Original Research Article

Mild-tourniquet induced ischaemia-reperfusion injury results in changes to haematological, haemostatic and inflammatory parameters

Abbreviations:
IRI – Ischaemia-Reperfusion Injury
vWF – von Willebrand Factor
PT – Prothrombin Time
IL – Interleukin
CVD – Cardiovascular Diseases
NO – Nitric Oxide
EDTA - di-potassium ethylene diamine tetra-aceticacid
ELISA – Enzyme-Linked Immunosorbent Assay
WBC – White Blood Cell
RBC – Red Blood Cell
MCV – Mean Cell Volume
HcT – Haematocrit
Plts – Platelets
APTT – Activated Partial Thromboplastin Time
ANOVA – One-Way Analysis of Varience
GCX – Glycocalyx
HNE – Human Neutrophil Elastase

Abstract

Background

Ischaemia-reperfusion injury (IRI) is an underlying condition in cardiovascular diseases such as arthrosclerosis and stroke, and also occurs after orthopaedic and transplant surgery. These clinical conditions are extremely prominent in the United Kingdom so knowledge of underlying processes such as IRI is very important to the survival of the patient. This study aimed to determine the effects of mild tourniquet induced IRI on specific haematological, haemostatic and inflammatory parameters.

Patients and Methods

An *in vivo* model of mild tourniquet induced IRI was performed on 15 volunteers with tourniquet pressure between 20-40 mmHg for up to 10 minutes (n=15). Venous blood samples were taken prior to IRI, then at 7 minutes and 48 hours reperfusion. The parameters investigated were full blood counts, von Willebrand factor (vWF), sE-selectin, prothrombin time (PT), Interleukin-6 (IL-6), IL-8 and IL-10.

Results

The results demonstrated a significant increase in vWF following reperfusion (p=0.005) and increasing trends for IL-6, IL-8 and sE-selectin (p=>0.05). Decreasing trends were observed for PT, white blood cell and platelet counts (p=>0.05).

Discussion and Conclusion

The study demonstrated thatbrief periods of IRI caused changesin haematological, haemostatic& inflammatory parameters. Following mild tourniquet induced IRI the haemostatic parameters vWF and sE-selectin increased which demonstrates activation of the endothelium, supported by an increase in pro-inflammatory cytokines IL-6 and IL-8. The data presented in this report has enabled suggestions of causal roles between haemostasis and inflammation, which appear to both participate in haematological changes observed by decreased white blood cell and platelet counts.

The results of this study provide a basis for exploration of other haematological, haemostatic and inflammatory parameters which may increase knowledge and understanding of the relationship between these systems during IRI. Further knowledge may highlight areas of therapeutic intervention for the underlying occurrence of IRI in pathological conditions, such as Cardiovascular Disease (CVD) and surgeries that involve the application of a tourniquet.

Keywords: *IRI, vWF, cytokine, inflammation, endothelium*

Background

Organs and tissues require oxygenated blood to support cellular viability butthe restriction or disruption of this nutritional blood supply is deemed as ischaemia, which can result in cellular dysfunction and necrosis **[1]**. Short term ischaemia causes only mild, reversible cellular damage if blood flow is returned promptly **[2]**. Yet peculiarly restoring blood flow to prevent permanent injury can result in greater injury to tissues and cells than that of the original

ischaemia. This event is known as ischaemia-reperfusion injury (IRI) and can produce damage at a local and systemic level **[3]**.IRI is a common underlying clinical process that occurs in diseases such as stroke, myocardial infarction and atherosclerosis whereby blood passage is restricted and then reperfused during treatment **[4]**. Cardiovascular diseases (CVD) are the leading cause of death in the United Kingdom, accounting for one in three of all deaths totalling 191,000 each year **[5]**.Other occurrences of IRI include surgical procedures that involve the use of a tourniquet to create a bloodless field, such as orthopaedic knee and hip surgeries, and organ transplant whereby the ischaemic donated organ is reperfused once positioned within the recipient.

The factors causing IRI can be divided between biochemical changes during the period of ischaemia and those that occur upon reperfusion of the oxygenated blood. The disruption of oxygenated blood to tissues and organs alters their metabolic activity, causing biochemical changes at the cell surface, within the cytosol and in mitochondria **[6, 7]**. These prior biochemical changes are important factors that predispose tissues to undergo free radical damage upon reperfusion of oxygenated blood. As the oxygenated blood comes into contact with the vascular endothelium, superoxide is produced which stimulates changes. Nitric oxide (NO) is an endothelium derived product that provides protective measures such as reducing reactive oxygen waste and inhibiting the production of pro-inflammatory cytokines. During IRI, the imbalance of superoxide radicals reduces NO and removes the protective buffer, creating an environment appropriate for a pro-inflammatory response to occur.

