan blue identifies all the dead cells by dying them blue and all the remaining viable cells can be counted. This allows accurate calculation of cell concentration.

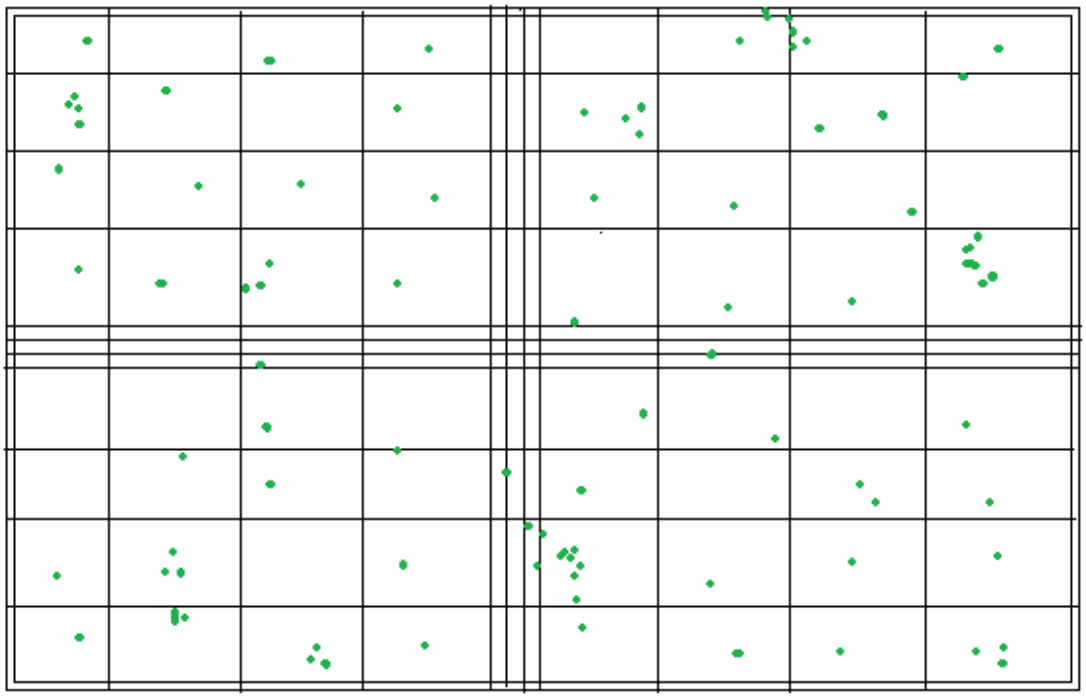


Figure 2.1; Cell counting using a Haemocytometer; this figure shows how a haemocytometer would appear under a light microscope. The cells only need to be counted in the four regions, each region has sixteen squares. Only the cells within the borders should be counted. After counting the cells there should be four values, one for each region.

2.7 Cell labelling

Cells were pelleted by centrifugation at 300g; 5minutes for HSC and 10 minutes for MSC and tumour cells, the time and speed was kept the same every time the cells were centrifuged. Media was discarded and 2ml of PBS was added ready for centrifugation. Then cells were re-suspended in 2 ml of PBS once again and added to a pre-prepared solution of 2ml of PBS with 2µl/5µm of CFSE. Cells were incubated at 37˚C for 10 minute. This was followed by a further addition of 6 ml of media and centrifugation. Cells were washed twice- each time with 2ml of media, followed by centrifugation each time. After the first wash cells were left to rest for 5minutes. Finally they were re-suspended in a desired volume ready for experimentation. Labelled cells were visible under fluorescent light (Figure 2.2). Carboxyfluorescein diacetete succinimidyl ester (CFSE) (purchased from Life Technologies) was used to label HSCs, MSCs and LLCs. For some experiments (Figure 3.6, 3.12, 3.17) the endogenous platelets were fluorescently labelled so that the thrombi formed could be clearly identified. This was done through injection into the carotid artery of a primary antibody against the platelet specific marker CD41 and a fluorescently labelled secondary antibody as shown in Table 2.1.

Table 2.1

Antibody	Conjugate	Clone	Host	Target	Isotype	Supplier	Conc	Volume
Anti-CD41	N/A	MwReg30	Rat	Mouse	IgG_1, κ	BD biosciences	0.5mg/ml	20µl
Polyclonal anti Rat IgG	Alexa-555	Polyclonal	Goat	Rat	IgG	life technologies	2mg/ml	40µl

2.8 Animals

In this study male C56BL/6 mice were used, at an average weight of 25g. Animals were housed at the BMSU (Biomedical service unit), University of Birmingham, under standard conditions according to Animals Scientific procedures Act 1986, under licence from the United Kingdom Home Office (PPL 40/3336) Animals were killed via Schedule 1- humane killing procedures; cervical dislocation.

2.9 Surgical procedures

Animals were anesthetised using an injection containing ketamine into the peritoneum (100 mg/kg) and Xylazine (50mg/kg). Then a cannula was inserted into the trachea. This allows the mouse to breathe more easily during the investigation. Cannulation of the artery chosen for the route of infusion (femoral artery/carotid artery) was performed; the surgical procedures were carried out on animals at the average weight of 25 grams. The cremaster muscle was exteriorised and pinned open after exposing the testis through a scrotal incision, and clearing any connective tissue. The cremaster was continuously superfused with bicarbonate buffered saline (physiological solution- (pH 7.4; 37˚C) – gassed with 5% CO_2/95% N_2) to avoid loss of moisture. Through the chosen cannulated route of infusion substances such as CFSE labelled cells (HSC, MSC and LLC), anaesthesia, saline were administered. The animal was then left for 30 minutes to allow stabilisation and then was monitored under an intravital microscope. This procedure was non-recovery (Figure 2.3).

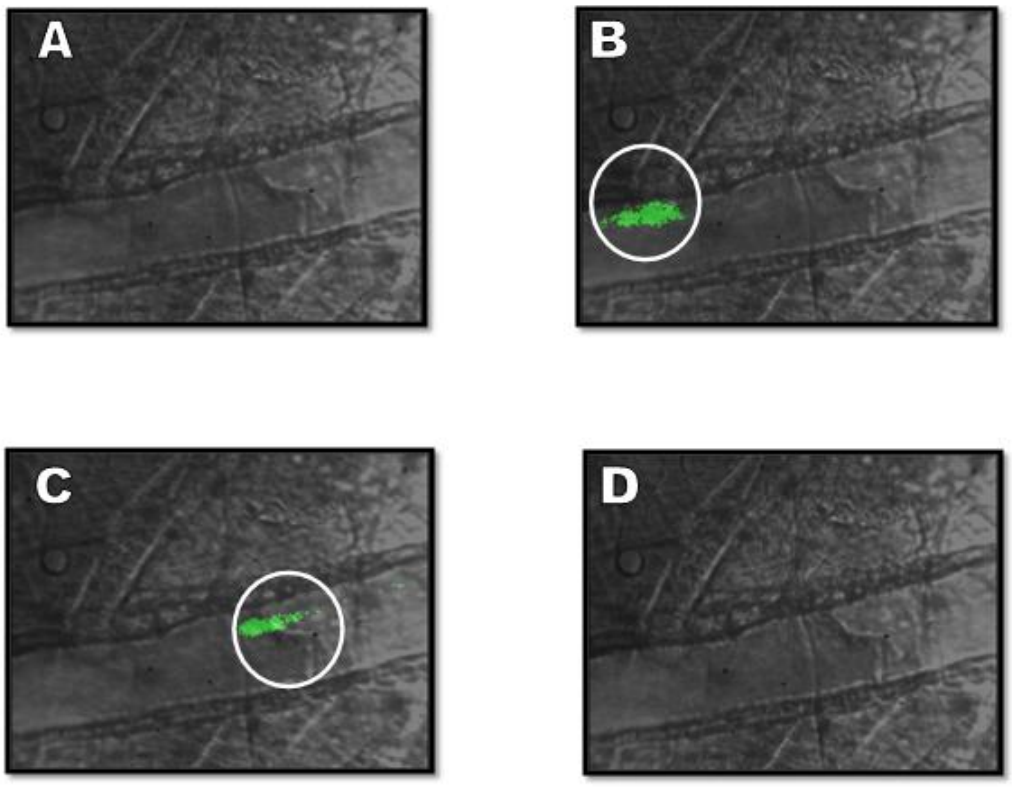


Figure 2.2; To ensure injected cells could be visualised trafficking through the cremaster microcirculation, they were fluorescently labelled using CFSE, a dye which fluoresces green upon activation. In preliminary studies, $1x10^6$ HPC-7 cells were injected via the femoral artery in the mice that had not undergone any additional procedures (ie. no laser ablation, TNFα stimulation, ischemia-reperfusion injury or any pre-treatment of cells with H_2O_2). (A) Arteriole before the cells were infused. (B) A cell as it enters the vessel. (C) The cell moved across the arteriole. (D) The cell has left this field of view.

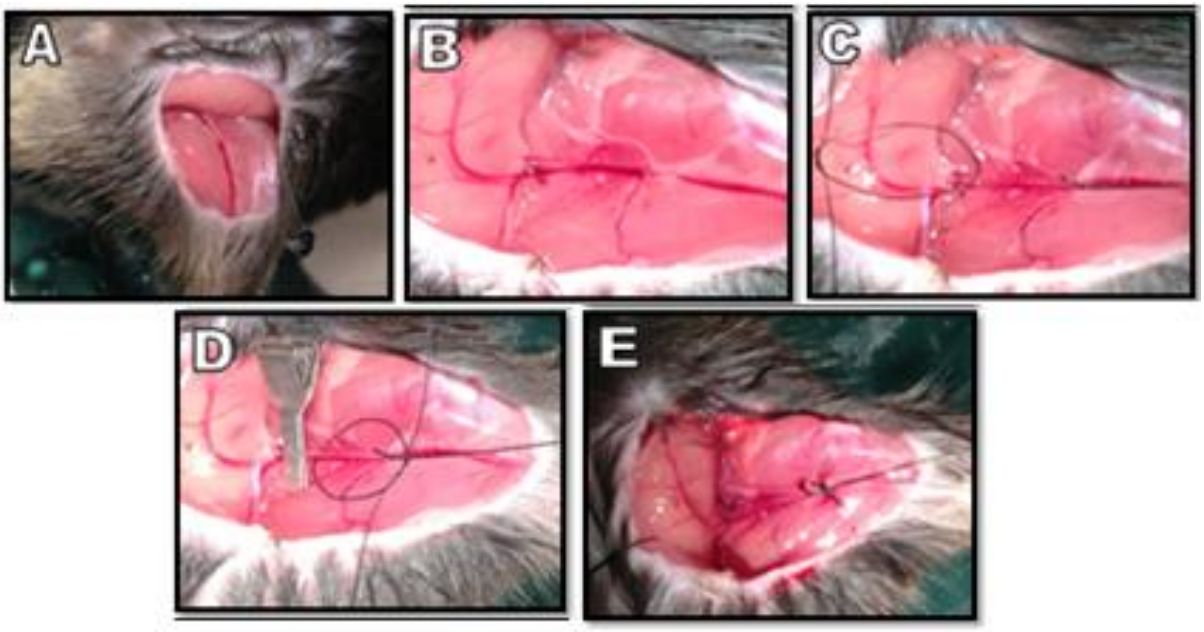


Figure 2.3; Surgical procedure of femoral cannulation in a mouse model; (A) Area was exposed through an incision above the thigh to expose the artery and the vein. (B) Fat tissue preventing access to the femoral was cleared. (C)(D) Connective tissue was cleared to expose the femoral artery for separation from the vessels and nerves that surrounded. The femoral artery was tied off towards the foot and a loose knot was positioned at the opposite end. The femoral was clamped. An incision was made three quarters of the way down the femoral. (E) Cannula was inserted and a double knot was tied over the cannula to secure its position and the clamp was removed followed by the removal of air bubbles from the cannula.

2.10 Intravital microscopy

Once the animal had been surgically prepared the microcirculation was examined under an intravital microscope (Olympus BX-61WI). Laser ablations were induced to form thrombi in the blood vessels within the cremaster, which would cause platelets to become adherent to the damaged area. For this investigation laser induced injury was beneficial as the degree of the injury could be controlled (laser power (75) the power at which the laser hits the tissue, FRAP (Fluorescence recovery after photo-bleaching) size (10) the amount of area the laser makes direct contact with on the tissue, FRAP (Fluorescence recovery after photo-bleaching) repetition (15)) the number of times the laser contacts the tissue using the same power and size. Therefore, the level of thrombi formation could also be controlled and kept the same throughout the experiment, which allowed results obtained to be comparable.

The labelled cells would then be perfused through in a single dose of $1x10^6$ cells when using HSC or LLC, and $5x10^5$ cells when using MSC. MSC were used at the concentration $5x10^5$ instead of $1x10^6$ because their level of growth was lower in comparison to HSC and LLC and therefore, it was difficult to obtain high numbers of MSCs.

