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Mild-Tourniquet Induced Ischaemia-reperfusion Injury Results in Changes to Haematological, Haemostatic and Inflammatory Parameters

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Authors' contributions

The design of the study and subject recruitment was carried out by authors RJE and SFH. While both authors SFH and PET were involved in the blood sampling procedures. Author RJE performed all of the analytical procedures, with help from author SFH during cytokine ELISA and PET during vWF and PT testing. Author RCC provided advisement regarding ELISA optimisation and interpretation of results. Authors SFH provided supervisory support during the study and RJE drafted the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Ischaemia-reperfusion injury (IRI) is an underlying condition in cardiovascular disease such as arthrosclerosis and stroke, and occurs during surgery that involves the application of a tourniquet. These clinical conditions are extremely prominent in the United Kingdom. This pilot-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 (n=15). Tourniquet pressure was set between 20-40 mmHg for 10 minutes and rendered the arm temporarily ischaemic. Baseline venous blood samples were taken prior to ischaemia, then following the release of the tourniquet at 7 minutes and 48 hours reperfusion. The parameters investigated included: full blood count, 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

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(p=0.005), and increasing trends of IL-6, IL-8 and sE-selectin concentrations (p=>0.05). Decreasing PT, white blood cell and platelet counts were observed following IRI but were not significant (p=>0.05).
Discussion and Conclusion: The study demonstrated that brief periods of IRI caused changes to haematological, haemostatic and inflammatory parameters. Specifically, a significant increase in vWF concentration was observed following tourniquet induced IRI. This suggests that changes to vascular integrity and that of endothelial activation may be occurring.
The results of this pilot-study provide a basis for further exploration of haematological, haemostatic and inflammatory parameters following IRI, which may increase our knowledge and understanding of a subject area that is not fully understood. Ultimately, further studies 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. These predictors, however, need further work to validate reliability in a clinical setting.

Keywords: IRI; vWF; cytokine; inflammation; endothelium.

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.

1. INTRODUCTION

Organs and tissues require oxygenated blood to support cellular viability but the 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 totaling 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.

Previous research investigating the effects of IRI on various haematological, haemostatic and inflammatory changes has encompassed some of the cell adhesion molecules, the cytokine cascade and endothelium derived molecules [4,8,9,10]. Specifically, interleukin-6 (IL-6) and IL-8 are inflammatory cytokines which have been reported to be up-regulated following IRI as described by Moro et al. and Huda, Solanki and Mathru [11,12] in a clinical setting von Willebrand 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 the causal relationship between haematology, haemostasis and inflammation in response to IRI.

This pilot-study aimed to investigate the effects of mild-tourniquet IRI on haematological, haemostatic and inflammatory markers. Full blood counts were used to determine if IRI caused any significant changes to haematological parameters. The haemostatic response was measured by investigating vWF, sE-selectin and prothrombin time (PT), whilst the cytokines IL-6, IL-8 and IL-10 were monitored to measure the inflammatory response following IRI.

2. METHODOLOGY

2.1 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. All recruited volunteers initially completed a health questionnaire and their blood pressure (BP) recorded. Any individuals with a history of diabetes or cardiovascular disease were excluded from the study, as were individuals with either low or high BP readings.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).

2.2 Blood Samples

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

2.3 Model of Ischaemia-Reperfusion Injury (IRI)

This model employed 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, which stood as 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.

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

Full blood counts were performed using a Coulter® MicoDiff18 automated cell counter (Beckman Coulter, U.K.).

2.5 Measurement of Endothelial and Haemostatic Function (sE-selectin, vWF and 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 the 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 the manufacturer's 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.

2.6 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 and D Systems Europe and involved using ELISA assays asdescribed by the manufacturer (R and D Systems, Catalogue # S6050; S8000C; S1000B).

2.7 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 $p \leq 0.05$.

3. RESULTS AND DISCUSSION

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

Following mild tourniquet induced ischaemia-reperfusion injury changes were 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 and Plts showed very little change from baseline values after ischaemia-reperfusion injury ($p=>0.05$).