Research into the haematological, haemostatic and inflammatory changes occurring during IRI has encompassed cell adhesion molecules, the cytokine cascade and endothelium derived molecules **[4, 8, 9, 10]**. Interleukin-6 (IL-6) and IL-8 are inflammatory cytokines which have been reported to up-regulate in response to IRI; IL-6 in a model of coronary occlusion by Moro et al (2007) and IL-8 after knee surgery reported by Huda, Solanki&Mathru (2004)**[11, 12]**.vonWillebrand Factor (vWF) and sE-selectin have also been reported to increase in concentration as a response to endothelial activation, a key concept of IRI **[13, 4, 8]**. However these papers largely focus on one of these areas, rarely exploring in the causal relationship between haematology, haemostasis and inflammation in response to IRI.

This study will investigate the haematological, haemostatic and inflammatory response to mild tourniquet induced forearm IRI exploring the early and late response of specific molecules within each of the categories. Full blood counts will be used to determine if IRI causes any significant changes to haematological parameters. The haemostatic response will be measured using vWF, sE-selectin and prothrombin time (PT) whilst the cytokines IL-6, IL-8 and IL-10 will be monitored to measure the inflammatory response of IRI. It is expected that all of these

parameters will alter when stimulated by IRI based on previous research, but this study will attempt to determine if and how these parameters would impact the pathophysiology of IRI in a clinical setting.

Methods

Subject Volunteers

Ethical approval (Re: 771/13/RE/BS) for this study was permitted from the Faculty of Life Sciences Research Committee (FREC), University of Chester. 15 healthy volunteers were recruited for the study after informed consent (n=15). The volunteers participating in this study were aged between 20 and 45 years old (mean age 28.07 ± 7.25 years; gender 13 males and 2 females). None of the research participants had any history of diabetes or cardiovascular disease.

Blood Samples

Venous blood samples were collected into vacutainerscontaining di-potassium ethylene diamine tetra-aceticacid (EDTA), tri-sodium citrateand serum clot activator. Subject plasma was obtained by centrifuging whole blood samples at 450g for 15 minutes, following which all plasma samples were stored (-40^{o}C) until required for the ELISA assays or semi automated analysis.

Model of Ischaemia-Reperfusion Injury (IRI)

This model provided an adapted method of mild tourniquet induced forearm ischaemia-reperfusion injury**[4, 8, 14]**. Venous blood samples were taken prior to commencing the investigations from the contra-lateral arm. This was a control measurement (baseline) for that particular individual. A sphygmomanometer was then placed around the upper experimental arm and inflated to approximately 20–40 mmHg for ten minutes as described by others **[14, 4, 8]**. This procedure reduced blood flow to the arm (ischaemia). The cuff was then removed to allow full blood flow to the arm (reperfusion). Further blood samples were then collected at 7 minutes and 48 hours reperfusion.

Measurement of Haematological Parameters (WBC, RBC, MCV, Hb, HcT&Plts)

Full blood counts were performed using a Coulter® MicoDiff^{18} automated cell counter (Beckman Coulter, U.K.).

Measurement of Endothelial and Haemostatic Function (sEselectin, vWF &PT)

Measurement of sE-selectin was performed using commercially available kits supplied by R&D Systems Europe and involved using ELISA assay as described by manufacturer (R&D Systems, Catalogue # SSLE00).

Plasma vWF concentration was measured as described previously by a sandwich-type ELISA technique using rabbit anti-human vWF and rabbit anti-human vWF peroxidase conjugate (Dako, UK), **[15, 16, 4]**.

PT was measured using a Randox Monza semi-automated system as described by manufacturers instructions (Randox RX Monza Method Sheet: PTH 2752). Citrated samples were used to measure PT, which is a haemostatic test that measures the extrinsic coagulation pathway.

Measurement of Inflammatory Markers (IL-6, IL-8, IL-10)

Measurement of inflammatory markers (IL-6, IL-8, IL-10) was performed using commercially available kits supplied by R&D Systems Europe and involved using ELISA assays as described by manufacturer (R&D Systems, Catalogue # S6050; S8000C; S1000B).