The program Slide Book was used to capture the images on the intravital microscope. The cells were visibly green under fluorescent light. Using the intravital microscope the labelled cells that were adherent to the platelets forming the thrombi would be identified and using the data obtained (visual images) quantification was done offline. For all the results and data obtained the whole cremaster was scanned using the intravital microscope. Cells were characterised to form temporary adhesions if after adherence they unattached. The experimental model used in this investigation was beneficial, as valuable qualitative data could also be obtained. From this identification of obvious differences in the quality of the

cremaster could be recognised and this may be of use in the analysis of the quantitative data obtained. Additionally, adherence of cells through the whole cremaster could be imaged. The effects of TNFα can also be identified via rolling leukocytes, therefore the efficiency of the intrascrotal injection can be visually confirmed.

2.11 Data analysis

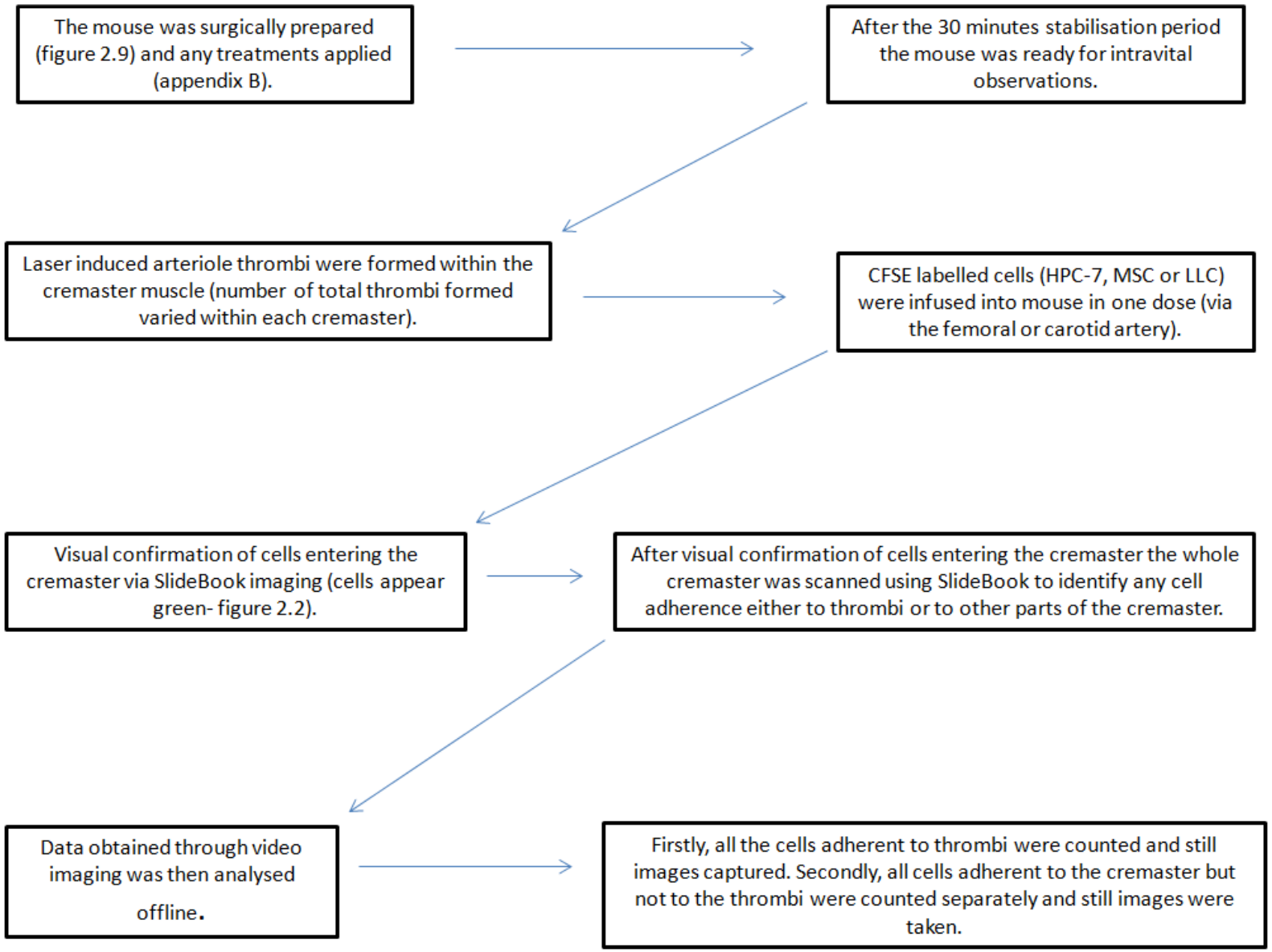
The data was normalised because the number of thrombi formed in each cremaster varied. Method of normalisation; (highest number of thrombi formed during the investigation/actual number of thrombi formed within the cremaster)*number of adherent cells to thrombi. The data was expressed as mean ± SEM. For statistical analysis the following was used; unpaired t-tests; one way ANOVA; Bonferroni (Post Hoc test). The program used was PRISM.

2.12 Method Development

Initial experiments were carried out using naive cells, infused into a naive cremaster; MSC (N=3), HPC-7 (N=4), LCC (N=5). The second set of experiments were carried out using pre-treated stem cells and TNFα treated cremaster, MSC (N=4) and HPC-7 (N=4). These were pre-treated with H_2O_2 for an hour before inserting them through the cannulated femoral artery. The animal was given 200µl/500ng TNFα via an intrascrotal injection 4 hours prior to ablation. During this second set of experiments the LLC (N=5) were perfused through 500ng TNFα treated cremaster, but the LLC were not pre-treated. The TNFα treatment used resulted in a more inflamed microenvironment within the cremaster microcirculation.

Previously the cells had no reason to become adherent, providing this environment increases their chances to become adherent. The route of infusion was investigated using the stem cells; MSC (N=4), HSC (N=4). These experiments investigated the difference in cell adherence when different routes of infusion were used therefore the carotid artery was cannulated instead of the femoral artery. The final sets of experiments were looking at the adherence of the cells when a different type of injury was induced; Ischemia for 30minutes and reperfusion for 90minute. When using the HSCs and MSCs the cells were pre-treated with H_2O_2 as in the previous experiments but LLC were not pre-treated. For these final set of experiments the cremaster was also not treated with TNFα; MSC (N=4), HSC (N=5), LLC (N=4).

Method summary



3.0 RESULTS

3.1 Naive HPC-7 show low adherence to thrombi generated in arterioles

Studies were conducted using naive HPC-7 (N= 4), during which 15, 16, 16, and 15 thrombi were formed giving a total of 62 arteriole thrombi. Route of infusion used was the femoral artery and the following slide book settings were used: FRAP; size (7)-(10), repetition (15); laser power (75). Naive HPC-7'S were perfused to examine their interaction with platelets. Naive HPC-7 showed some adherence to thrombi formed. However, the number of HPC-7 adherent was minimal in comparison to the number of HPC-7 perfused through the mouse model. Only 2 HPC-7 were adherent to 1 thrombi out of 62, and 4 cells out of $1x10^6$ were adherent within the capillaries in the cremaster (adherent cells to thrombi/field: 0.6 ± 0.62; Figure 3.3).

3.2 HPC-7 Show temporary adhesion to thrombi when stimulated with H_2O_2 and TNFα treated cremaster

Studies were conducted using HPC-7 that were pre-treated with H_2O_2 and the cremaster was treated with TNFα (N= 4), during which 9, 15, 15 and 20 thrombi were formed giving a total of 69 arteriole thrombi and the femoral artery was used as the route of infusion. Additional studies were conducted using HPC-7 that were also pre-treated with H_2O_2 and the cremaster was also treated with TNFα (N= 4), during which 15, 14, 20 and 15 thrombi were formed giving a total of 59 arteriole thrombi but on this occasion the carotid artery as the route of infusion. Only 5 HPC-7 were adherent to 1 thrombi out of 69 thrombi, and 3

cells out of $1x10^6$ were adherent within the capillaries in the cremaster; only 1 HPC-7 was adherent to 1 thrombi out of 59 thrombi. HPC-7 became adherent to thrombi before breaking their attachments, possibly due to the pressure of blood flow in the arterioles (adherent cells to thrombi/field: 1.5 ± 1.50; Figure 3.4).

Additional experiments were conducted in which HPC-7 were pre-treated with H_2O_2 and were perfused into an ischemia reperfusion injury mouse model (N=4), during which 16, 15, 15 and 15 thrombi were formed giving a total of 61 arteriole thrombi and the carotid artery was used as the route of infusion. 2 cells out of $1x10^6$ were adherent within the capillaries in the cremaster (adherent cells to cremaster/field: 0.5 ± 0.29; Figure 3.6). The following slide book setting were used for all these experiments: FRAP; size (10), repetition (15); laser power (75).

3.3 Naive and pre-treated MSC show low adherence to thrombi generated in arterioles, however they do become trapped within capillaries in the cremaster

Studies were conducted using naive MSCs (N=4), during which 15, 15, 15 and 15 thrombi were formed giving a total of 60 arteriole thrombi. The femoral artery was used as the route of infusion. Additional studies were conducted using H_2O_2 pre-treated MSCs and TNFα treated cremaster (N=4), during which 17, 15, 14 and 15 thrombi were formed giving a total of 61 arteriole thrombi and the femoral artery was used as the route of infusion. Another set of experiments were conducted in which MSCs were also pre-treated with H_2O_2 and were perfused into TNFα treated cremaster (N=4), during which 15, 14, 20 and 15 thrombi were formed giving a total of 64 arteriole thrombi but, on this occasion carotid artery was

used as the route of infusion. The following slide book setting were used: FRAP; size (10), repetition (15); laser power (75). Only 1 MSC was adherent to 1 thrombi out of 64 thrombi.

These particular stem cells did not show much signs of adherence to thrombi even after being stimulated with H_2O_2 and the cremaster stimulated with TNFα (adherent cells to thrombi/field: 0.3 ± 0.33; Figure 3.9 and 3.10). However, due to the size of these specific stem cells, they did have the tendency to become stuck within small capillaries (Figure 3.12).

Another study was conducted using H_2O_2 pre-treated MSCs (N=4), during which 15, 15, 15 and 16 thrombi were formed giving a total of 61 arteriole thrombi. The carotid artery was used as the route of infusion. MSCs once again did not become adherent to any of the thrombi generated even after inducing ischemia reperfusion injury prior to thrombi formation. However, cells did appear to be adherent within the capillaries (adherent cells to cremaster/field: 0.5 ± 0.29; Figure 3.12). The following slide book setting were used: FRAP; size (10), repetition (15); laser power (75). Naive MSC; 1 out of $5x10^5$ were adherent within the capillaries in the cremaster. MSC H_2O_2 pre-treated and TNF-α treated cremaster (femoral infusion); 1 out of $5x10^5$ were adherent within the capillaries in the cremaster. MSC H_2O_2 pre-treated and TNF-α treated cremaster (carotid infusion); 1 out of $5x10^5$ were adherent within the capillaries in the cremaster. MSC H_2O_2 pre-treated and ischemia reperfusion; 2 out of $5x10^5$ were adherent within the capillaries in the cremaster.

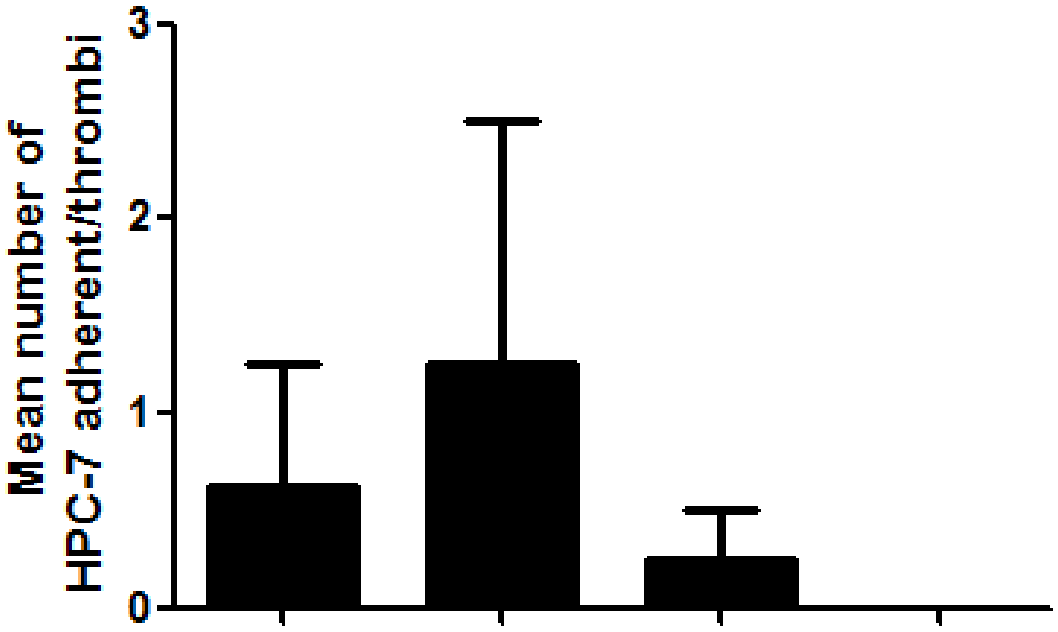
3.4 No significant difference identified when perfusing cells through the carotid artery instead of the femoral artery

Results from this investigation described earlier were used to investigate any differences in route of infusion. To confirm that the stem cells not becoming adherent was not due to the chosen route of administration, experiments were carried out using carotid artery cannulation (Figure 3.5 and 3.11). The cells were pre-treated with H_2O_2 and the mouse was injected using an intrascrotal injection in the testes with TNFα. Inserting cells via the femoral artery may have been causing cells to perfuse through too quick, possibly due to the force at which the cell were perfused, and also due to the femoral being a closer and more direct route into the flow within the cremaster. Therefore, the stem cells may have not been getting an opportunity to become adherent, due to the force of the cells allowing a low level of contact to the damaged vessels as they are perfused. Perfusing through the carotid artery as an alternative would not disturb the flow as much as when perfusing cells via the femoral. The cells would now be more likely to become adherent without any resistance due to flow pressure. However, these experiments had shown no significant difference in stem cell adherence levels, as mentioned earlier.

