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Effects of exercise intensity on clot microstructure and mechanical properties in healthy individuals

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Abstract

Background: Exercise is well established to lead to exercise-induced hypercoagulability, as demonstrated by kinetic coagulation markers. It remains unclear as to whether exercise-induces changes lead in clot development and increased polymerisation. Fractal dimension (d_f) has been shown to act as a marker of clot microstructure and mechanical properties, and may provide a more meaningful method of determining the relationship between exercise-induced hypercoagulability and potential clot development.

Methods: d_f was measured in 24 healthy individuals prior to, after 5 minutes of submaximal exercise, following maximal exercise, 45 minutes of passive recovery and following 60 minutes of recovery. Results were compared with conventional markers of coagulation, fibrinolysis and SEM images.

Results: Significantly increased d_f was observed following exercise, returning to resting values following 60 minutes of recovery. The relationship between d_f and mature clot microstructure was confirmed by SEM: higher d_f was associated with dense clots formed of smaller fibrin fibres immediately following exercise compared to at rest. Conventional markers of coagulation confirmed findings of previous studies.

Conclusion: This study demonstrates that d_f is a sensitive technique which quantifies the structure and properties of blood clots following exercise. In healthy individuals, the haemostatic balance between coagulation and fibrinolysis is maintained in equilibrium following exercise. In individuals with underlying vascular damage who participate in exercise, this equilibrium may be displaced and lead to enhanced clot formation and a prothrombotic state. d_f may therefore have the potential to not only quantify hypercoagulability, but may also be useful in screening these individuals.

Introduction

Exercise exerts a physiological effect on the coagulation system, leading to exercise-induced hypercoagulable phase, which has been noted to occur in healthy individuals undertaking a range of different exercise modalities [1-3]. The effect of exercise intensity on haemostasis and clot propensity is still poorly understood. Whilst many previous studies have investigated the effect of exercise on individual components of haemostasis such as markers of clot initiation, propagation and fibrinolysis [1,2,4-6], it remains unclear as to whether changes to kinetic pathways of coagulation lead to clot development and increased clot polymerisation, and if exercise intensity has an effect on these changes.

Standard kinetic markers of initiation, propagation and amplification in coagulation have been investigated with regard to exercise-induced hypercoagulability. Markers of clot initiation such as activated partial thromboplastin time (APTT) have been investigated, with both increased and decreased APTT reported by different studies [1,4,6-8]. FVIII is well established to increase following exercise, which may in part be due to acute inflammatory response stimulated by exercise [7-10]. Similarly, thrombin generation, a marker of clot propagation, has also generated conflicting results, with Summan et al. reporting an increase in thrombin generation following exercise [11], but this has not been determined in other studies [8].

Fibrinolysis, an important aspect of coagulation in clot modification or breakdown, has also been investigated in response to exercise, with several studies reporting an increase in fibrinolytic activity via an increase in the fibrin degradation product D-dimers [5,12-15]. In addition, inhibition of fibrinolysis by the plasminogen activator inhibitor-1 (PAI-1)has been demonstrated via a decrease in plasminogen activated inhibitor-1 (PAI-1) activity [16-18]. However it is not known if apparent increases in fibrinolysis are as a natural response intrinsic effect of coagulation change of exercise or as a result of exercise or due to increased fibrinolytic activity due to clot formation as a result of exercise-induced hypercoagulability. These findings suggest there may be an increase in clots formed as a result of exercise-induced hypercoagulability, and that increased fibrinolysis maintains the equilibrium of normal haemostasis in healthy individuals. Given the conflicting results generated from previous studies, there is a need for a more meaningful method of determining the relationship between exercise-induced changes in haemostasis and its effect on potential clot development, fibrin organisation, clot structure and fibrinolysis.

We have previously demonstrated that a new biomarker, fractal dimension (d_f), capable of quantifying changes to the inter-dependent variables of haemostasis, clot microstructure and clot development in a more sensitive manner than conventional markers of clot initiation and propagation in healthy individuals [19]. In addition, d_f has also been shown to quantify altered physiological effects and the relationship

between kinetic changes and clot formation in patients with known vascular inflammatory disease in hypo and hypercoagulable states [19-23] haemodilution [24] and in response to different temperatures [25]. Furthermore, d_f of the incipient, early blood clot was shown to be related to structure of the mature blood clot [19-24], as shown by scanning electron microscopy (SEM), a measure not previously investigated in exercise. At high d_f values, SEM images demonstrated tight clots comprised of thin fibrin fibres, in contrast to those at lower d_f values, comprised of thicker fibrin fibres with larger porous spaces [19-24]. It was therefore hypothesized that blood taken from participants following intense exercise would have increased d_f compared to those obtained from blood taken before exercise. Scanning electron microscopy and computational modelling were also undertaken in order to relate changes in the early, incipient clot structure to those of the mature blood clot and associated fibrin mass.