Statistical Analysis

During this study, all results were presented as mean ± standard errors (SE) or median ± Iqr. Where data were normally distributed, repeated measures one-way analysis of variance (ANOVA) between samples test was employed adopting a 5% level of significance. Post hoc testing was conducted using the Tukey test for pairwise comparisons between means. Data that did not comply with normality were analysed using the Friedman test. Where the Friedman test resulted in statistical significance, subsequent tests were performed using the Wilcoxon test. Statistical significance was accepted when $p \leq 0.05$.

Results

Measurement of Haematology (WBC, RBC, MCV, Hb, Hct and Plts) Parameters

Following mild tourniquet induced ischaemia-reperfusion injury changes can be observed in several haematological parameters **(Table 1).** WBC, RBC and Hct demonstrated a decreasing trend from baseline at both 7 minutes and 48 hours reperfusion (p=>0.05). MCV, Hb&Pltsshowed very little change from baseline values after ischaemia-reperfusion injury (p=>0.05).

Measurement of Endothelial and Haemostatic Function (sE-selectin, vWF, PT or APTT)

sE-selectin concentration

The results are expressed as pg/ml and represent the changes in sE-selectin concentration following mild tourniquet induced ischaemia-reperfusion injury **(Figure 1)**. This parameter was measured as marker of endothelial activation. Following ischaemia-reperfusion a trend of increasing sE-selectin was observed (p=>0.05, as determined by the Friedman test). SE-selectin increased from baseline (33.46 ± 18.12) at 7 minutes reperfusions (35.13 ± 17.06) peaking at 48 hours reperfusion (38.55 ± 24.48).

vWF

The results are expressed as IU/ml and represent the changes in vWF concentration following mild tourniquet induced ischaemia-reperfusion injury **(Figure 2)**. This parameter was measured as marker of endothelial activation. Following ischaemia-reperfusion a significant change in vWF was observed (p=0.005, as determined by ANOVA). vWF concentration increased from baseline (1.92 ± 0.48) at 7 minutes reperfusions (3.02 ± 0.78). Following 48 hours reperfusion vWF decreased but remained highter than those of basal values (2.59 ± 0.67). Upon further analysis, pairwise comparisons showed significant differences between baseline *vs* 7 minutes reperfusion (p=0.004).

Prothrombin Time (PT)

The results are expressed as seconds and represent the changes in PT following mild tourniquet induced ischaemia-reperfusion injury **(Figure 3)**. This parameter was measured as marker of haemostatic function, specifically investigating the extrinsic pathway. Following ischaemia reperfusion a decrease in PT was observed from baseline (12.93 ± 3.23) at 48 hours reperfusion (12.49 ± 3.23). This change was not significant (p=>0.05, as determined by paired t-test).

Measurement of Inflammatory Markers (IL-6, IL-8, IL-10)

IL-6

The results are expressed as pg/ml and represent the changes in IL-6 concentration following mild tourniquet induced ischaemia-reperfusion injury **(Figure 4)**. This parameter was measured as marker of inflammatory response. Following ischaemia-reperfusion a trend of increasing IL-6 was observed (p=>0.05, as determined by the Friedman test). IL-6 increased

from baseline (1.22 ± 0.56) at 7 minutes reperfusions (1.52 ± 0.51) peaking at 48 hours reperfusion (1.58 ± 0.15).

IL-8

The results are expressed as pg/ml and represent the changes in IL-8 concentration following mild tourniquet induced ischaemia-reperfusion injury **(Figure 5)**. This parameter was measured as marker of inflammatory response. Following ischaemia-reperfusion a trend of increasing IL-8 was observed (p=>0.05, as determined by the Friedman test). IL-8 increased from baseline (1.1 ± 0.31) at 7 minutes reperfusions (1.57 ± 0.31) peaking at 48 hours reperfusion (1.88 ± 0.06).

IL-10

The results are expressed as pg/ml and represent the changes in IL-10 concentration following mild tourniquet induced ischaemia-reperfusion injury **(Figure 6)**. This parameter was measured as marker of inflammatory response. Following ischaemia-reperfusion, IL-10 decreased from baseline (2.23 ± 0.62) at 7 minutes reperfusions (1.96 ± 0.54) increasing above baseline at 48 hours reperfusion (2.65 ± 0.74). The changes observed in IL-10 concentration were not significant (p=>0.05, as determined by the Friedman test).