3.5 Naive Lewis carcinoma cells do not adhere to thrombi generated within arterioles

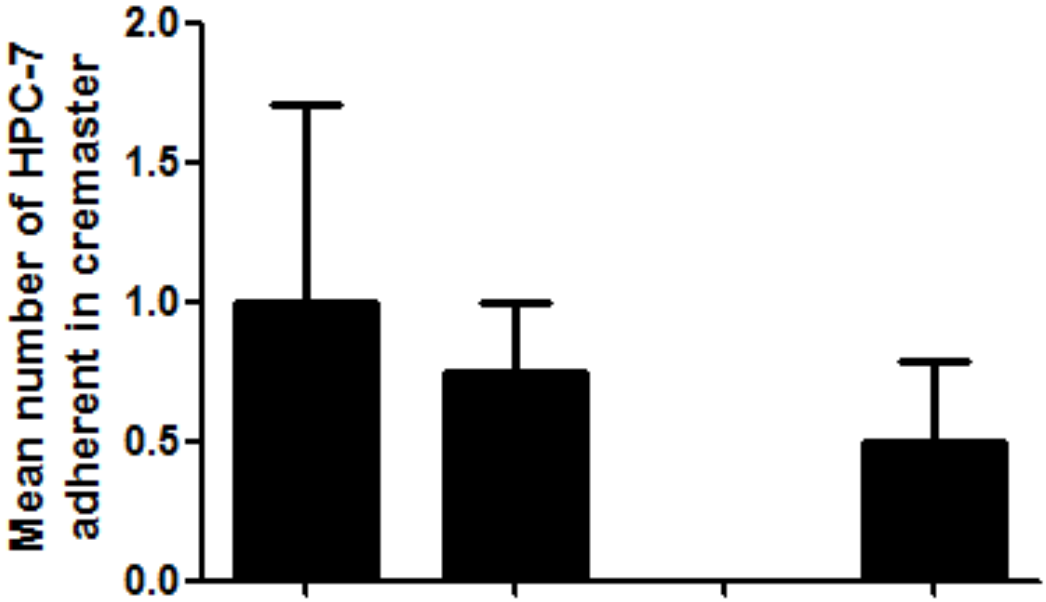
Studies were conducted using naive LLCs and a naive cremaster (N=5), 15, 12, 19, 15 and 20 thrombi were formed giving a total of 81 arteriole thrombi. Another set of experiments were carried out using naive LLC and TNFα treated cremaster (N=5), during which 15, 15, 12, 16 and 15 thrombi were formed giving a total of 73 arteriole. LLC were perfused through

the mouse model without any treatment to the cells or the mouse and they were also perfused through after stimulating the cremaster. LLC did not show any adherence within the cremaster in any case, as the cells were identified moving past large thrombi without any interaction observed (Figure 3.15 and 3.16). However, inducing ischemia reperfusion in the mouse model resulted in adherence of cells within the capillaries. In this additional study naive LLC were used without any treatment to the cremaster (N=4), during which 15, 15, 15 and 15 thrombi were formed giving a total of 60 arteriole. 3 out of $1x10^6$ were adherent in the capillaries within cremaster; (adherent cells to cremaster/field: 0.75 ± 0.25; Figure 3.17). The following slide book settings were used for all the experiments: FRAP; size (10), repetition (15); laser power (75) and the carotid artery was used as the route of infusion for all the LLC experiments.



Femoral infusion	+	+	-	-
Carotid infusion	-	-	+	+
Cremaster (TNF - α)	-	+	+	-
Cells (H_2O_2)	-	+	+	+
Ischemia reperfusion	-	-	-	+

Figure 3.1; HPC-7 stem cells show low adherence to thrombi after laser injury induced thrombi formation. Animal received 1 x 10^6 HPC-7 cells, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. No significant differences were seen between the following comparisons; (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, femoral infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, carotid infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, IR – injury, carotid infusion). No cell adherence was observed after H_2O_2 pre-treated cells, IR – injury, carotid infusion.



Femoral infusion	+	+	-	-
Carotid infusion	-	-	+	+
Cremaster (TNF - α)	-	+	+	-
Cells (H_2O_2)	-	+	+	+
Ischemia reperfusion	-	-	-	+

Figure 3.2; HPC-7 stem cells show low adherence to the cremaster. Animal received 1 x 10^6 HPC-7 cells, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. No significant differences were seen between the following comparisons; (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, femoral infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, carotid infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, IR – injury, carotid infusion). No cell adherence was observed after H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, carotid infusion.

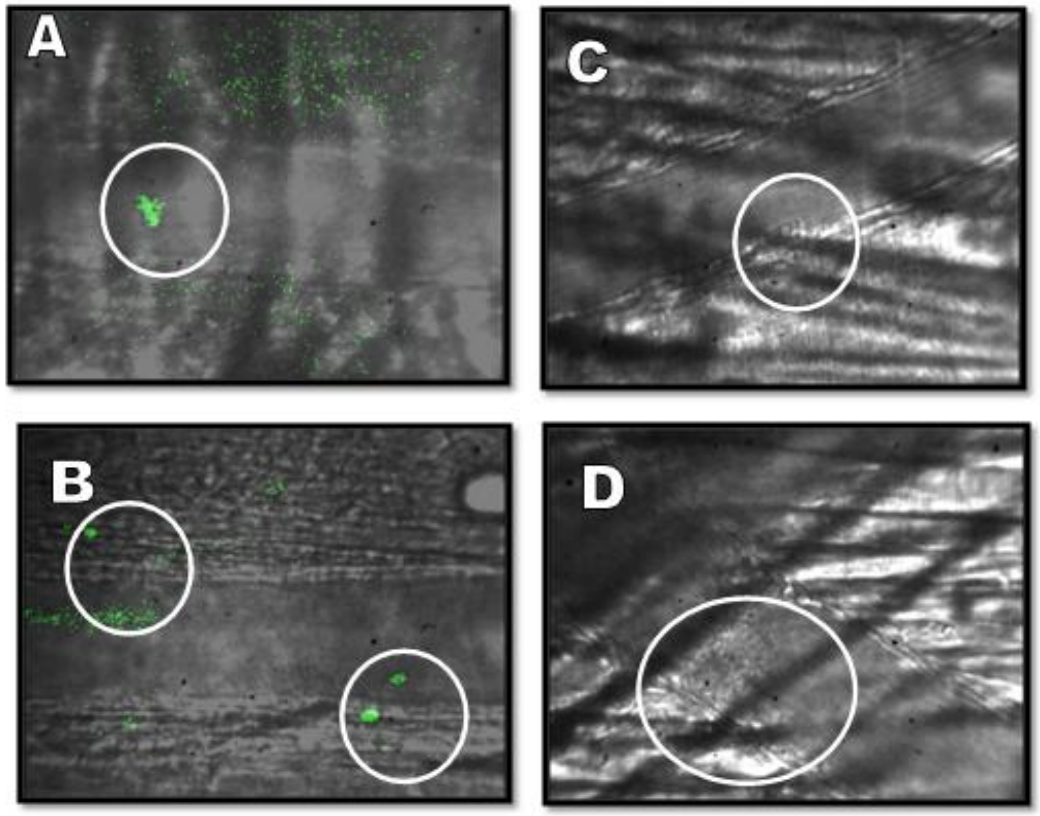


Figure 3.3.**Naive HPC-7**, **naive cremaster** (A) Three HPC-7 adherent to the arteriole in the absence of a thrombi. (B) Two HPC-7 adherent to a thrombi generated in the arteriole. One HPC-7 trapped within a capillary. (C) No HPC-7 adherent to thrombi generated in the arteriole. (D) No HPC-7 adherent to thrombi generated in venule.

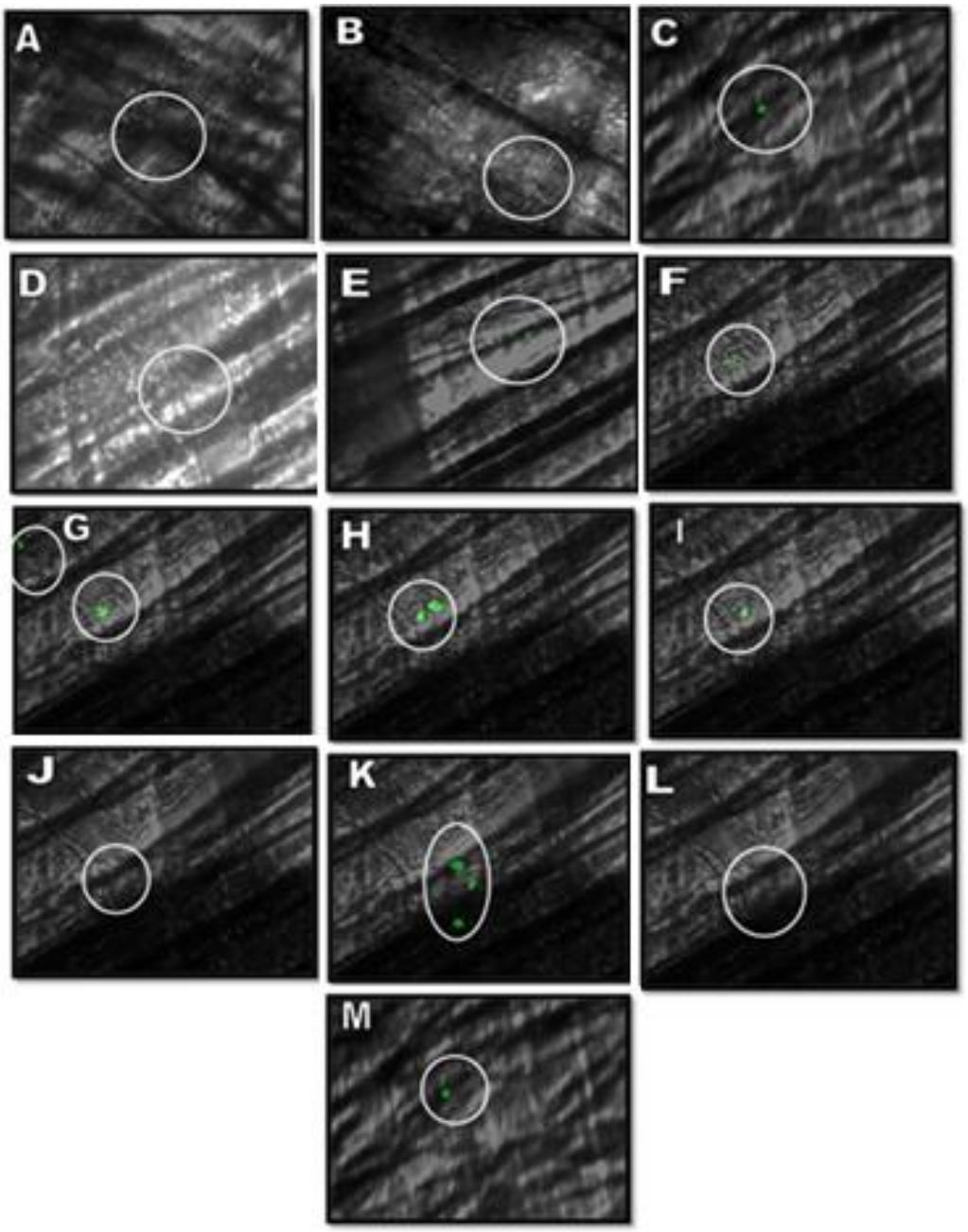


Figure 3.4; Pre – Treated HPC-7, femoral infusion (A)(B)No HPC-7 adherent to thrombi's generated in arteriole's. (C) 1 HPC-7 trapped within a capillary. (D) No HPC-7 adherent to thrombi's generated in arteriole's.(E)1 HPC-7 trapped within a capillary.(F) 1 HPC-7 adherent to thrombi generated in arteriole. (G) Another HPC-7 moving towards the thrombi. (H) 2 HPC-7 are now adherent to the thrombi. (I) The second HPC-7 that became adherent to the thrombi in (H) has now unattached and moved away with flow. (J) The remaining adherent HPC-7 has also unattached itself and moved away with flow. (K) 3 HPC-7 adherent to thrombi. (L) All 3 HPC-7 have unattached and moved along with flow. (M) 1 HPC-7 trapped within a capillary.