Materials and Methods

Healthy participants were recruited to the study from January 2014 to May 2014, following a formal screening process and medical history review. The study received formal ethical approval by the University of South Wales Human Research Ethics Committee and all procedures were carried out in accordance with the Declaration of Helsinki of the World Medical Associated and revisions thereof. All participants gave written, informed consent.

Collection of samples

To account for diurnal variation all samples were collected at the same time each day. Participants were cannulated using an 18G intravenous cannula in a forearm antecubital vein. Fasting blood samples were obtained following 30 minutes of rest post cannulation at baseline ('Pre'), after 5 minutes exercise at 35W ('Submaximal'), immediately following exercise to exhaustion ('Maximal'), after 45 minutes rest ('Passive Recovery +45 min') and after 1hour rest ('Passive recovery +60min). The first 3-5mls were discarded as waste and whole venous blood transferred immediately for rheometric analysis. Further samples were transferred into vacuum-sealed tubes containing sodium citrate (3.2%) or K_2EDTA (Greiner Bio-one, Stonehouse, UK) for measurement of standard markers of coagulation and fibrinolysis.

Maximal Exercise Test Design

Prior to exercise, all participants were fasted for 4 hours, and water intake not monitored but kept to a minimum throughout. Following previous familiarization sessions, participants then seated on an electronically braked, semi-recumbent cycle ergometer (Corival; Lode BV, Groningen, The Netherlands) prior to an incremental exercise test to exhaustion. Workload was initially was set at 35 W for 5 mins

(70rpm) and increased by 35 W/min until participants could no longer meet the required power output, at which point the session was ceased.

Cardiorespiratory measurements

Heart rate was monitored throughout the exercise bout using a lead II electrocardiogram (Dual BioAmp, AD Instruments, Oxford, UK). Online breath-by-breath respiratory gas analysis was performed using a metabolic cart (MedGraphics CPX/D; Medical Graphics Corporation; St. Paul, Minn, USA) with minute volumes of oxygen/carbon dioxide uptake (VO_2/VCO_2) calculated via the Haldane equation. Maximal performance was confirmed if participants were unable to maintain the required cadence for > 10 s despite verbal encouragement, a respiratory exchange ratio (RER) in excess of 1.10 arbitrary units (AU) and Borg rating of perceived exertion of 20 points.

Metabolic Measurements

Lactate

Whole blood lactate was blood lactate was measured using an automated electrochemical analyser (Analox PLM5 Champion, London, UK).

Conventional markers of coagulation and fibrinolysis

Citrated blood samples were centrifuged at 600 *g* for 10 minutes at 4°C. The time-based kinetic markers of clot initiation (PT, APTT, fibrinogen and factor VIII) propagation (thrombin generation) and fibrinolysis (D-dimer and PAI-1) were measured as follows. PT, APTT and fibrinogen concentration were measured using an ACL TOP 700 CTS analyser (Werfen, Warrington, UK). Haematocrit was analysed using the micro-haematocrit centrifuge method, and the Coulter-counter method used to measure haemoglobin (Beckman Coulter, High Wycombe, UK). Factor VIII was performed as one-stage factor assays on the ACL TOP 500 according to manufacturer's instructions using Instrumentation laboratory calibration plasma, quality control material and factor deficient substrate plasma (Werfen, Warrington, UK).

Thrombin generation was measured using the Thrombin Generation Assay. Fluorogenic substrate Z-G-G-R-AMC and TGA Trigger reagent were added to citrated plasma and measured using a Ceveron Alpha analyser (all reagents and analyser from Technoclone GmbH, Vienna, Austria). TGA® software was used to calculate individual thrombin generation curves. D dimer analysis was carried out on an ACL TOP 700 CTA (Werfen, Warrington, UK) using the HS Latex immunoturbidimetric assay. PAI-1 antigen was measured using an ELISA assay performed according to manufacturer's instructions (Hyphen Biomed, Quadratech, Epsom, UK).

Rheometric analysis

The gel point measurements (T_{GP}, $G`_{GP}$ & d_f), which are the focus of this study, were obtained immediately from coagulating blood, as previously described [19-24]. Briefly, 6.6ml of unadulterated, whole blood is transferred to a double-gap concentric cylinder geometry of controlled stress rheometer (DHR-2 hybrid rheometer, TA Instruments, DE, USA) at 37°C. Sequential frequency measurements were then carried out in the linear viscoelastic range and the gel point measures calculated as previously described [19]. All sampling was carried out by appropriately trained, experienced members of the research team, and anonymised data reviewed independently by three haemorheologists blinded to the sample origin. Mean results were used for further analysis.