Discussion

This study aimed to determine whether ischaemia-reperfusion injury, using a mild tourniquet induced forearm model, resulted in changes to haematological, haemostatic and inflammatory parameters. Another aim was to explore whether any causal link between the parameters and IRI could be observed. The study demonstrated that vWF changed significantly in response to IRI, whilst IL-6, IL-8 and sE-selectin displayed increasing trends. The reperfusion of oxygenated blood to ischaemic tissue is known to activate the endothelium creating a pro-inflammatory and pro-coagulation state **[9, 17]**. The increasing trend of inflammatory cytokines IL-6 and IL-8 in addition to the significant increase of vWF and increasing sE-selectin concentration support the idea that the endothelium is activated during IRI.

The haemostatic parameters sE-selectin, vWF and PT were considered in this model of IRI. The endothelial derived molecule sE-selecin demonstrated an increasing trend of concentration following reperfusion which is supported by Domanski et al. (2006)**[10]**. They found that upon renal reperfusion of the donated organ, sE-selectin increased significantly from baseline at 3 minutes reperfusion. Yu, Hu, Li & Wen (2011) also demonstrated a significant increase of sE-

selectin immediately following total hip replacement and 24 hours post operatively**[13]**. Whilst these two papers reported significant increases of sE-selectin following reperfusion, the trend observed in this report correlates with their pattern of results. The change in vWF concentration during this study was significant, with further analysis demonstrating a significant increase from baseline at 7 minutes reperfusion ($p=0.004$). The same trend was observed by Hughes et al. (2007; 2010) who reported increasing vWF concentrations during early reperfusion that then decreased but remained elevated above basal levels**[4, 8]**.

The endothelium is the interface between blood and surrounding tissues, composed of a monolayer of endothelial cells **[18]**. The endothelial surface is covered by the glycocalyx (GCX), composed of heparin sulphate proteoglycans, which supports homeostasis of the blood vessel wall. The conditions that arise during ischaemia, and particularly reperfusion, cause this GCX layer to partially shed. Activation of the endothelium occurs upon GXC shedding, causing a conversion to a pro-inflammatory and pro-coagulation state which disseminates injury **[9, 17]**.The activation of the endothelium can be demonstrated by the increase of sE-selectin and vWF following ischaemia-reperfusion in this study. sE-selectin is exclusively expressed by activated endothelial cells which are also the main source of vWF production **[19, 20]**. During IRI, the imbalance of superoxide radicals reduces nitric oxide, an endothelium derived product,upon which vWF stimulation is enhanced in humans **[21, 22]**. vWF possesses binding and bridging functions that can cause damage if present in plasma at high levels by increasing platelet aggregation and thrombus formation **[23]**. The findings of the present study support this notion, with circulating platelets decreasing from baseline at 7 minutes and 48 hours reperfusion **(Table 1)** whilst the prothrombin time decreased **(Figure 3)**. Whilst the changes in these parameters are not significant in this study, it must be observed that the model of IRI was very mild. A point of note in regards to the present study is that the vWF samples were assayed in EDTA rather than citrate which is known to provide less stable results **[24, 25]**. Therefore whilst the values reported in this paper are higher than expected, the purpose was to expose a change after reperfusion which has been achieved.

The inflammatory changes observed in the present study are in agreement with other research exploring the impact of IRI in a variety of settings**[9, 11, 26]**). Moro et al. (2007) performed coronary occlusion on rats demonstrating that IL-6 significantly rose following reperfusion, continuing to increase for days after surgery**[11]**. Whilst the increasing trend of IL-6 in this report was not significant, the form of IRI was more severe in the Moro et al. (2007) paper and more likely to induce significant results. Other studies exploring the cytokine's response in orthopaedic surgery have demonstrated significant increases in IL-6 following reperfusion **[9, 26, 27]**. Despite differences in models of IRI, reperfusion times and in the case of Moro et al. (2007) using rats, each of these studies demonstrate an increase in IL-6 following reperfusion

which supports the results of this study. Several of these studies also explored impact of IRI on IL-8, with Huda et al. (2004) demonstrating a significant increase of IL-8 after 4 hours reperfusion following elective knee surgery**[12]**. In contrast to the pro-inflammatory cytokines, the anti-inflammatory cytokine IL-10 was shown to decrease immediately following reperfusion in this study. This finding is in contrast to Zhao et al. (2005) who demonstrated a rapid increase of IL-10 following liver transplant reperfusion between identical twins**[28]**. This deviation may be because the model used in the present paper was too mild to induce an accurate IL-10 response. Due to the isograft, Zhao et al. (2005) were able to perform the transplant without immunosuppression drugs which enabled the observation of the natural cytokine response to transplant. However this is only applicable to isografts, and not during any other transplant.