Table 1. Effect of IRI on various haematological parameters

Parameter	Baseline	7 minutes reperfusion	48 reperfusion	p-value (Significance p=<0.05
WBC ($x10^9$/L)	6.43±1.66	6.37±1.64	6.11±1.58	p=0.439
RBC ($x10^{12}$/L)	5.12±1.32	4.98±1.29	4.97±1.28	p=0.298
MCV (fL)	91.4±81.3	90.9±81.8	91.2±81.6	p=0.06
Hb(g/dL)	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 ($x10^9$/L)	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 the points represent mean/median ± SE/Iqr, as determined by ANOVA or Friedman respectively. Significance accepted p=<0.05, (n=15).

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

3.2.1 sE-selectin concentration

The results are expressed as pg/ml and represent changes in sE-selectin concentration following mild tourniquet induced ischaemia-reperfusion injury Fig. 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). Specifically, sE-selectin increased from baseline (33.46±18.12), at 7 minutes reperfusion (35.13±17.06) and peaking at 48 hours reperfusion (38.55±24.48).

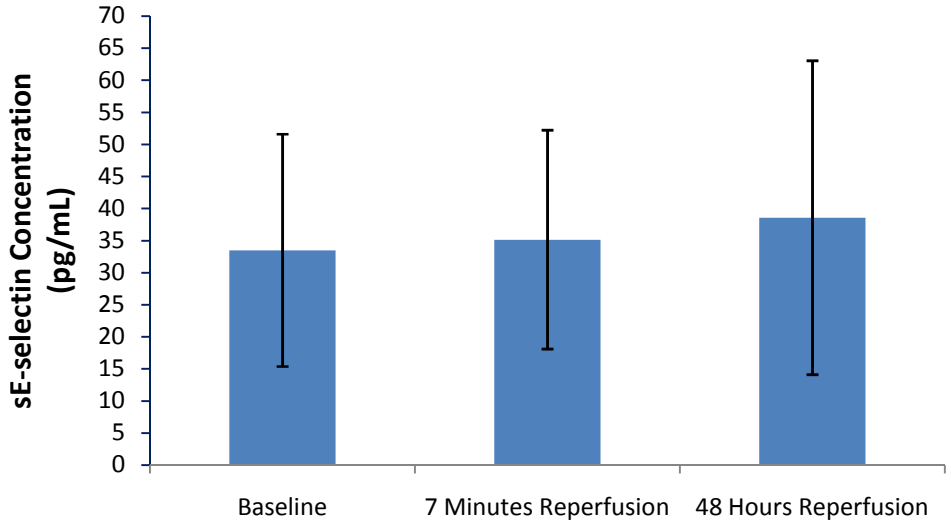

Fig. 1. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on sE-selectin concentration

The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15)

3.2.2 vWF

The results are expressed as IU/ml and represent the changes in vWF concentration following mild tourniquet induced ischaemia-reperfusion injury Fig. 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). Specifically, vWF concentration increased from baseline (1.92±0.48) and during 7 minutes reperfusion (3.02±0.78). Following 48 hours reperfusion, vWF concentration decreased but remained higher 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).