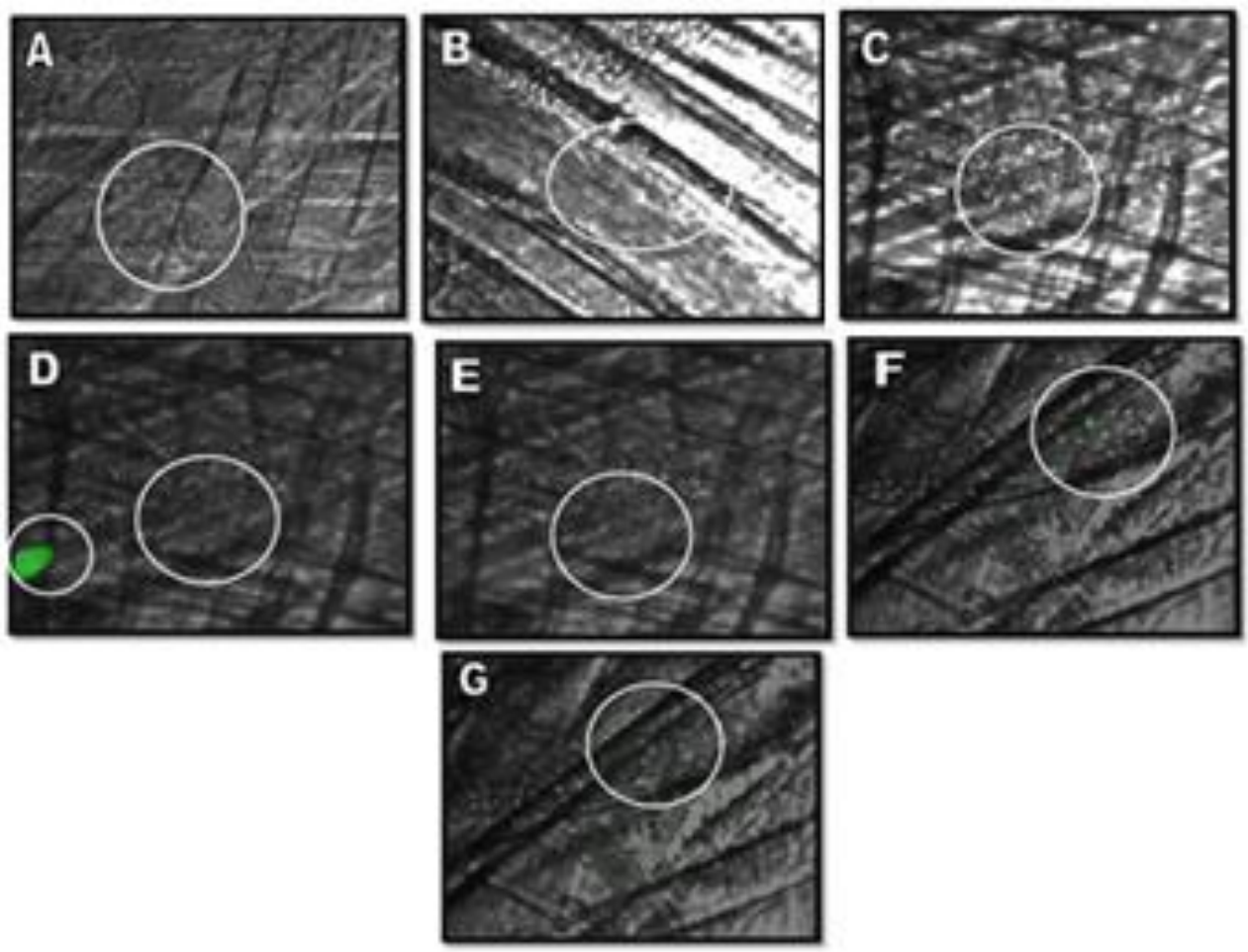
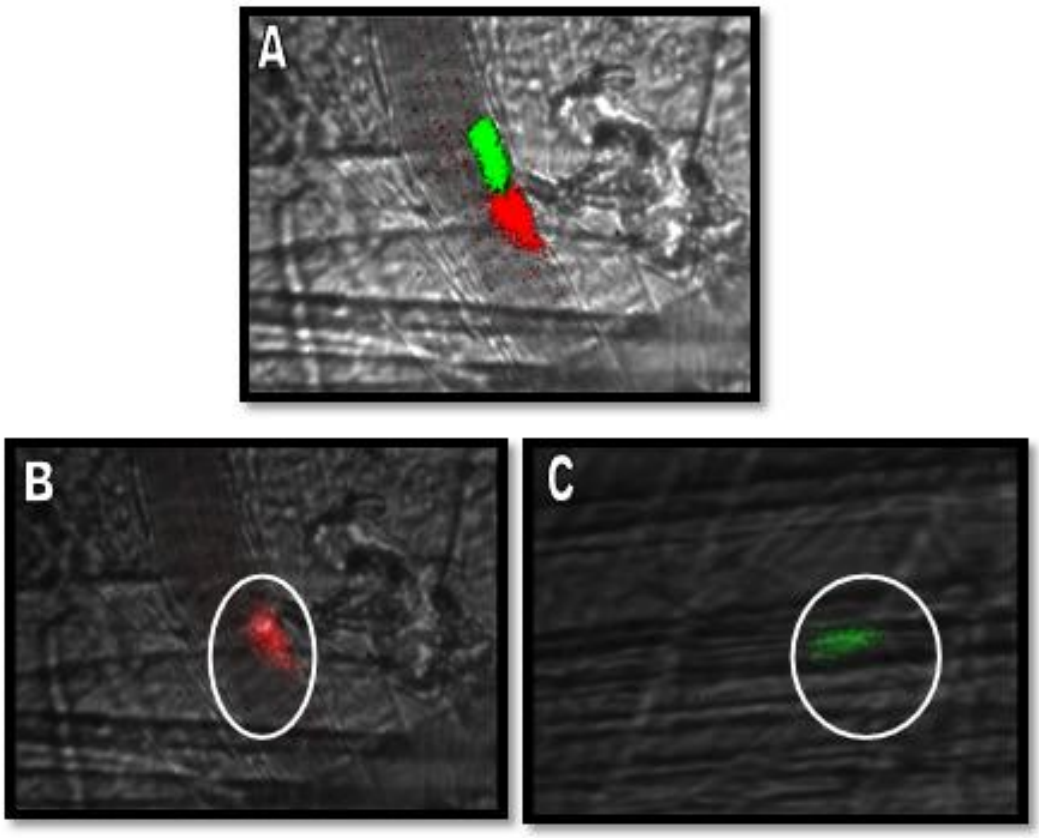
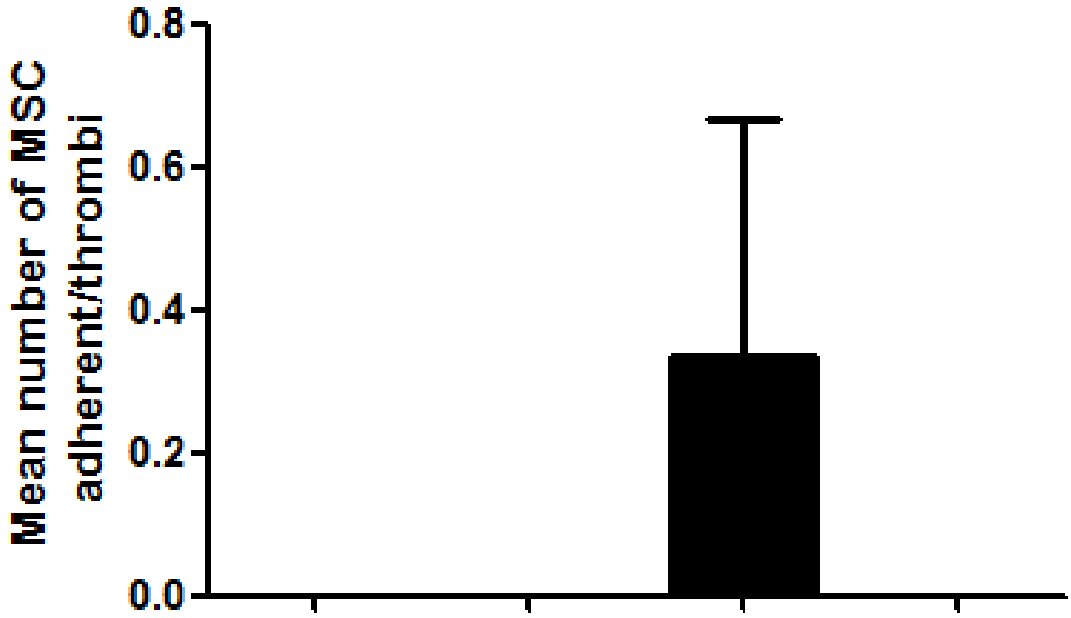


Figure 3.5; Pre-Treated HPC-7, **carotid infusion** (A)(B)(C)No HPC-7 adherent to thrombi generated in arteriole. (D) HPC-7 moving towards the thrombi. (E) No HPC-7 adherent to thrombi generated in arteriole. (F) 1 HPC-7 adherent to the thrombi. (G) The HPC-7 unattached and no longer adherent to the thrombi



F**igure 3.6; Pre – treated HSC – Ischemia reperfusion injury**(A) Cells move into the arteriole and pass the labelled thrombi. (B) No cells are adherent to the thrombi. (C) Cell adherent within capillary.



Femoral infusion	+	+	-	-
Carotid infusion	-	-	+	+
Cremaster (TNF - α)	-	+	+	-
Cells (H_2O_2)	-	+	+	+
Ischemia reperfusion	-	-	-	+

Figure 3.7; MSC stem cells show very low adherence to thrombi after laser injury induced thrombi formation. Animal received 5 x 10^5 MSC cells, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. Cell adherence was only observed after H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, carotid infusion. No cell adherence was observed after any of the other treatments.

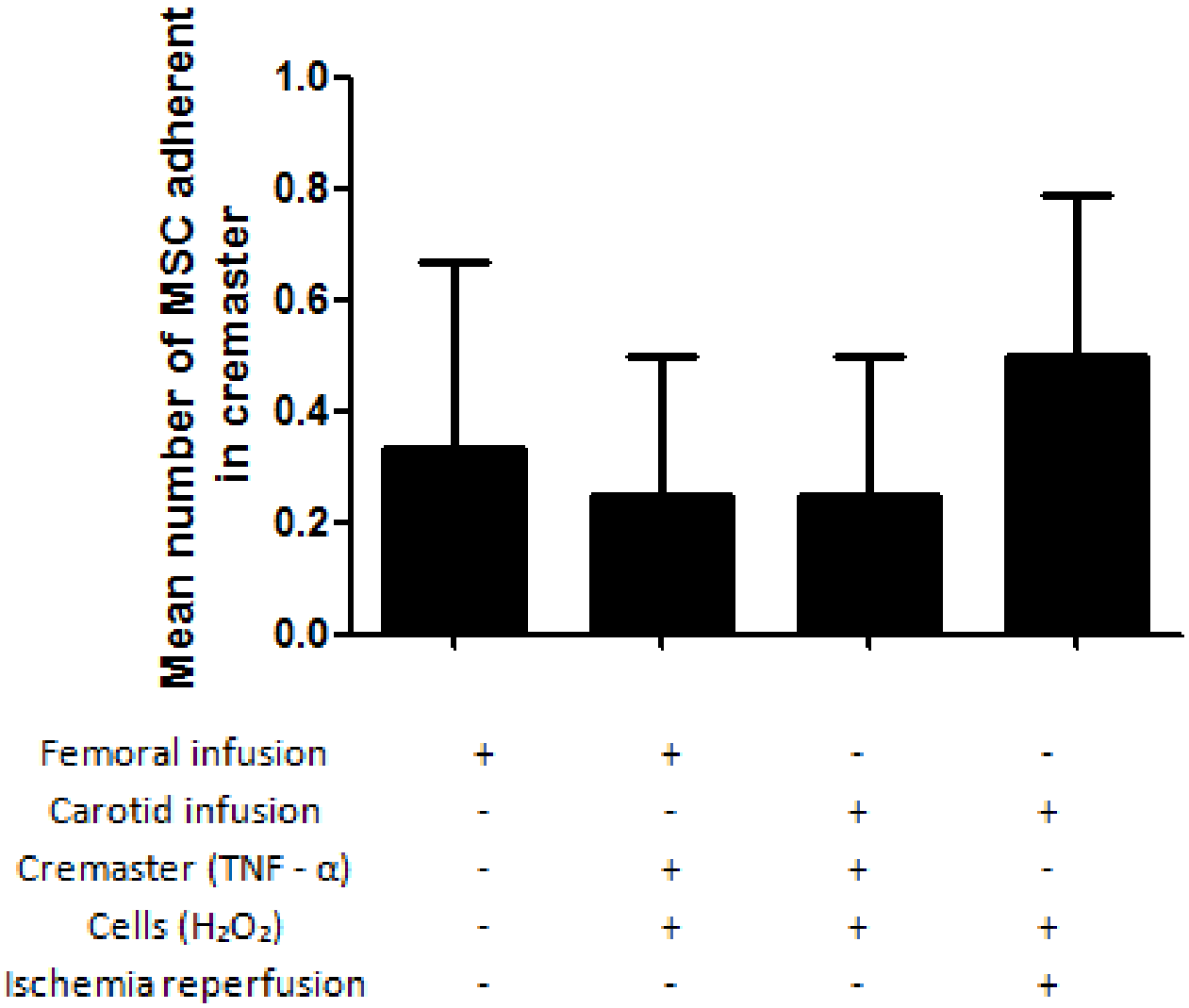


Figure 3.8; MSC stem cells show low adherence to the cremaster. Animal received 5 x 10^5 MSC cells, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. No significant differences were seen between the following comparisons; (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, femoral infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, TNF-α pre-treated cremaster, carotid infusion), (Naive cells, naive cremaster vs. H_2O_2 pre-treated cells, IR – injury, carotid infusion).