The effects of ischaemia-reperfusion at a cellular level provide many mechanisms upon which an inflammatory response may be stimulated.Cytokines are released in a cascade, with earlier cytokines such as TNF-α causing subsequent inflammatory cytokines like IL-6 and IL-8 to be released **[29]**.IL-6 and IL-8 both have common cells of origin; macrophages and endothelial cells, which together cause endothelial activation, neutrophil chemoattraction and release. IL-6 is also responsible for up-regulation of adhesion molecules that contribute to neutrophil adhesion to the endothelium, thought to contribute to unsuccessful organ transplant **[30]**. The results of this paper demonstrate an increase in IL-6 over the course of reperfusion measurements, but also show a decrease in white blood cells **(Table 1)**. During an inflammatory response the number of white blood cells would be expected to increase,yet the results of this paper indicate that leukocytes are becoming trapped and activated.Chemoattractants, such as IL-8, increase the adherence of neutrophils to the endothelium, which occurs within minutes of reperfusion **[31]**. Activated neutrophils release proteases such as human neutrophil elastase (HNE) from granules causing necrosis, whilst also impacting microvessles, endothelial permeability and capillary plugging. The loss of the endothelial permeability barrier causes haemorrhage, whilst platelet adhesion causes a loss in antithrombotic activity **[32]**. As vWF has already been implicated in the increase of thrombus formation, the combination of haemostatic and inflammatory changes are the likely cause of IRI pathology, which is clinically relevant as excessive clot formation following surgery is a concerning post-surgical complications. In contrast to the inflammatory cytokines, IL-10 has been suggested to hamper endothelial activation which in turn would reduce adhesion molecules**[33].** However Rabb&Bonventre (1999) based this finding on an administered dose of IL-10 as a therapeutic agent to IRI, meaning it does not truly demonstrate the effect of naturally produced IL-10 post reperfusion. In the present study IL-10 was seen to decrease upon early reperfusion, but increase above baseline at 48 hours **(Figure 6)**. This suggests that

IL-10 does not play a role in the down-regulation of pro-inflammatory cytokines following early reperfusion and could possibly be hampered by the significant increase in concentration of vWF. The pattern of change for IL-10 and vWF are opposite, upon decrease of vWF the IL-10 concentration is observed to increase above baseline. This implies an anti-inflammatory response cannot be initiated whilst haemostatic parameters are significantly increased.

There were several limitations of this study, particularly the amount of reperfusion samples that were able to be obtained following reperfusion. As a cannula was unable to be utilized, only two reperfusion time samples were able to be collected which meant possible changes occurring between 7 minutes and 48 hours were not able to be observed. Whilst the parameters measured in this study enabled a demonstration of inflammatory, haemostatic and haematological changes in response to IRI, there are several other parameters that could have been included. TNF-α is plays a predominant role in early inflammation, released in large quantities from macrophages and endothelial cells within minutes of stimulation **[34, 35]**. Cell surface adhesion molecules, such as CD11b or CD62L, and other haemostatic parameters like fibrinogen would provide further information to determine causal effects. The time and pressure employed in this study to induce mild forearm ischaemia was extremely low in comparison to clinical settings like orthopaedic surgery where tourniquet pressure is approximately 250-300 mmHg for more than an hour**[36, 37]**. The results obtained in this study would not be clinically significant but as changes in haematological, haemostatic and inflammatory parameters were observed it demonstrates the effects of mild IRI, which would be an underlying process in clinical disorders. This study provides clear evidence of increases in haemostatic function and activation of the endothelium following IRI which was supported by the increasing trend of inflammatory markers. That mild IRI induced in this study can cause changes in these parameters suggests the data is useful to appreciate the underlying processof IRI in CVD and following tourniquet applied or transplant surgery.