Computational analysis of fibrin mass

In order to investigate changes in d_f and the previously reported increase in fibrin mass of the mature clot [20], computational analysis was carried out. Random fractal aggregates are generated using a numerical technique where d_f is presented as a fixed priori [26,27]. On a set length of scales, over which the structure is deemed to be fractal, a box-counting measure algorithm is used to determine hypercubes required to encompass the aggregate. At each length scale the number of required hypercubes are randomly chosen and importantly linked using a simple random walk in the embedding dimension, ensuring connectivity of the random fractal aggregate on all considered length scales. This is highly efficient and overcomes the limitations on the achievable magnitude of the d_f encountered by alternative techniques and provides a visual illustration of clot structure, based on the d_f values measured on whole blood, allowing the corresponding fibrin mass to be calculated.

Scanning electron microscopy of mature clot structure

12µl of whole blood to clot for 15 minutes at 37^{o}C and resulting clots were washed with cacodylate buffer and fixed with gluteraldehyde, before point-critical dehydration with ethanol (30-100%) and hexamethyldisilazane (Sigma Aldrich, UK). Samples were coated with gold palladium, imaged using a Hitachi Ultra-high resolution FE-SEM S-4800, and fibre width of randomly selected regions calculated as previously described [28].

Statistical analysis

Sample size was calculated based on a mean difference in d_f of 0.05 (an expected change based on pilot data) between rest and maximal exercise, with an estimated standard deviation of 0.04 to achieve a power of 0.85 and significance set to a value of 0.05. Data was assumed to be normally distributed as

demonstrated by Shapiro-Wilk tests for further analyses. The statistical software PRISM® version 5.00 was used to analyse all data. One way-analysis of variance (ANOVA) was used to compare the mean difference between the time points, followed by a Bonferroni-corrected paired sample t-tests. Statistical significance was set at 5% ($p < 0.05$). All numerical data are presented as mean values ± standard deviation (SD).

Results

Participants

A total of 27 participants were recruited, of which 3 were excluded (Two were excluded due to failure in completing both the exercise test and recovery sessions, one was excluded due to underlying medical reasons). Exclusion criteria included: 1) individuals with acute or chronic diseases or inflammatory conditions known to effect coagulation, (i.e. malignancy, hepatic and/or renal dysfunction), 2) individuals taking anti-platelet or anti-coagulation treatment or any other drug known to affect coagulation, 3) individuals with a family history of either bleeding or thromboembolic disorders. All participants were non-smokers and abstained from taking nutritional supplements such as oral anti-oxidants or anti-inflammatories 14 days previously. They were also asked to refrain from physical activity, caffeine and alcohol and to follow a low nitrate/nitrite diet 24 h prior to formal experimentation to avoid any vascular (endothelial) confounds. The data shown are for twenty four healthy subjects (Mean ±SD: 3 females, Age 26 ± 7, Height (m) 1.75 ± 0.09, Mass (kg) 86.2 ± 20.1, Body Mass Index 28 ± 5.4).

Cardiorespiratory measures

Cardiorespiratory measures for the 24 participants who completed the maximal exercise test are shown in Table 1. All measures obtained are within the normal range expected of young, untrained healthy individuals.

Parameter	Rest	Submaximal Exercise	Maximal Exercise
Work (watts)	0	5	269 ± 48
VO_2 (mL/kg/min)	3.8 ± 0.7	7.8 ± 1.5	35.6 ± 7.3
RER	0.90 ± 0.11	0.85 ± 0.09	1.23 ± 0.1
VE BTPS (L/min)	10.6 ± 3.4	18.1 ± 3.5	138.6 ± 33.7
VE/VO_2	33 ± 9	28 ± 7	47 ± 10
VE/VCO_2	37 ± 8	34 ± 6	38 ± 7
HR (BPM)	76 ± 14	92 ± 14	188 ± 12
Lactate (mM)	1.6 ± 0.9	-	8.0 ± 1.3

Table 1. Cardiorespiratory measures taken at rest, after the initial submaximal phase and following maximal exercise. VO_2: Volume of oxygen uptake; RER: Respiratory exchange ratio; VE BTPS: Minute ventilation, body temperature and pressure, saturated; VE/VO2 and VE/VCO2: Ventilatory equivalent ratio for oxygen and carbon dioxide; HR: Heart Rate, beats per minute. (Data ±SD).