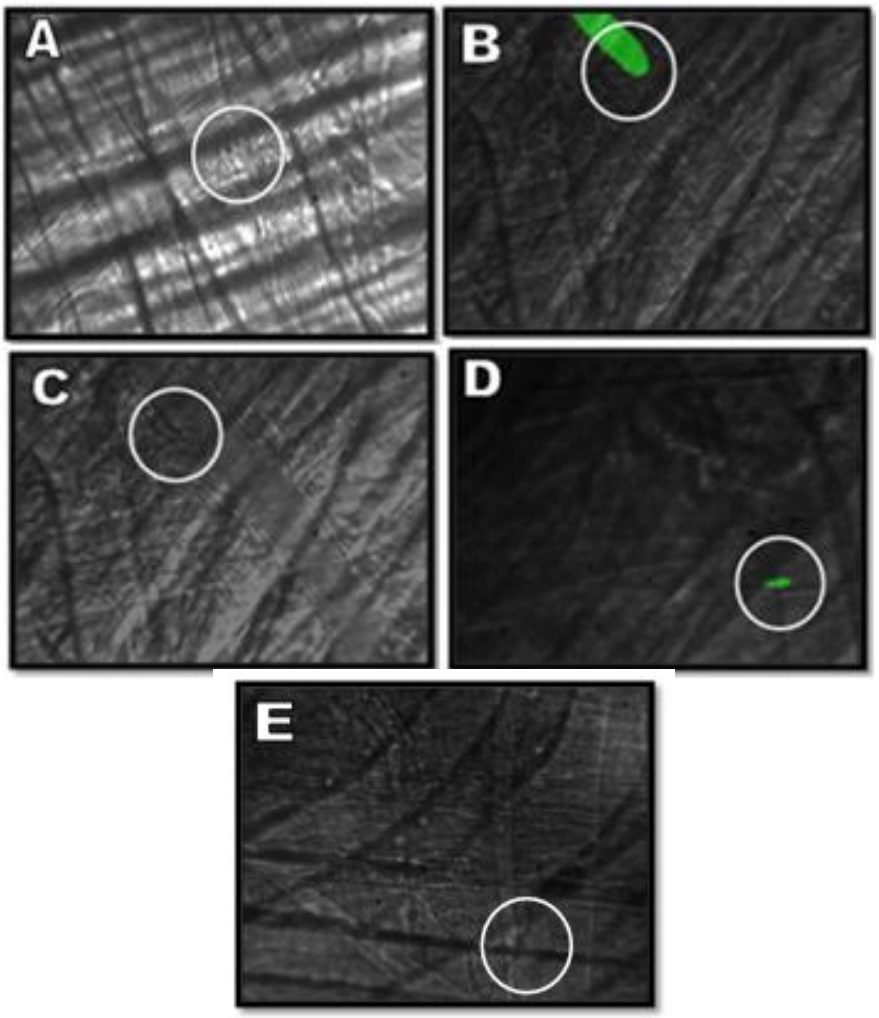


Figure 3.9; **Naive MSC** (A) Thrombi prior to the perfusion of MSC (B) MSC move into the arteriole passing the thrombi. (C) No MSC adherent to thrombi generated in arteriole. (D) 1 MSC trapped within a capillary.(E) No MSC adherent to thrombi in arteriole.

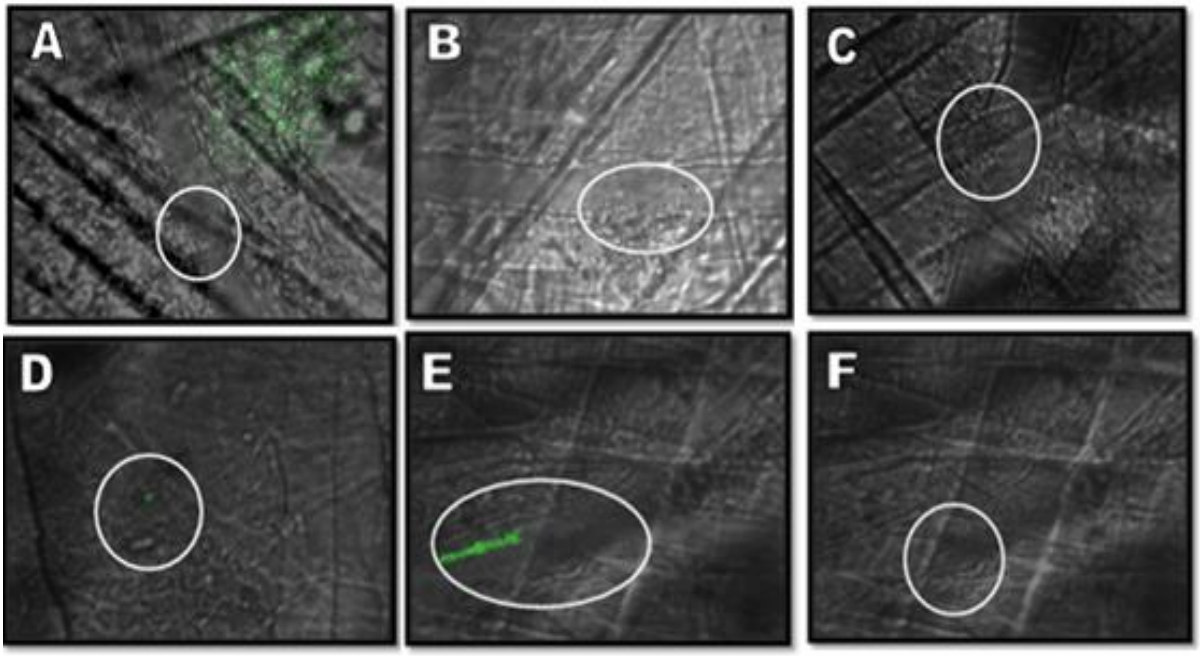


Figure 3.10; Pre – treated MSC, femoral infusion (A)(B)(C)No MSC adherent to thrombi's generated in arteriole's. (D) 1 MSC trapped within a capillary. (E) MSC move into the arteriole passing the thrombi. (F) No MSC adherent to thrombi generated in arteriole.

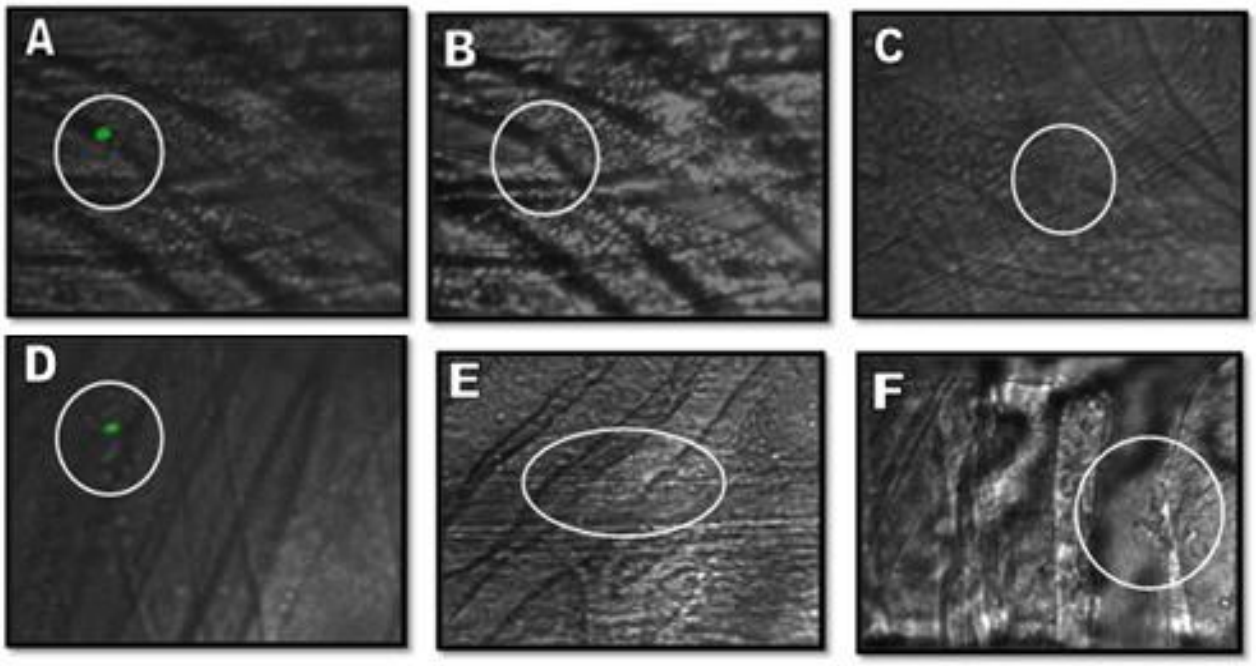
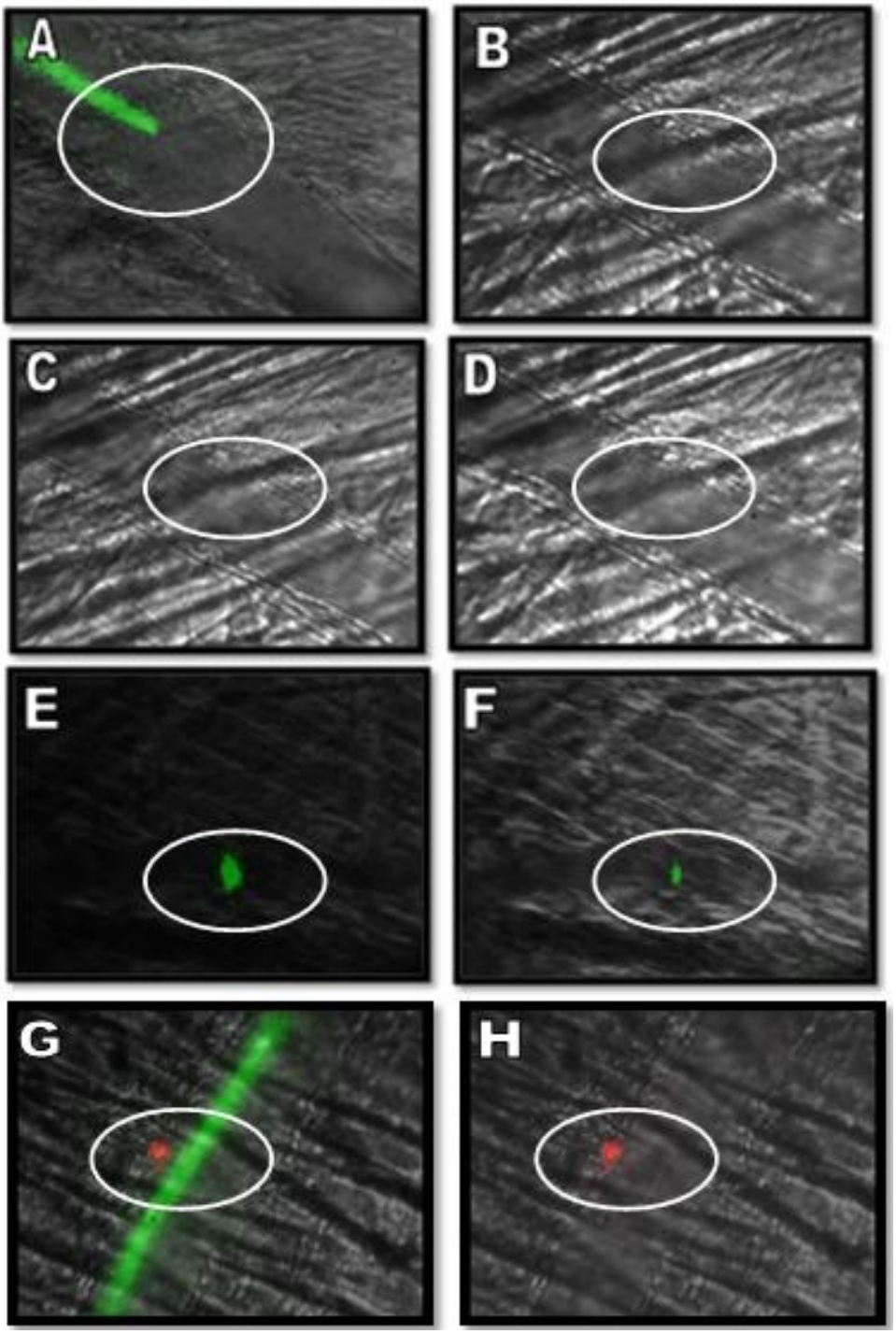


Figure 3.11; Pre-Treated MSC, carotid infusion (A) 1 MSC adherent to the thrombi. (B) The MSC unattached and no longer adherent to the thrombi. (C) No MSC adherent to thrombi generated in arteriole. (D) 1 MSC trapped within a capillary.(E)(F) No MSC adherent to thrombi generated in arteriole.