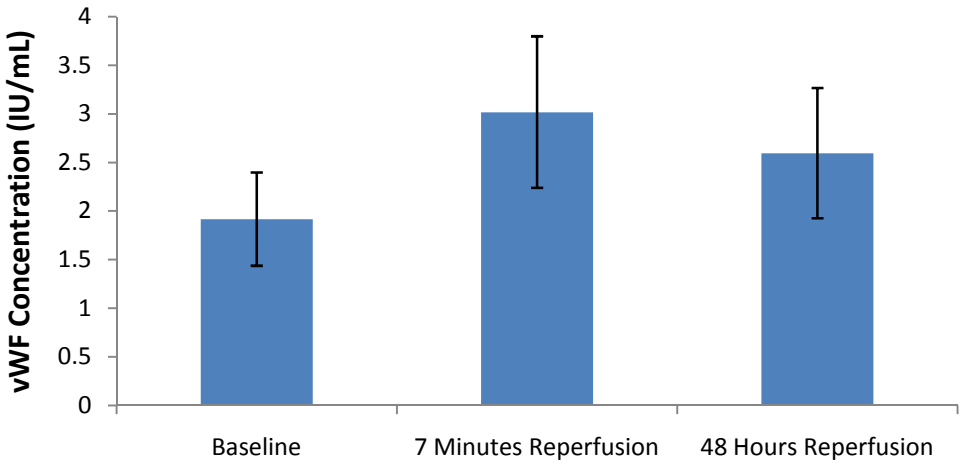

Fig. 2. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on vWF concentration

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).

3.2.3 Prothrombin Time (PT)

The results are expressed as seconds and represent the changes in PT following mild tourniquet induced ischaemia-reperfusion injury Fig. 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) and at 48 hours reperfusion (12.49±3.23). This change was not significant (p=>0.05, as determined by paired t-test).

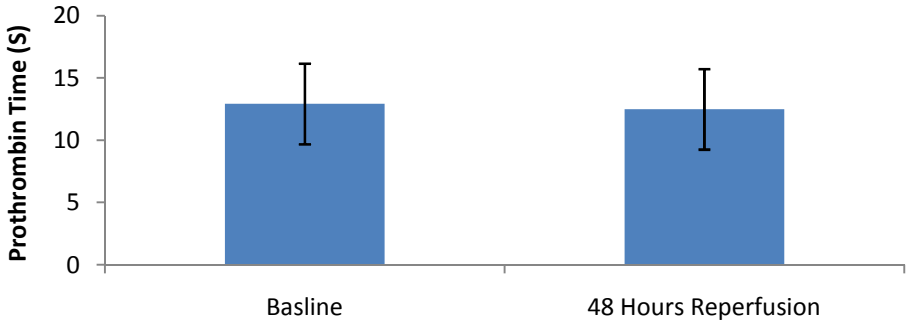

Fig. 3. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on prothrombin time.

The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

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

3.3.1 IL-6

The results are expressed as pg/ml and represent changes in IL-6 concentration following mild tourniquet induced ischaemia-reperfusion injury Fig. 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), during 7 minutes reperfusion (1.52±0.51) and peaking at 48 hours reperfusion (1.58±0.15).

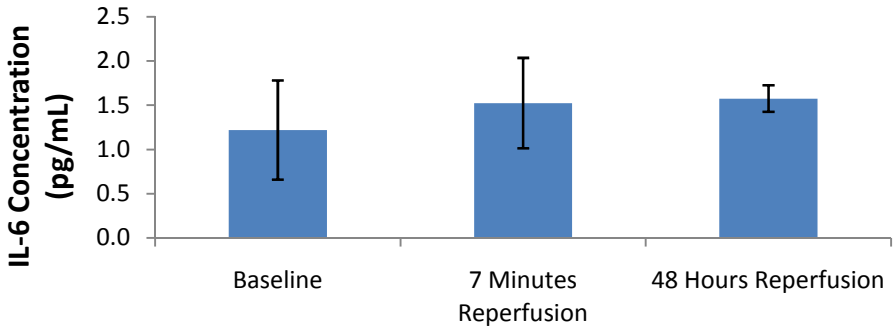

Fig. 4. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-6 concentration

The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15).

3.3.2 IL-8

The results are expressed as pg/ml and represent changes in IL-8 concentration following mild tourniquet induced ischaemia-reperfusion injury Fig. 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), during 7 minutes reperfusion (1.57 ± 0.31) and peaking at 48 hours reperfusion (1.88 ± 0.06).