Conventional markers of coagulation and fibrinolysis

No significant differences were observed in either PT or fibrinogen during the study period (Table 2). Changes to the markers of clot initiation APTT and FVIII were observed following maximal exercise compared to resting levels (31.2 ± 3.8s v 27.8 v 3.7s, and 247.1 ± 129.3 v 110.8 ± 70.0 iu/L; both $p<0.05$), and remained shortened for the recovery period. FVIII also remained elevated at least 1 hour after exercise completion. Levels of haematocrit and haemoglobin also appeared to increase following maximal exercise, whilst blood volume decreased (Table 2), but all remained within healthy ranges for the study duration and fibrinogen levels did not significantly change. With the exception of FVIII, all values had returned to resting levels within 1hour post exercise. Whilst an increase in D-dimer was observed after maximal exercise compared to at rest (300.5 ± 604.2 v 110.9 ± 101.8 ng/ml), this failed to reach significance ($p=0.084$). No significant differences were observed in PAI-1 antigen for any time point throughout the study duration. Likewise, no significant differences were observed in clot propagation measured via thrombin generation throughout the study duration, as shown in Table 3. Correlation analyses between

FVIII and thrombin generation parameters did also not identify significant relationships between these markers at any point during the study duration (Table 4).

	Rest	Submaximal	Maximal	Rec 45	Rec 60
PT (sec)	11.2 ± 0.7 (6.3)	11.3 ± 0.6 (5.3)	10.7 ± 0.6 (5.6)	11.1 ± 0.8 (7.2)	11.1 ± 0.9 (8.1)
APTT (sec)	31.3 ± 3.8 (12.1)	31.9 ± 3.8 (11.9)	27.8 ± 3.7* (13.3)	26.5 ± 3.4 (12.8)	26.5 ± 2.8 (10.6)
Clauss fibrinogen (g/L)	2.4 ± 0.4 (16.7)	2.4 ± 0.5 (20.8)	2.6 ± 0.5 (19.2)	2.3 ± 0.4 (17.4)	2.3 ± 0.3 (13.0)
Haematocrit (%)	44.9 ± 3.5 (7.8)	47.5 ± 3.4 (7.2)	49.9 ± 3.6 (7.2)	46.2 ± 3.7 (8.0)	44.3 ± 3.3 7(.4)
Haemoglobin (g/L)	150.2 ± 12.0 (8.0)	156.5 ± 12.1 (7.7)	162.7 ± 12.8 (7.9)	151.2 ± 14.6 (9.7)	142.9 ± 11.8 (8.3)
D dimer (ng/ml)	110.9± 101.8 (91.8)	85± 64.1 (75.4)	300.5± 604.2 (201.1)	112.0± 94.9 (84.7)	106.6± 48.7 (45.6)
Factor VIII:C (iu/L)	110.8± 70.0 (63.1)	113.1± 60.5 (53.5)	247.1± 129.3* (52.3)	256.1± 156.8 (61.2)	227.5± 108.0 (47.5)
vWF Antigen (iu/L)	94.7± 32.5 (34.3)	96.4± 35.4 (36.7)	155.6± 52.3 (33.6)	153.1± 42.6 (27.8)	150.3± 52.3 (34.8)
PAI-1 Antigen (ng/ml)	5.9± 3.8 (63.0)	6.0± 3.6 (59.3)	6.3± 3.5 (56.1)	5.8± 3.6 (62.9)	5.4± 2.3 (43.3)
Plasma Volume (%)	100	93.2± 5.1	85.6± 6.8	98.6± 8.9	108.0± 8.1

Table 2. Changes observed in standard laboratory markers of haemostasis at rest, post exercise and during recovery. With the exception of a significantly shortened APTT (compared to at rest), no other changes were observed in standard laboratory markers. (Data ±SD (CV); *denotes significant result, $p<0.05$).

	Rest	Submaximal	Maximal	Rec45	Rec60
Lag time (min)	3.7±1.2 (33.2)	3.7±1.5 (40.2)	3.4±1.2 (36.0)	3.3±1.5 (45.9)	3.2±1.4 (43.4)
Time to peak (min)	8.4±5.2 (62.1)	7.7±3.4 (44.6)	6.7±2.5 (37.5)	6.7±2.9 (42.7)	6.1±2.6 (40.0)
Peak (Nm)	319.7±132.6 (41.5)	322.9±140 (43.5)	363.3 ±111.7 (30.8)	353.8±127.4 (36.0)	364.7±146.4 (40.1)
Endogenous Thrombin Potential (ETP, nM/min)	103.8±61.0 (58.7)	109.3±67.9 (62.1)	128.0±60.8 (47.5)	129.3±65.1 (50.4)	132.6±78.5 (59.2)
Area under the curve (AUC, nM)	2355.9±584.0 (24.8)	2214.4±401.6 (18.1)	2378.5±356.6 (15.0)	2440.2±407.9 (16.7)	2398.0±404.2 (16.9)

Table 3. Changes observed in thrombin generation parameters at rest, post exercise and during recovery. No significant changes were observed in thrombin generation parameters over the study duration. (Data ±SD (CV)).