F**igure 3.12; Pre – treated MSC – Ischemia reperfusion injury** (A) MSC's perfused through cremaster (arteriole). (B)(C)(D) Thrombi generated in arteriole with no MSC's attached. (E)(F) Cells stuck within the capillaries (G) Cells move into the arteriole and past the labelled thrombi. (H) No cells are adherent to the thrombi

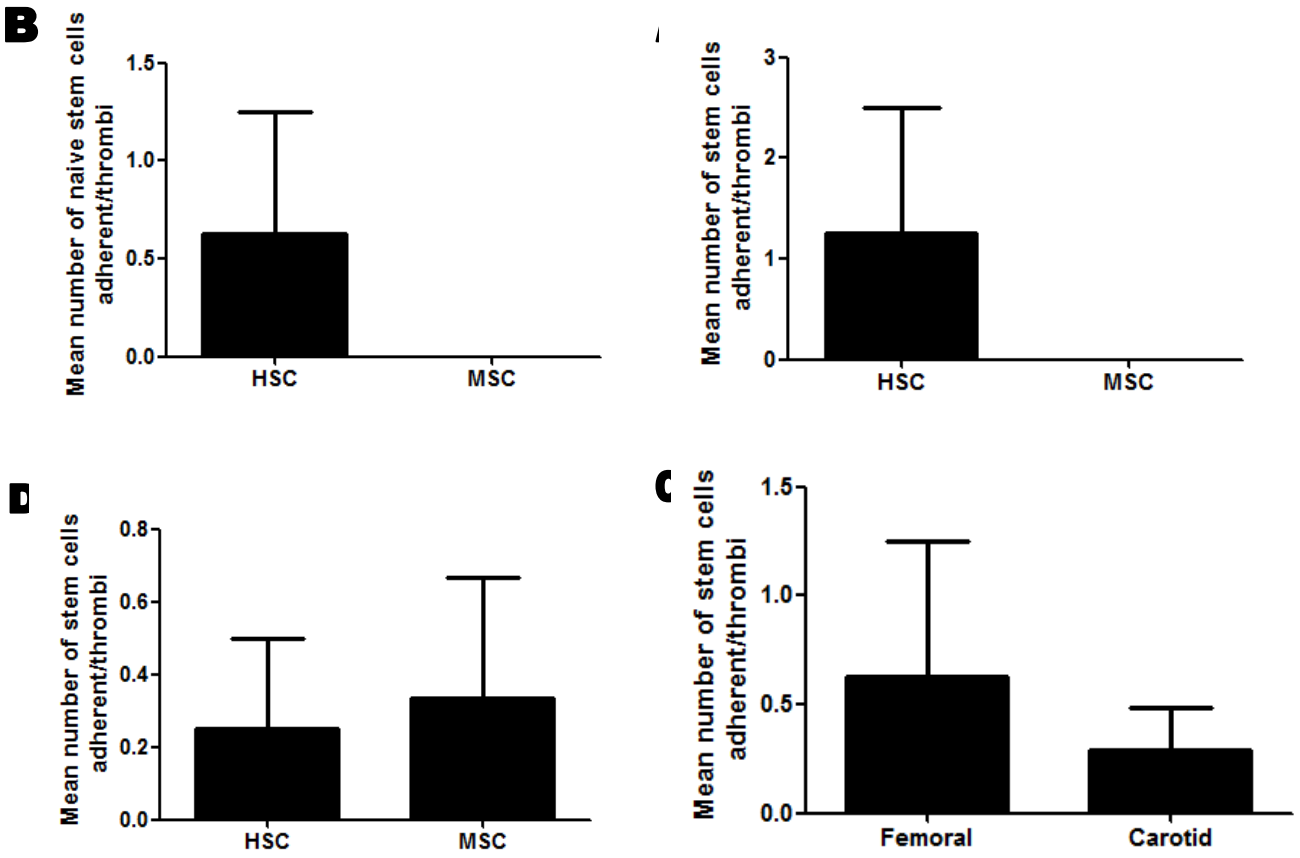


Figure 3.13; No significant differences between HPC-7 and MSC adherence to the cremaster. (A) HPC-7 stem cells are more likely to be adherent to thrombi in the cremaster after laser injury induced thrombi formation, in comparison to MSC stem cells. (B) HPC-7 stem cells are more likely to be adherent to thrombi cremaster in laser injury induced thrombi formation, in comparison to MSC stem cells, when infused through the femoral artery as no MSC adherence was observed in comparison. (C) HPC-7 stem cells adherent to the thrombi in the cremaster after laser injury induced thrombi formation, in comparison to MSC stem cells; infused through the carotid artery; not significantly different . (D) Overall, in the conditions of H_2O_2 pre-treated cells and TNF-α pre-treated cremaster, femoral infusion resulted in more adherence in comparison to carotid infusion. Animal received 1 x 10^6 HPC-7 cells or 5x10^5, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. No significant differences were seen in (A), (B), (C) and (D).

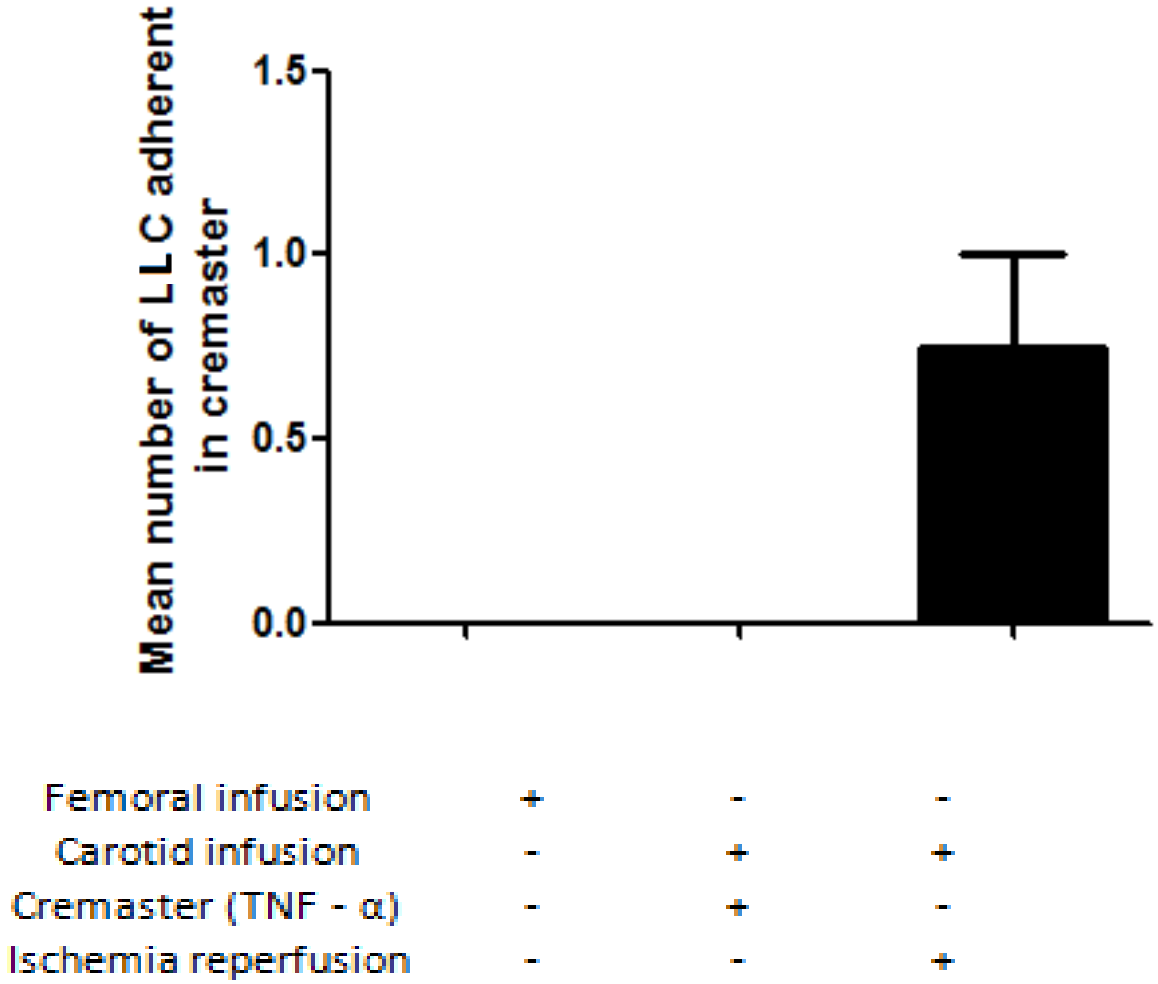
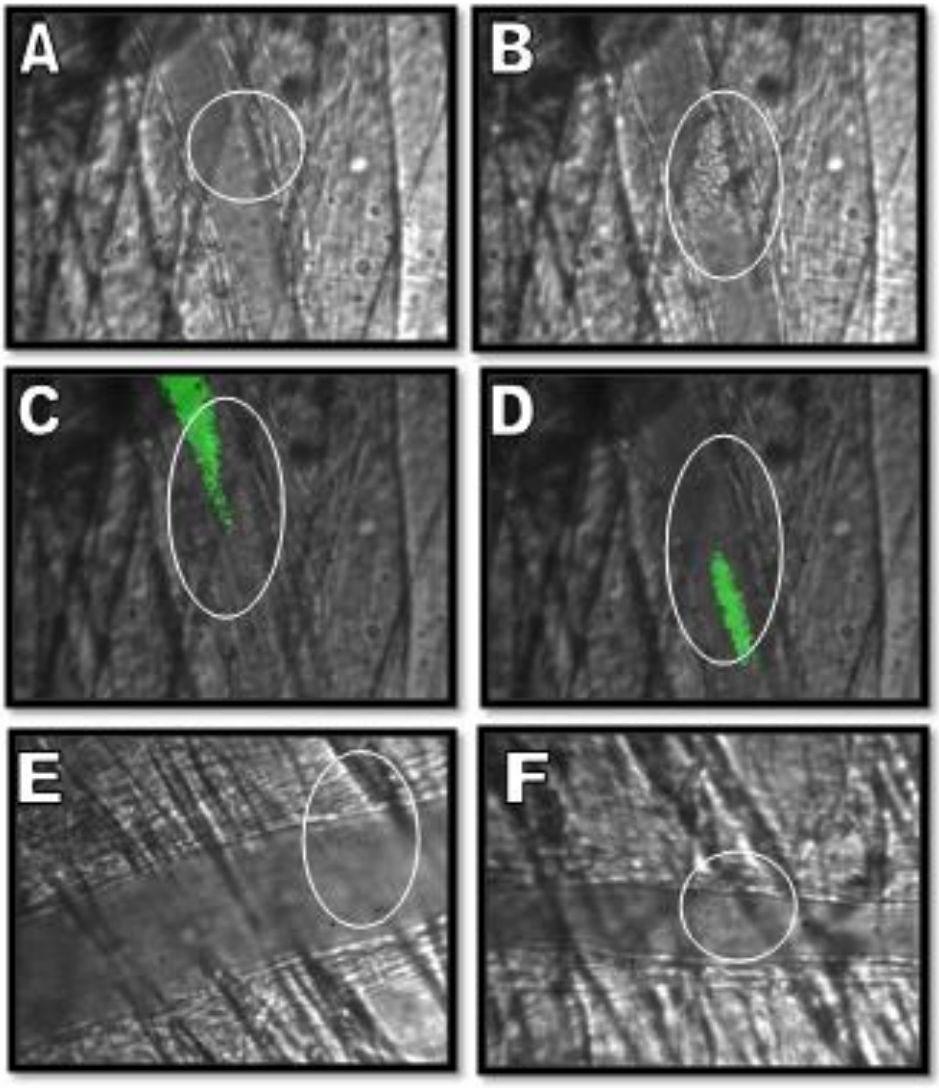
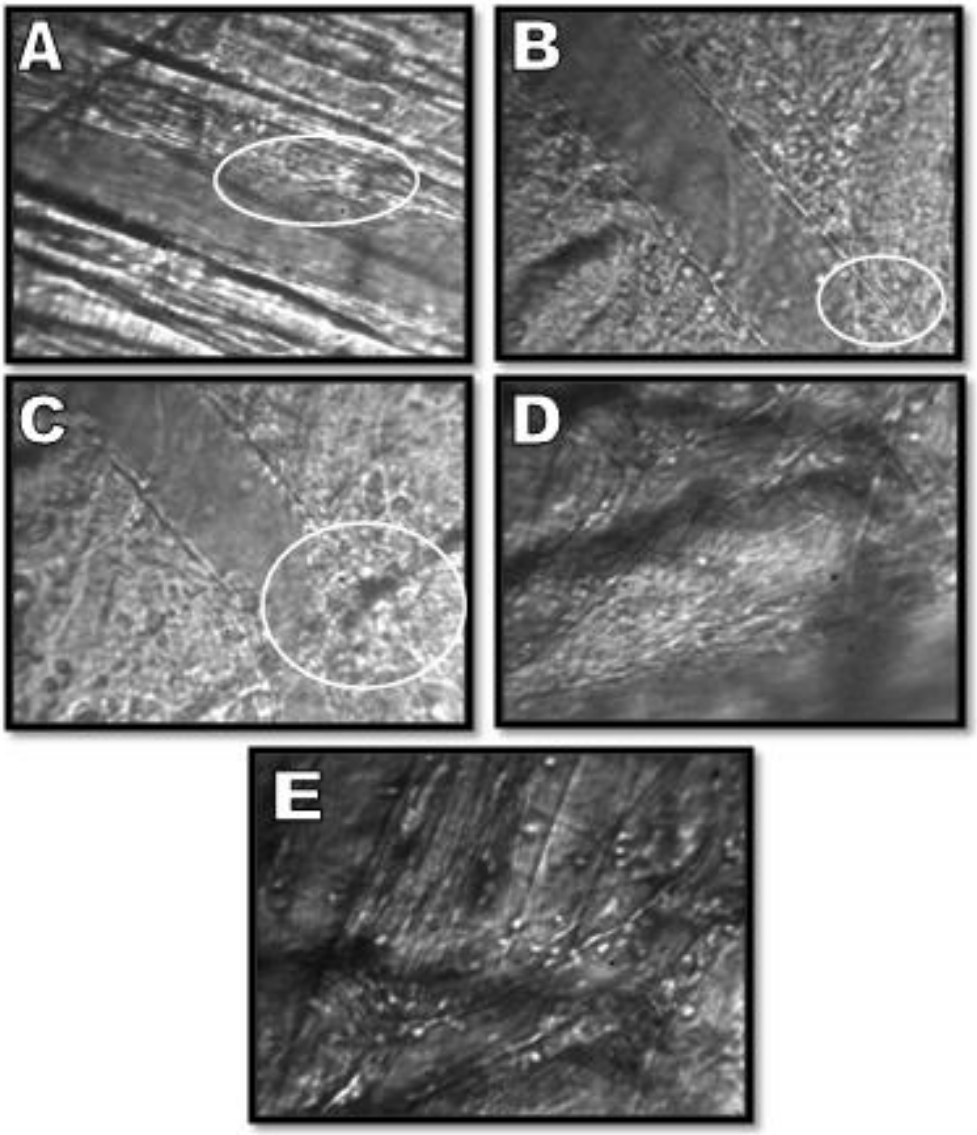