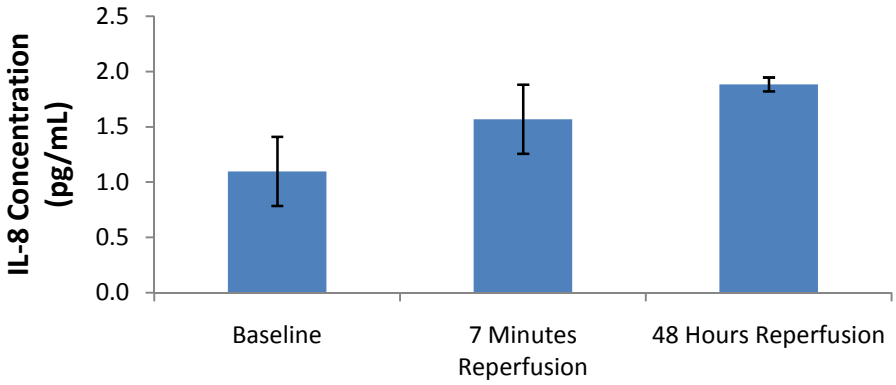

Fig. 5. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-8 concentration

The points represent median ± Iqr, p=>0.05 as determined by the Friedman test. (n=15)

3.3.3 IL-10

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

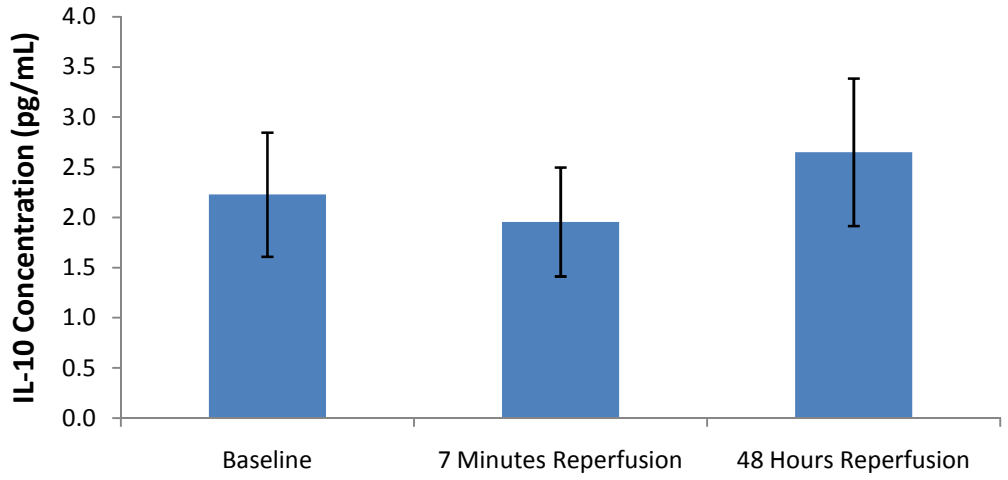

Fig. 6. Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-10 concentration

The points represent mean ± SE, p=>0.005 as determined by ANOVA, (n=15)

3.4 Discussion

This pilot-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 links between the parameters and IRI could be observed. The study demonstrated that vWF concentration changed significantly (p=0.005) following IRI, whilst IL-6, IL-8 and sE-selectin also increased but were not significant. The reperfusion of oxygenated blood to ischaemic tissue is known to activate the endothelium creating a pro-inflammatory and pro-coagulation state [9,17]. In agreement with other, changes to the inflammatory cytokines, IL-6 and IL-8, in addition to the observed changes to vWF and sE-selectin in our study, support the premise of endothelial activation following IRI.

The endothelial derived molecule sE-selectin demonstrated a trend of increasing concentration following IRI, which was in agreement with the report published by Domanski et al. [10]. Specifically, they found that upon renal reperfusion of the donated organ, sE-selectin increased significantly from baseline at 3 minutes reperfusion. Yu, Hu, Li and Wen [13] also demonstrated a significant increase in sE-selectin immediately following total hip replacement and up to 24 hours post operatively. Whilst these two papers reported significant increases of sE-selectin following reperfusion, the trend observed in this report correlates with their pattern of results. Further evidence of endothelial activation was supported by the significant changes in vWF following during the present study. A similar

observation has previously been reported by Hughes et al. [4,8], who have also demonstrated an increase in vWF concentration in non-surgical models of IRI.