		Lag time (min)	Time to peak (min)	Peak (Nm)	Endogenous Thrombin Potential (ETP, nM/min)	Area Under the Curve (AUC, nM)
FVIII:C (iu/L)	**Rest**	-0.285 (0.198)	-0.153 (0.497)	0.009 (0.967)	-0.000 (0.999)	0.043 (0.850)
	Submaximal	-0.328 (0.137)	-0.300 (0.174)	0.093 (0.679)	0.122 (0.587)	-0.070 (0.758)
	Maximal	-0.117 (0.605)	-0.080 (0.723)	-0.141 (0.533)	-0.097 (0.666)	-0.204 (0.363)
	Rec 45	-0.315 (0.154)	-0.285 (0.198)	0.282 (0.203)	0.251 (0.261)	0.020 (0.930)
	Rec60	-0.360 (0.143)	-0.391 (0.108	0.220 (0.381)	0.265 (0.287)	-0.066 (0.793)

Table 4. Correlation analyses between FVIII:C and thrombin generation parameters. No significant correlations were identified between FVIII:C and parameters of thrombin generation for any time point throughout the study. (Data presented as r value, (p value)).

Rheometric analysis (d_f)

Mean resting values of d_f obtained prior to exercise were 1.72 ± 0.05, consistent with values reported in our previous study of healthy individuals [19], increasing to 1.75 ± 0.05 following 5 minutes of low intensity, submaximal exercise (Figure 1a; p>0.05). In contrast to conventional markers, a significant increase in d_f was observed following maximal exercise compared to resting levels (1.79 ± 0.05 v 1.72 ± 0.05, p<0.05), which returned to basal levels within 1 hour of recovery. A number of patients had increased d_f values of up to 1.86 following maximal exercise. G`$_{GP}$ followed a similar pattern to d_f, significantly increasing following maximal exercise, but returning to resting levels within 1 hour post exercise (Figure 2a). Whilst T_{GP} appeared to increase in a non-significant manner following submaximal exercise, it shortened following maximal exercise, again returning to resting levels within 1 hour (Figure 2b).

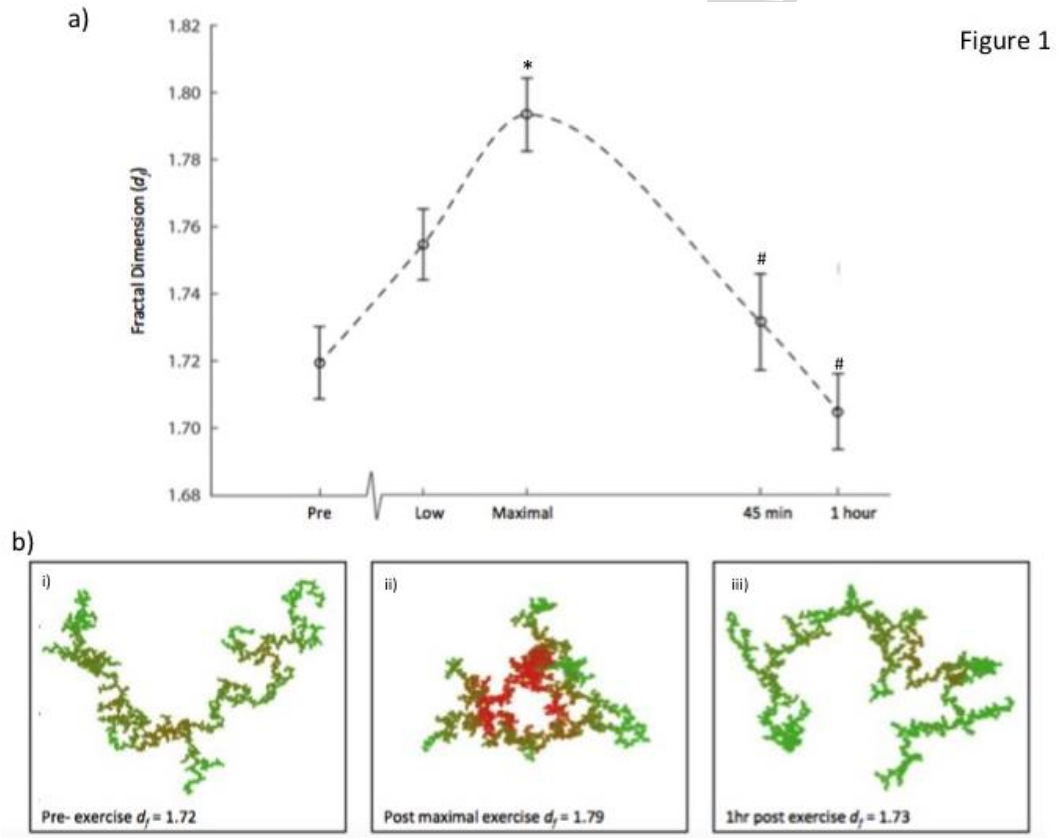

Figure 1. Graph illustrating the changes observed in fractal dimension (d_f) at rest, post exercise and during recovery. Significantly increased d_f (a) was observed following maximal exercise compared to at rest, returning to basal values by 60 minutes post exercise. (* Significantly different from Rest, p<0.05; † Significantly different from Low intensity exercise; p<0.05; # Significantly different from High Exercise Intensity, p<0.05).