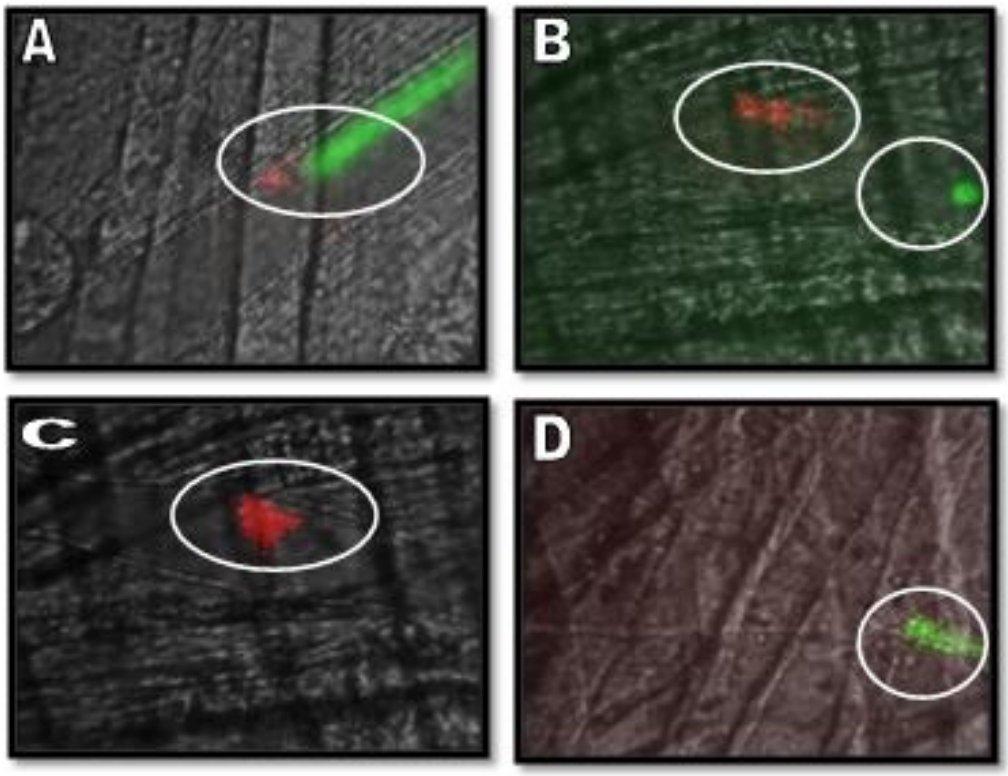
Figure 3.14; Lewis lung carcinoma cells show very low adherence to the cremaster. Animal received 1 x 10^6 LLC cells, stained with CFSE. Results are presented as mean cells per cremaster ± SEM. Cell adherence was only observed after IR – injury, carotid infusion. No cell adherence was observed after any of the other treatments.



F**igure 3.15; Naive LLC** (A) Thrombi beginning to form in arteriole. (B) Thrombi from (A) Further developed. (C) Lewis cells infused. (D) Cells move past thrombi and do not become adherent. (E)(F) No tumour cells adherent to thrombi's generated in other arterioles.



F**igure 3.16; LLC – Mouse TNF-α treated** (A)(B)(C)(D) Thrombi generated in arteriole with no cells attached. (E) Rolling cells in arteriole as a result of TNF-α treatment.



F**igure 3.17; LLC – Ischemia reperfusion injury** (A)(B)Cells move into the arteriole and past the labelled thrombi. (C) No cells are adherent to the thrombi. (D) Cell adherent within capillary.

4.0 DISCUSSION

From this study it has been identified that platelets are not very successful at capturing trafficking cells from flow. This investigation confirmed low levels of interactions between platelets and HPC-7, and between platelets and MSC. However, no interactions between platelets and LLC were observed. Furthermore, data also demonstrated some adherence at very low levels of all three cell types within capillaries in the cremaster.

$1x10^6$ naive HPC-7 were infused through a naive cremaster after laser induced thrombi formation. Very low adherence was observed. This could be due to the fact that, neither the cremaster, nor the HPC-7 were stimulated. Therefore, there was no reason for adherence to thrombi to occur.

Stimulating both the cremaster and the stem cells was seen as the most appropriate experimental development. $1x10^6$ HPC-7 were pre-treated with H_2O_2, and infused through the cremaster, which was stimulated with TNF-α. Adherence to thrombi was still observed to be low after quantification. The adherence observed with HPC-7 was temporary as a few seconds of adherence, was followed by the unattachment of the HPC-7. However, adherence was still greater than that identified in the naive cremaster using naive HPC-7. Interestingly, the increase identified was not statically significant. It is a possibility that the low levels of adherence could be a result of the cremaster not being stimulated enough.

Ischemia reperfusion injury has been identified to simulate the cremaster at a higher level than TNF-α. Therefore, the next development was to use an ischemia reperfusion model. $1x10^6$ HPC-7 were pre-treated with H_2O_2 and were infused through the cremaster after 30

minutes ischemia and 90 minutes of reperfusion. From data obtained, no adherence was identified however; studies previously carried out using similar techniques had shown high levels of adherence of HPC-7 within the cremaster in the absence of thrombi (Kavanagh *et al.*, 2010; Kavanagh *et al.*, 2013(a))**.** The major difference was the use of laser induced injury for thrombi formation. Directing towards the possibility of laser induced injury to be causing possible changes to the conditions within microenvironment. This may be resulting in the possible alteration of the adhesive ability of the cremaster.

Literature has shown, HSC derived from the bone marrow were observed to show a significant increase in adherence levels in ischemia reperfusion injury in the cremaster; due to unknown factors released during IR injury of renal injury. TNF-α enhanced recruitment of HSC in the cremaster significantly, but 1L-1β enhanced recruitment better than TNF-α (Kavanagh *et al.,* 2011(a)).

In Another study a specific type of HSC (HPC-7) were infused into C57BL/6 male mice and while observing the cremaster significant levels of adherence was observed following ischemia for 30 minutes and reperfusion for 90 minutes when comparisons were made with the sham cremaster. Furthermore, TNF-α significantly increased adhesion of HPC-7 in the cremaster however, the dose of TNF-α used was different to the dose used in this investigation as it was much lower at 100ng/200µl (Kavanagh *et al.,* 2013(b)). Other experiments which would have been a beneficial control would be saline injected mice in comparison to TNF-α injected mouse.

Infusion through the femoral using pre-treated HPC-7 and IR injury was an important experiment which would be a direct comparison to other previous studies. In this study HPC-7 adherence seen was low in comparison to literature. One of the main differences

being femoral infusion instead of carotid infusion, resulting in higher levels of adherence previously identified.

$5x10^5$ naive MSC were infused through a naive cremaster after laser induced thrombi formation. No adherence was observed possibly due to the same reason concluded with the HPC-7; no stimulation of the stem cells or the cremaster, giving no reason for adherence to thrombi to occur.

The cremaster and the stem cells were both stimulated for the next set of experiments. $5x10^5$ MSC were pre-treated with H_2O_2, and infused through the cremaster, which was stimulated with TNF-α. Adherence to thrombi was identified, but at a very low level.

Ischemia reperfusion was the final experimental development made to provide a more adhesive microenvironment for the MSC. $5x10^5$ MSC were pre-treated with H_2O_2 which were infused through the cremaster after 30 minutes ischemia and 90 minutes of reperfusion. From data obtained, once again like the HPC-7 no adherence was identified. Other than the possible reasons identified previously, specific to the MSC, it is possible that due to their larger size they were becoming caught in capillaries. Data obtained showed that MSC were getting caught up in capillaries in all four of the experimental conditions investigated. Most likely the MSC are also becoming adherent within other organs, to confirm this after the experiment the organs could have been removed and observed to identify any MSCs. However, this experimental step was not carried out due to time constraints. MSCs being stuck within capillaries and possibly many organs would lower the concentration of MSCs circulating past the thrombi. Therefore, have a lower chance to become adherent. To support this finding, much literature has shown that MSC do not seem to adhere to damaged tissue (Kavanagh *et al*., 2013(b)). However, there is literature that

contradicts these findings as studies have also demonstrated MSCs to have the ability to home in injured tissues (O'Loughlin *et al.,* 2013).

In vitro studies have identified how AMSC (Adipose tissue derived mesenchymal stem cells) should be used rather than BM derived MSC in further clinical research. This was proposed due to the finding that after passaging multiple times AMSC were observed to have a faster proliferation rate in comparison to BMSC. The study also gave supporting evidence of MSC to be used as a 'therapeutic delivery vehicle'. However, research has not been carried out to fully confirm the efficiency of MSC as a therapeutic agent (Courtney *et al.,* 2013).

Literature has also shown how BM-derived MSC may be effective in treatment of non-healing diabetic foot ulcers. The study carried out used $1x10^6$ MSC in a rabbit ear ulcer model. Increased angiogenesis was found after treatment, in comparison to untreated wounds. From this it can be concluded that using BMSC rather than AMSC may still be effective. To be certain further research could be carried out using AMSC and BMSC looking at diabetic foot ulcers to identify if there is any significant difference between the two. Furthermore, the findings from this study would need to be further researched before any clinical application can be made for diabetic patients. It may be a possibility that if this therapy is fully developed, it may be of use in healing wounds other than those seen in diabetics, such as in patients with coagulation related problems (O'Loughlin *et al.,* 2013).

Overall, between the two stem cells, HPC-7 were adherent within the cremaster and to the thrombi at a greater number in comparison to the MSC. However, through statistical analysis no significant difference between the two stem cells was found. However, similar intravital studies have seen significance in HPC-7 recruitment (Kavanagh *et al*., 2010; Kavanagh *et al*., 2011; (a) Kavanagh *et al*., 2013 (a)).