Conclusion

The present study aimed to determine haemostatic, haematological and inflammatory changes that may occur in response to mild tourniquet induced IRI. The data has revealed evidence that even during brief and mild periods of IRI, changes in haematology, haemostasis and inflammation parametersoccurred. Following mild tourniquet induced IRI the haemostatic parameters vWF and sE-selectin increase demonstrating an activation of the endothelium which is supported by an increase in pro-inflammatory cytokines. The data presented in this report has enabled suggestions of causal roles between haemostasis and inflammation, which

appear to both participate in haematological changes. This study provides a basis for further exploration of haemostatic,haematological and inflammatory parameters which will enhance understanding of the relationship between the three during IRI. Continuing research in this area may highlight areas of therapeutic intervention for the underlying occurrence of IRI in pathological conditions such as atherosclerosis or stoke, and after orthopaedic surgery or transplant.

Competing interests

The author(s) declare that they have no competing interests.

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Table:

Table 1: Effect of IRI on various haematological parameters. The points represent mean/median ± SE/Iqr, as determined by ANOVA or Friedman respectively. Significance accepted p=<0.05, (n=15).

Parameter	Baseline	7 minutes reperfusion	48 reperfusion	p-value (Significance p=<0.05)
WBC	6.43 ± 1.66	6.37 ± 1.64	6.11 ± 1.58	p=0.439
RBC	5.12 ± 1.32	4.98 ± 1.29	4.97 ± 1.28	p=0.298
MCV	91.4 ± 81.3	90.9 ± 81.8	91.2 ± 81.6	p=0.06
Hb	15.1 ± 12.6	14.9 ± 11.7	15 ± 12.3	p=0.692
Hct	46.06 ± 11.89	44.87 ± 11.57	44.52 ± 11.5	p=0.115
Plts	215 ± 162	214± 164	211 ± 161	p=0.819

Legend: WBC – white blood cells; RBC – red blood cells; MCV – mean cell volume; Hb – Haemoglobin; Hct – haematocrit; Plts - platelets

Figures:

Figure 1: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on sE-selectin concentration. The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

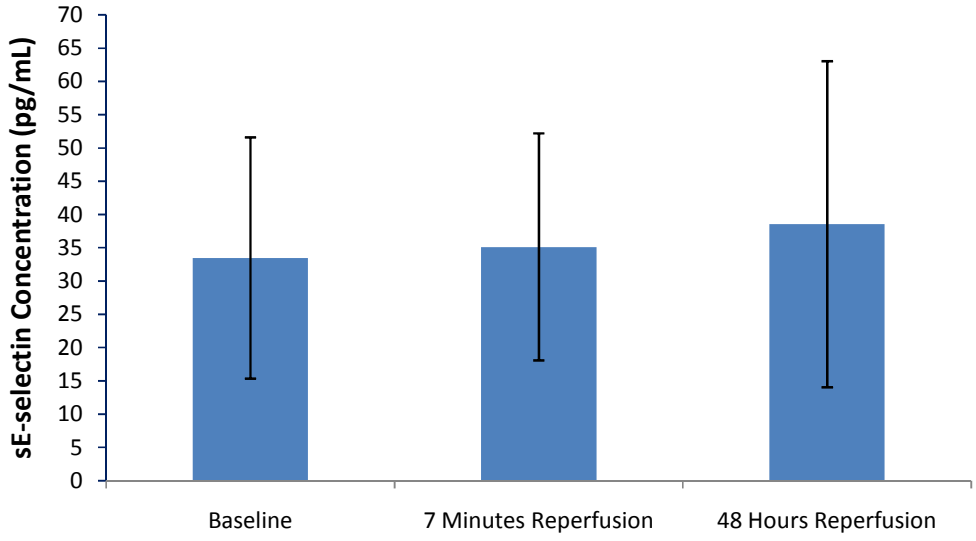

Figure 2: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on vWF concentration. The points represent mean ± SE, p=0.005 as determined by ANOVA. Upon further analysis, pairwise comparisons showed significant differences between baseline *vs* 7 minutes reperfusion (p=0.004), (n=15).