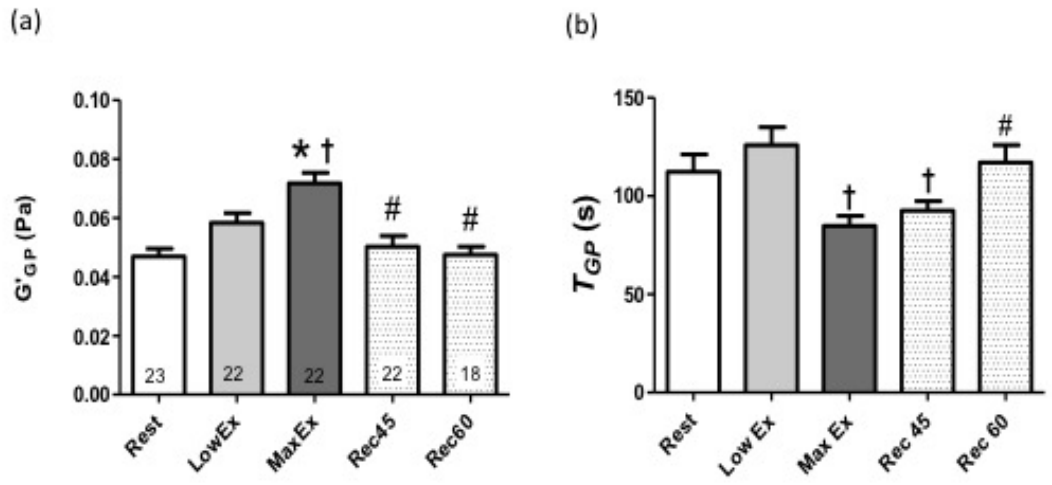

**Figure 2. Graph illustrating the changes observed in $G`_{GP}$ and $T_{GP}$ at rest, post exercise and during recovery.** Significantly increased $G`_{GP}$ observed following maximal exercise (a) compared to at rest, while a significant shortening of T_{GP} (b) was observed compared to following warm up exercise. (* Significantly different from Rest, $p<0.05$; † Significantly different from Low intensity exercise; $p<0.05$; # Significantly different from High Exercise Intensity, $p<0.05$).

Computational analysis of fibrin mass

As illustrated in Figure 1b (i), computational analysis of d_f values prior to exercise demonstrates a relatively open structure with few red nodal areas, indicating few areas of fibrin cross connectivity. Following submaximal exercise, fibrin mass associated with the clot increased by 150% compared to pre-exercise. In contrast to basal values, computational analysis of d_f values obtained following maximal exercise demonstrates highly dense clot structures with an increase in red nodal areas and an estimated increase in fibrin mass of up to 268%. In those with the highest d_f values observed in the study (1.86), the increase in polymerised mass was potentially estimated to be up to 590% (Figure 1b (ii)). This increase in red nodal areas indicates additional areas of fibrin cross connectivity, suggesting clots of highly dense, polymerised structures with increased mechanical strength. By 1 hour post exercise, d_f values had returned to pre-exercise levels, as mirrored by computational models at this time point (Figure 1b (iii)), demonstrating open, porous structures with fewer areas of red nodal areas.

Scanning electron microscopy of mature clot structure

Clots formed from blood from participants prior to exercise revealed open porous structures formed of fibres with an average width of 0.19 ± 0.09 μm (Figure 3a)**.** Following maximal exercise, SEM images

demonstrated clots with a highly branching fibrin network, formed of thinner fibres (0.09 ± 0.02 μm; Figure 3b) than those described previously. SEM images of clots formed from blood obtained up to 1 hour after exercise demonstrated structures to those formed before exercise, with fibrin fibres of similar widths (0.18 ± 0.08 μm; Figure 3c).