In recent in vivo studies it was observed that MSC expressed adhesion molecules CD49, CD18, β7 and CD62L, on their cell surface (Kavanagh *et al*., 2013 (b)). However, adhesion was not seen to the endothelium even when pre-treated with H_2O_2 and/or TNF-α. Any adherence that was observed was in non injured lung tissue (Kavanagh *et al*., 2013 (b)).This also supports the finding from this investigation.

On the other hand, although results from this investigation has shown little HPC-7 adherence; much literature supports significant adherence of these specific stem cells within organs in mouse models. It was also found that HSC have a higher adherence level due to the intergrins CD18 expressed which are important for HSC adherence (Kavanagh *et al*., 2010; Kavanagh *et al*., 2011; (a) Kavanagh *et al*., 2013 (a)).

Adhesion molecules play an important role in mediating interaction between cells. Neutrophils have been recognised to interact with platelets through P-selectin and b_2 integrin CD11b/CD18, it possible that some or all these adhesion molecules may be absent from the cell surface of LLC, HPC-7 and MSC, further resulting in the inability to mediate interaction with platelets (Brown *et al*., 1998; Li., 2008; Smyth *et al*., 2009; Yang *et al.,* 1999). Therefore, resulting in low levels of adherence that were observed through the results obtained.

$1x10^6$ naive LLC were infused through a naive cremaster after laser induced thrombi formation. In the next experiment $1x10^6$ naive LLC were infused through the cremaster which was stimulated with TNF-α; followed by the final set of experiments consisting of the infusion of $1x10^6$ naive LLC into a cremaster which had undergone 30 minutes ischemia and 90 minutes of reperfusion. None of the three experimental conditions investigated displayed any LLC adherence to the thrombi formed. However, in the ischemia reperfusion

model LLC were identified to be adherent within other areas of the cremaster, but at a very low level.

The reasons why LLC are not displaying any interaction with platelets is not very clear. Much literature has reported high levels of interaction between tumour cells and platelets further leading to a role in cancer metastasis. Many of the studies focused on cancer-platelet interactions are using many different cancer cell lines. It may well be a possibility that this particular cell line does not interact with platelets. Therefore, metastasis seen within this type of cancer may be mostly influenced by other microenvironmental factors, other than platelets.

Additionally, other reasons for not observing any platelets-tumour interactions could be in relation to the chosen method of investigation. Other studies demonstrating their interaction have used alternative methods such as observing disseminated tumour cells in blood samples from cancer patients with tumour development (Buergy *et al*., 2012; Placke *et al.,* 2012). In vitro investigation using isolated tumour cell (B16F1- melanoma cells) from C57BL/6 mice was also used to demonstrate tumour-platelet interactions and the increased metastatic ability (Amano *et al.*, 2013). Alternatively, mouse models have also been used to show restoration in metastatic potential after platelet depleted animals were injected with tumour cells intravenously. These investigations were more directed towards investigating the platelet-tumour interaction in terms of the mouse model and samples used (Buergy *et al*., 2012).

In comparison, it is possible that the method of investigation used for this study was not effective as the concentration of tumour cells infused were a fraction of what would be in an actual tumour mouse model therefore, resulting in having a lower chance to interact

with platelets. Alternatively, an ideal model to confirm any interactions between LLC and platelets would be using a LLC tumour platelet depleted mouse model, in which platelets can be injected and the level of metastasis observed.

Due to time constraints many experiments which would have been important for this investigation were not carried out. Looking at a few alternative cancer cell lines would have been valuable, as LLC did not interact with platelets but much literature has shown interaction between platelets and tumour cells has been observed on many occasions.

The data obtained through all the investigations were subject to factors that may have influenced their outcome. Two routes of infusion were investigated; femoral artery cannulation and carotid artery cannulation. After statistical analysis it was clear that there wasn't any significant difference between the two routes. However, it was observed that femoral artery infusion had shown a higher level of adherence in comparison to carotid artery infusion. The reason for this could be that the femoral was a more direct route into the cremaster for the cells to be infused through. Therefore, less cells were more likely to be lost elsewhere in the mouse model. Using femoral artery for cell infusion instead of the carotid artery maximised the percentage of cells entering the cremaster as the cremaster muscle itself has a lower level of blood flow in comparisons to other parts of the body and after the cells have passed once through the cremaster it is likely that the number of cells circulating within the cremaster are at a very low level, consequently lowering the likeliness of any cellular interactions.

After the cells were infused through the mouse model overall adherence identified was quite low, therefore there is a likely possibility that the cells may be trafficking to other

parts of the mouse model other than the cremaster muscle, such as the heart lungs and the kidneys as they have the highest percentage of blood flow (Elad and Einav., 2004).

For this investigation decisions were made to help focus on the research question. The formation of thrombi in an arteriole was smaller in size, in comparison to a thrombi formation in a venule. Therefore, thrombi were mainly generated in arterioles rather than the venules as a part of the investigation. The larger the injured area the more the stem cells were expected to adhere. Also, it is possible that the stem cells would have also become adherent to the platelets that are already attached to the injured area.

During this investigation, were all the other types (LLC and HPC-7) were infused at $1x10^6$ but the MSC were infused at $5x10^5$; this is a possible limitation when comparing to HPC-7 adherence. Also, having a low number could cause an obvious decrease in the level of adherence due to having only $5X10^5$ cells infused. However, in a recent study it was also found that increasing the number of MSC from $5x10^5$ to $1x10^6$ did not increase the level of adherence (Kavanagh *et al.,* 2013 (b)). However, the degree of interaction could have been increased if the cells were administrated in multiple doses, resulting in a gradual increase in the circulating cells therefore increase the possibility of any interactions.

Another factor to consider during this investigation was the cremaster muscle. Observations made from the preparation were limited and directed more from the center of the tissue. This was to avoid the damaged regions at the edges of the tissue. When the cremaster muscle is prepared many interconnecting vessels are a damaged which alters the flow distribution. This type of damage could have affected the results obtained, additionally the damage caused in each cremaster could have affected the flow distribution differently each time (Bagher *et al*., 2011).

However, this particular vascular bed was good to study microcirculation via intravital microscopy because the preparation was versatile allowing experimental manipulations. Images from this preparation were of good quality and blood flow direction could easily be identified therefore laser injury used for this investigation was easily directed toward the arteriole walls as required. This vascular bed was better than other vascular beds for this particular investigation as cellular interactions could be more clearly identified. The cremaster muscle is a thin layer which can easily be prepared to study microcirculation in comparison to other vascular beds. The same preparation can also be done in other animal models such as in rats and hamsters. The preparation was also time efficient. However, the cremaster muscle is not commonly associated with types of injury requiring tissue regeneration or cancer therapy. Therefore, finding a suitable way to visualise cellular interactions in other vascular beds that are associated with injury may be more beneficial for therapeutic reasons (Bagher *et al*., 2011).

The results expected in line with other literature, would have been to show homing of many stem cells dependant on the size of the injury. This would show that stem cells have the potential to adhere to the damaged tissue when and if required. However, the homing was expected to not be fully efficient and through method development techniques to enhance this process would have been examined and investigated. On the whole, this method would have been used primarily to confirm that stem cells do adhere to injured tissue, and the next step would be to investigate how to enhance the homing method of the stem cells. In terms of cancer cells, expected results were to see high levels of interaction between platelets and LLC. Having the understanding that they aid metastasis, methods of decreasing the interactions would have been investigated.

However, findings that were obtained from the investigation carried out can help research move forward. As now it is clear that stem cells show very low adherence to platelets in the conditions demonstrated and the reasons for this should be investigated further. Additionally, it is also clear that not all types of tumour cells interact with platelets in the same way. Therefore, further investigation of tumour cell lines and their interactive ability with platelets should be investigated, to recognise the different adhesive mechanisms that mediate their interactions. It is a possibility that certain tumour cells can be grouped in terms of their style of interaction. This would further be useful in the development of specific therapies to halt platelet- tumour interactions.

5.0 CONCLUSION

This investigation required a great deal of preparation. However, studying stem cells and investigating their homing techniques is important. Stem cells have shown potential in repairing damaged tissues rapidly in diabetic patients and also in many types of bone injuries and injured organs. It has also been identified that types of stem cells have a potential in seeking out damaged cells before they become cancerous. Using different techniques to understanding the underlying mechanism of how stem cells actually adhere to the damaged tissues would lead to a better understanding, and a direction towards the development of techniques aiding their homing. Additionally, it would lead to using stem cells in therapy in patients with many different types of diseases such as, cancer, bone disease and diabetic wounds. In terms of LLC no interactions were observed. As this was the first time LLC-platelet interactions were investigated further research using LLC tumour mouse models should be carried out to confirm the results from this study. Although this investigation has not provided any significance in platelets interacting with stem cell or tumour cells, much literature has demonstrated their interactions and resulting possibility of therapeutic effects.

	Cell Culturing- Material			
Cell Lines		**HSC**	**MSC**	**LLC**
Base media	Stem Pro	45ml		
	MEM		47ml	
	DMEM		47ml	500ml
P/S		0.5ml	0.5ml	5ml
Alglutamine		0.5ml	0.5ml	5ml
Stem cell growth factor		20µl		
Growth factor T			10µl	
SPS		1.3ml		
FBS			0.5ml	
				50ml

	HPC-7

SLIDE BOOK SETTINGS THROMBI EXOGI

Sham = Naive cells – Naive cremaster (Femoral)-infusion	✓ N=4
Treated = cells - H_2O_2 / Cremaster -TNFα (Carotid)-infusion	✓ N=4
Treated = cells - H_2O_2 / cremaster- TNFα (Femoral)-infusion	✓ N=4
Treated = Naive cells / cremaster- TNFα (Carotid)-infusion	x
I/R Injured Cremaster = cells- H_2O_2 (Carotid)- infusion	✓ N=5
I/R Injured cremaster = Naive cells (Carotid)- infusion	x

(Overall Total N= 46)

LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISED (
75	10	15		15	0	0	0
75	10	15		15	0	0	0
75	10	15		15	0	0	0
75	10	15		15	0	0	0
				15.00	0.00		

SLIDE BOOK SETTINGS				THROMBI			EXOGI
LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISED (
75	10	15		15	0	0	0
75	10	15		16	0	0	0
75	10	15		15	0	0	0
75	10	15		15	0	0	0
				15.25	0.00		

LLC-

MOUSE	WEIGHT (g)

SLIDE BOOK SETTINGS

SLIDE BOOK SETTINGS				THROMBI			EXOGI
LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISED (
75	10	15		15	0	0	0
75	10	15		15	0	0	0
75	10	15		16	0	0	0
75	10	15		15	0	0	0
				15.25	0.00		

				LASER POWER	FRAP SIZE	FRAP REPITIONS		AR
	1	30		75	10	15		
	2	29		75	10	15		
	3	29		75	10	15		
	4	27		75	10	15		
	5	24		75	10	15		
Averages		27.80						
SD								
SEM								

SLIDE BOOK SETTINGS	THROMBI	EXO

				SLIDE BOOK SETTINGS				
	MOUSE	WEIGHT (g)		LASER POWER	FRAP SIZE	FRAP REPITIONS		AR
LLC-TNFα Carotid infusion	1	27		75	10	15		
	2	25		75	10	15		
	3	26		75	10	15		
	4	25		75	10	15		
	5	25		75	10	15		
Averages		25.60						
SD								
SEM								

LASER POWER	FRAP SIZE	FRAP REPITIONS	ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISE
75	7	7	15	0	0	0
75	10	15	16	0	2	2.5
75	10	15	16	0	0	0
75	10	15	15	3	0	0
			15.50	0.75		0.63
						1.25
						0.63

SLIDE BOOK SETTINGS			THROMBI			EXO
LASER POWER	FRAP SIZE	FRAP REPITIONS	ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISE
75	10	15	9	9	0	0
75	10	15	15	0	0	0
75	10	15	15	0	0	0
75	10	15	20	0	5	5
			14.75	2.25		1.25
						2.50
						1.25

SLIDE BOOK SETTINGS			THROMBI			EXO
LASER POWER	FRAP SIZE	FRAP REPITIONS	ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMALISE
75	10	15	15	0	0	0
75	10	15	20	0	0	0
75	10	15	14	0	0	0
75	10	15	20	1	1	1
			17.25	0.25		0.25
						0.50
						0.25

	LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMA
	75	10	15		15	3	0	
	75	10	15		15	1	0	
	75	10	15		15	0	0	
					15.00	1.33		
	SLIDE BOOK SETTINGS				THROMBI			
	LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMA
	75	10	15		17	1	0	
	75	10	15		15	0	0	
	75	10	15		14	0	0	
	75	10	15		15	0	0	
					15.25	0.25		
	SLIDE BOOK SETTINGS				THROMBI			
	LASER POWER	FRAP SIZE	FRAP REPITIONS		ARTERIOLE	VENULES	ARTERIOLE THROMBI	NORMA
	75	10	15		15	0	1	1.333
	75	10	15		16	0	0	
	75	10	15		17	0	0	
	75	10	15		15	0	0	
					15.75	0.00		0
								0
								0

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