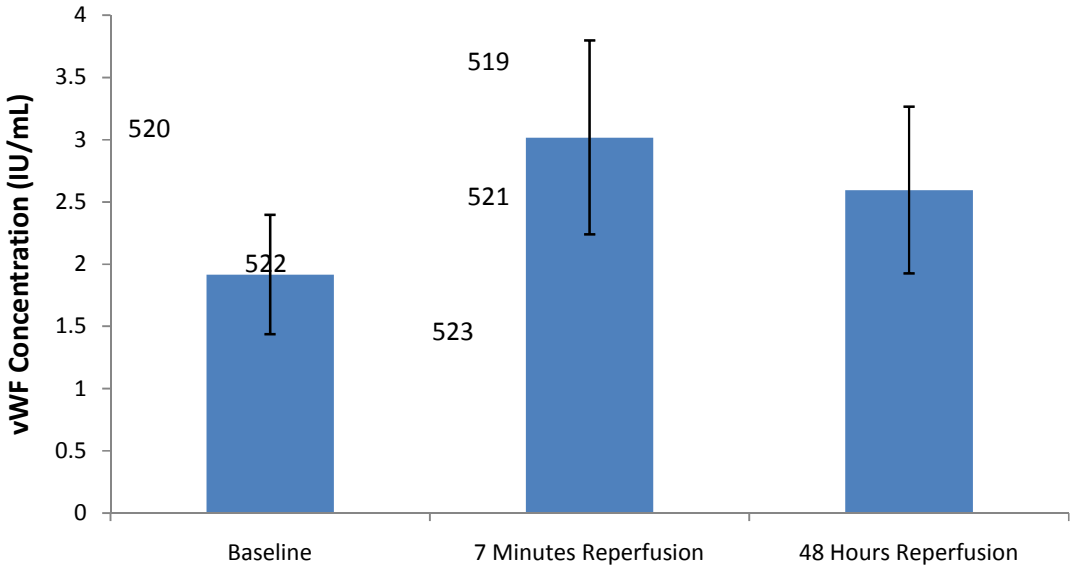

Figure 3: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on prothrombin time. The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

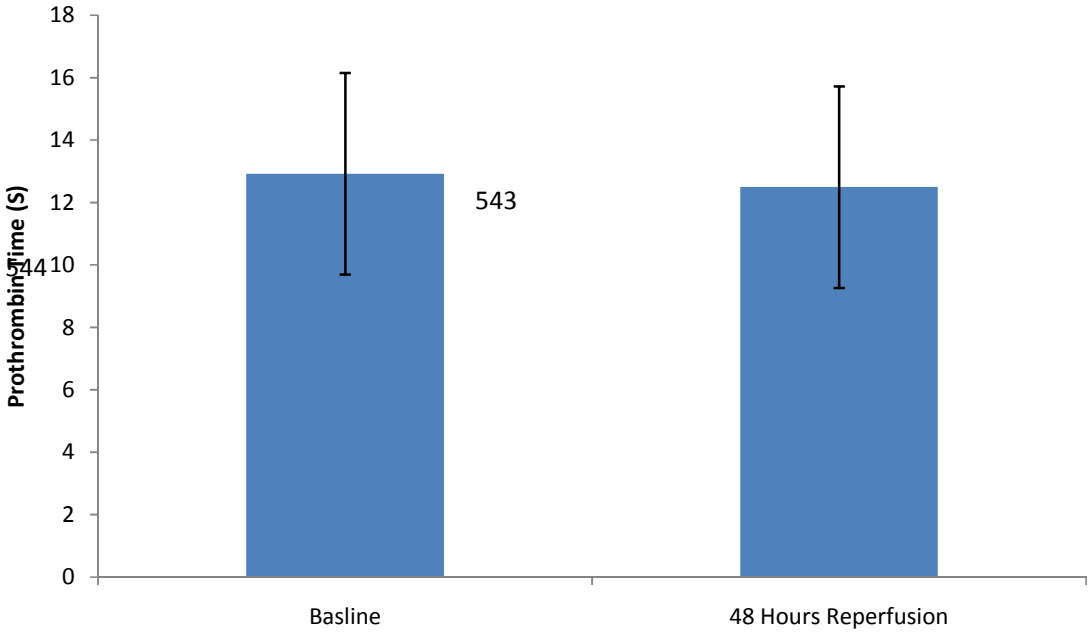

Figure 4: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-6 concentration. The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