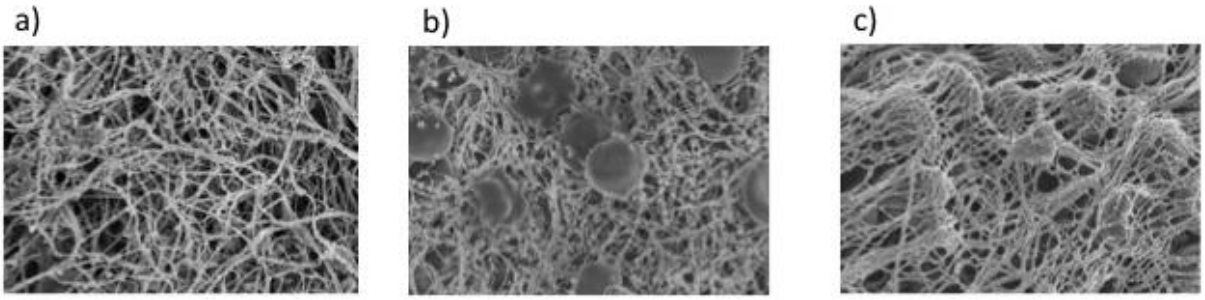

Figure 3: SEM micrographs of mature blood clots. Scanning electron micrographs of mature clots at a) rest, d_f=1.72, b) following maximal exercise, d_f=1.75 and c) following 60 minutes of passive recovery, d_f = 1.73, all at x5.00k magnification using a Hitachi Ultra-high resolution FE-SEM S-4800. Clots were allowed to form at 37°C then imaged at room temperature.

Discussion

Despite the well-established effects of exercise on conventional kinetic markers of haemostasis, it still remains unclear as to whether the hypercoagulable phase of exercise leads to initiation of clot formation with altered structure and increased polymerisation. This study is the first to demonstrate that haemorheology and fractal dimension (d_f) can assess the kinetic pathway changes and global effects of exercise-induced physiological changes on haemostasis with resultant clot formation following both submaximal and maximal exercise in a primarily male healthy population. As demonstrated by rheometric analysis, 5 minutes of submaximal exercise was sufficient to significantly increase d_f, which was further increased following maximal exercise. These changes were confirmed by increases in $G`_{GP}$, a measure of functional clot properties including viscoelastic strength, polymerisation and crosslinking, following both submaximal exercise and maximal exercise, suggesting that the resulting clot has increased viscoelastic strength following both exercise intensities. We have previously reported that d_f has accurately quantifies the effects of acute and chronic vascular inflammatory changes [21-23], sheer stress [29], temperature [25] and haemoconcentration/dilution [24] on clot structure, all of which are established to occur during high intensity exercise [1,2].

This study is consistent with findings of previous studies demonstrating that exercise leads to changes in kinetic markers of clot initiation and propagation, as indicated by a significant decrease in APTT [1,4,6-8], probably due to plasma volume shift [30,31]. As observed in previous studies [1-5], no change was observed in PT, suggesting that no vascular damage occurs as a result of the exercise performed. More importantly, an increase in FVIII immediately following maximal exercise, which remained for elevated for the study duration, consistent with previous studies and persisting inflammatory response as a result of increased exercise intensity. With the exception of FVIII, all standard markers of clot initiation and propagation returned to baseline levels within 1 hour of cessation of exercise. However it is not understood if these changes to kinetic and concentration based markers of haemostasis translate to clot formation and polymerisation following exercise. Interestingly, whilst previous studies have identified an increase in thrombin generation along with increased FVIII [32, 33], we did not observe any significant differences in thrombin generation or correlations between these parameters and FVIII:C in the current study. Additional studies on larger populations would be required in order to investigate the mechanism by which changes occur, which were outside the scope of the current proof of context study.

Fibrinolysis and its activity has also been investigated with respect to exercise, with apparent increases reported via an increase in the fibrin degradation product D-dimers and a decrease in an inhibitor of fibrinolysis, PAI-1 [5,12-18], findings also observed in the current study. It is unclear as to whether increases in fibrinolysis are due to exercise *per se* or as an indirect response to exercise-induced hypercoagulability, and subsequently increased clot formation. This study demonstrated that participation in exercise resulted in clot formation and subsequent breakdown, which were mirrored by increases in D-dimer, which may suggest an apparent increase in clot formation. However in healthy individuals, particularly male, such as those in the current study, efficient fibrinolysis maintains the haemostatic equilibrium, preventing inappropriate clot formation and balancing exercise-induced hypercoagulability.

We previously demonstrated d_f to be highly sensitive to changes in fibrinogen concentration, observed when blood was diluted by 20% [24], whilst conventional kinetic markers (PT and APTT) were only significantly changed when diluted by 40% [24]. Following participation in maximal exercise, a 15% decrease in plasma volume was observed, accompanied by an apparent 8% increase in fibrinogen concentration. Whilst d_f appeared to be sensitive to the effects of haemoconcentration and increased fibrinogen concentration immediately following participation in maximal exercise, we also observed an increase following 5 minutes of submaximal exercise, despite no changes observed in fibrinogen concentration at this time point. Previous studies have stated that the fibrinogen concentration is critical in maintaining normal clot function and strength [34-36], but in addition to our previous studies [23,24], we

believe this further reaffirms that structural organisation of fibrinogen is more important in determining the effects of coagulation change and resultant clot structure and function than fibrinogen concentration assays.