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]. It is proposed that activation of the endothelium is aided by the increase of sE-selectin and vWF which was observed following ischaemia-reperfusion in this study. sE-selectin, an adhesion molecule responsible for recruitment of neutrophils, monocytes and lymphocytes, 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 Fig. 3. With regards to the present study, samples for vWF analysis were assayed in blood collected in EDTA rather than citrated tubes, which have previously been reported to provide higher results than blood collected in citrate tubes [24, 25]. However, the aim of the present study was to determine the effects of IRI on vWF and not to compare the effects of anti-coagulants on VWF, and thus was relevant to this study.

The inflammatory changes observed in the present study are in agreement with other research exploring the impact of IRI in a variety of clinical settings [9,11,26,27]. Moro et al. [11] performed coronary occlusion on rats, and demonstrated that IL-6 significantly rose upon reperfusion for several days after surgery. Our results, although not significant, also demonstrated an increase in IL-6 following IRI up to 48 hours reperfusion and are in agreement with Moro et al. [11]. Other studies, exploring the effects of IL-6 in a clinical setting have demonstrated similar findings of increased IL-6 concentration [9,26,27]. Huda et al. [12] demonstrated a significant increase of IL-8 after 4 hours reperfusion following elective knee surgery. Although not significant, a similar pattern of results were seen in the present study, which demonstrated an increased IL-8 concentration following IRI up to 48 hours. It can therefore be appreciated that following mild tourniquet induced IRI, changes to IL6 and IL-8 may be supporting a pro-inflammatory environment. In contrast to the pro-inflammatory cytokines (IL-6 and IL-8), the anti-inflammatory cytokine IL-10 was shown to decrease immediately following reperfusion in the present study. This finding is in contrast to Zhao et al. who demonstrated a rapid increase of IL-10 following liver transplant 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.

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 such as 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 micro-vessels, 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 may be the likely cause of IRI pathology, which is clinically relevant as excessive clot formation following surgery is a concerning post-surgical complication. In contrast to the inflammatory cytokines, IL-10 has been suggested to hamper endothelial activation, which in turn would reduce adhesion molecules [33]. In the present study IL-10 was seen to decrease upon early reperfusion, but increased above baseline at 48 hours Fig. 6. This may suggest 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, although, in order to confirm this more studies would need to be undertaken.

There were several limitations of this study, particularly the amount of reperfusion samples that were able to be obtained following tourniquet induced ischaemia. However, due to time constriction recruiting more subject volunteers for the study would have been beneficial and may have helped provide statistical significance to some of the parameters that were measured in the study. Whilst the parameters measured in this study provided information regarding the haematological, haemostatic and inflammatory response following IRI, there are several other parameters that could have been included. Specifically, TNF-α, which plays a predominant role in early inflammation, cell surface adhesion molecules, such as CD11b or CD62L, and other haemostatic parameters such as fibrinogen [34,35]. The duration of rendering the arm ischaemic and the set tourniquet pressure employed in the present study was relatively short and very mild in comparison to a typical clinical setting. For example, during lower limb orthopaedic surgery tourniquet pressure is set to approximately 250-350 mmHg for periods of up to 2 hours [36,37]. However, despite the acknowledged limitations of this study, the main aim was to determine the effects of effects of a non-surgical model of mild IRI on specific haematological, haemostatic and inflammatory parameters. Generally, the present study achieved this and provides a sound platform to continue research into this area.

4. CONCLUSION

The study demonstrated that brief periods of IRI caused changes to haematological, haemostatic and inflammatory parameters. Specifically, a significant increase in vWF concentration was observed following tourniquet induced IRI. This suggests that changes to vascular integrity and that of endothelial activation may be occurring.

The results of this pilot-study provide a basis for further exploration of haematological, haemostatic and inflammatory parameters following IRI, which may increase our knowledge and understanding of a subject area that is not fully understood. Ultimately, further studies 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. These predictors, however, need further work to validate reliability in a clinical setting.

CONSENT

Not applicable.

ETHICAL APPROVAL

Ethical approval (Re: 771/13/RE/BS) for this study was permitted from the Faculty of Life Sciences Research Committee (FREC), University of Chester.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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