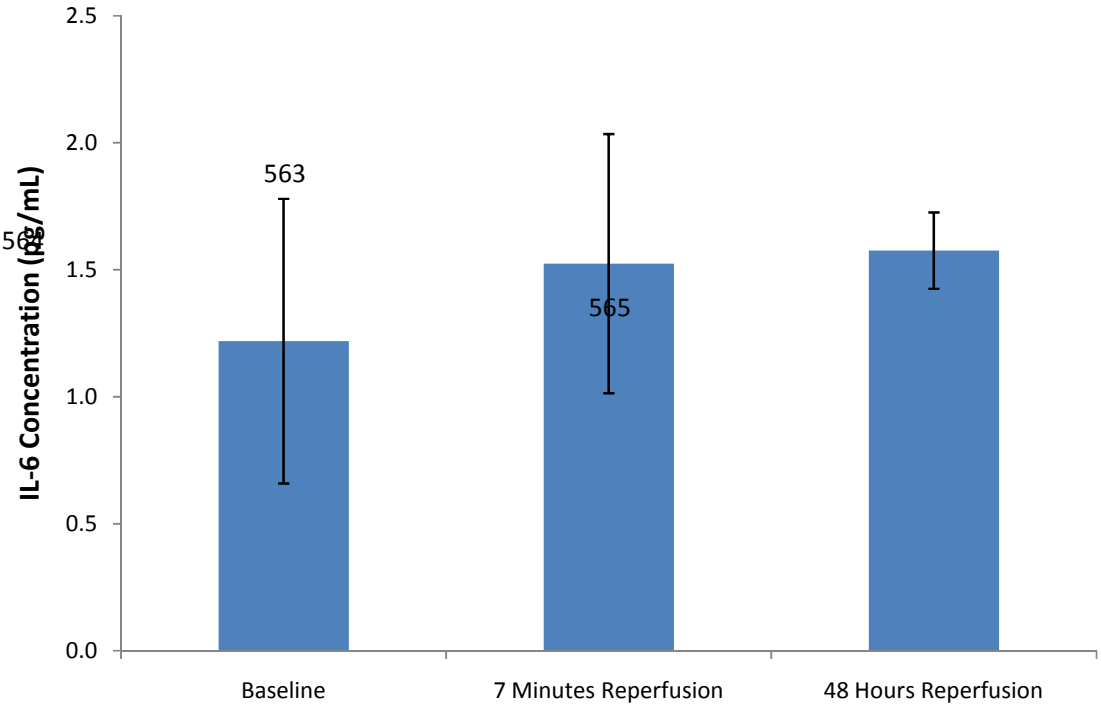

Figure 5: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-8 concentration. The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

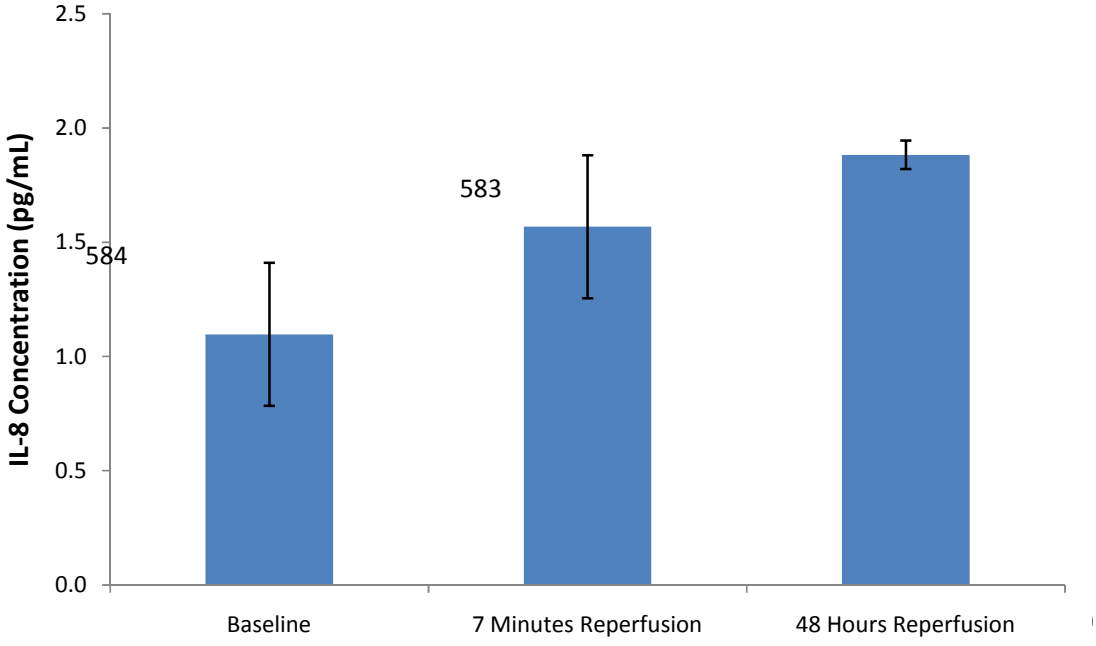

Figure 6: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-10 concentration. The points represent mean ± SE, p=>0.005 as determined by ANOVA, (n=15).