Computational models of d_f values obtained before, after and on recovery from maximal exercise also demonstrated changes in the structure and cross-linking of the incipient clot. Following submaximal exercise when d_f increased to 1.79, clots are comprised of highly dense areas with increased fibrin branching, leading to an increase of fibrin mass of up to 150%, further increasing in both cross-linking and fibrin mass to 590% post maximal exercise. SEM micrographs of blood clots further indicated changes in clot structure immediately following maximal exercise, which returned to baseline post recovery. Consistent with previous work [20,23], blood clots formed higher d_f values i.e. after maximal exercise were comprised of thinner fibrin fibres than those formed from blood obtained before, and 1 hour post exercise. These thinner fibres were arranged in a highly branching, highly branching network in contrast to the relatively open and porous structured formed from large fibrin fibres in lower values of d_f. These changes suggest an increase in clot density and fibrin mass as exercise intensity increased, generating clots with increased mechanical strength consistent with clot formation, compared to those produced at rest.

Given the benefits associated with exercise [37-39], its use is readily advocated as a recreational activity and in the rehabilitation of patients with vascular inflammatory disease, such as those who have experienced a myocardial infarction [40,41]. The current study provides new evidence that exercise induces transient hypercoagulability and quantifiable increases in clot structure which appear to be balanced by fibrinolysis in a primarily male population of healthy individuals. Increased coagulation potential and decreased fibrinolysis have been reported in those with vascular disease [42-44], indicating that this equilibrium may be displaced in these individuals, as a result of endothelial damage and plaque formation. Exercise-induced hypercoagulability may therefore lead to formation of pathological clots with highly dense structures, increasing the risk of thromboembolic events in these individuals. The threshold at which the equilibrium between the haemostatics pathways shift and the extent to which d_f is increased before increasing the risk of thromboembolic events is not yet known. It would therefore be beneficial to assess clot microstructure using the measures investigated in the current study in a population of patients with diagnosed vascular disorders or cardiac history who are participating in exercise rehabilitation programmes.

In conclusion, the current study provides new and important information on clot microstructure and mechanical strength in participants of a graded exercise programme in healthy individuals (primarily male)

with normal endothelium and haemostasis, demonstrating the ability of d_f to quantify these changes in a highly sensitive manner in which conventional haematological markers were unable to. Further studies will be required in order to investigate the role of d_f in quantifying exercise-induced changes to clot microstructure in a more heterogeneous population and patients with underlying vascular disease, further investigating its reproducibility and application in clinical care.

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Author Contributions

NAD: study design and analysis, blood sample collection, data collection (rheology and coagulation), drafting of the article, coagulation interpretation and statistical analysis; OL: blood sample collection, data collection (rheology), drafting of the article; JVB, CJM & DH: blood sample collection, data collection (physiological and coagulation); GRD: blood sample collection, data collection (rheology), revising the article for scientific and intellectual content; MJL: study design and analysis, interpretation of the data (rheology), revising the article for scientific and intellectual content; LD: Data collection and analysis (SEM Imaging); RHKM: study design and statistical analysis;; KH: revising the article for scientific and intellectual content, interpretation of the data (rheology); PRW: Study design and data analysis, revising the article for scientific and intellectual content (rheology); DMB: Collaboration, study design and revising the article for scientific and intellectual content (exercise physiology); PAE: Idea initiation, study design and data analysis, final approval of the version to be published. All authors read and approved the final manuscript.

Conflicts of interest

PRW and PAE currently hold a patent on a method of analysing coagulating blood; No other authors have anything to disclose.

Highlights

- Exercise induces a hypercoaguable phase, as measured by kinetic markers of coagulation and concentration based measures, but it is not known if exercise induces changes to clot formation and polymerization.

- Fibrinolysis has been observed to increase following exercise but it is not known if this is due to clot formation as a result of exercise induced hypercoagulability or exercise *per se*.

- Fractal dimension (d_f) is a technique previously demonstrated to quantify clot microstructure, shown to be associated with changes observed by scanning electron microscopy (SEM). Following intense exercise, d_f is significantly higher in individuals following maximal exercise, suggesting exercise-induced hypercoagulability produces clots with altered structure and polymerisation.

- Changes to coagulation as a result of exercise-induced hypercoagulability returned to resting levels within 1hour of rest following exercise, suggesting hypercoagulable changes are balanced by fibrinolysis in healthy individuals, maintaining the haemostatic equilibrium.

- Changes in d_f were associated with significantly increased fibrin mass and cross-linking, as demonstrated by computational modeling, and similar changes in SEM images of mature clot structures. This reaffirms the link between incipient and mature clot structure.