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The procoagulant effects of extracellular vesicles derived from hypoxic endothelial cells can be selectively inhibited by inorganic nitrite.

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Running title: Procoagulant effects of hypoxic endothelial cell-derived vesicles

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-- Essentials

- Endothelial-derived extracellular vesicles (EVs) are elevated in cardiovascular disease.
- EV produced following a hypoxic insult exhibit increased tissue factor and have reduced levels of tissue pathway factor inhibitor in comparison to normoxia-derived EVs.
- Endothelial-derived EVs produced under hypoxic conditions demonstrate increased pro-coagulant behaviour in in vitro assays including fibrin polymer formation following stimulation with thrombin, platelet activity and the clot microstructure.
- This pro-coagulant effect of hypoxia-derived EVs is abrogated by pre-treatment of endothelial cells in hypoxia with inorganic nitrite.

**Key words:** Extracellular vesicles, nitrite, coagulation, thrombosis, haemostasis
Abstract

**Background:** Extracellular vesicles (EVs) derived from endothelial cells are elevated in cardiovascular disease and promote inflammation and coagulation. Hypoxia is often a key feature and is itself a potent stimulator of increased EV production. Inorganic nitrite (NO$_2^-$) has beneficial and protective effects that are enhanced in hypoxia.

**Objectives:** Investigate the impact of hypoxia on the functional capacity of EV derived from endothelial cells under hypoxia, and assess whether pre-treatment of endothelial cells with NO$_2^-$ can alter EV function.

**Methods:** Differential ultracentrifugation was used to isolate EV from the cultured endothelial cell line HECV (CEV), and from primary human umbilical cord derived endothelial cells (PEV), with time-resolved fluorescence used to assess EV protein composition. Clot formation was induced by thrombin and calcium in two assays; using an Alexa Fluor 594 human fibrinogen conjugate assay and standard turbidometry. Platelet aggregation was determined using multiple electrode aggregometry. Scanning electron microscopy was used to visualise fibrin clots.

**Results:** Hypoxia exposure (1% O$_2$) significantly increased CEV production in comparison to normoxia (21% O$_2$) (1825±72 EVs/cell vs 117±9 EVs/cell, p<0.001, respectively) but had no effect on CEV mean size (221±6 nm vs 203±4 nm, p>0.05). Hypoxia-derived PEVs contained significantly more tissue factor than normoxia-derived EVs (Relative Fluorescence Units (RFU) =7666±1698 vs 5958±1644, p<0.001, respectively) and less tissue factor pathway inhibitor (RFU=9799±2353 vs 19723±2698, p<0.05). Hypoxia significantly increased CEV induced fibrin polymer formation compared to normoxia (% area =46.98±0.97 vs 36.36±0.72, p<0.05). Pre-treatment of endothelial cells with NO$_2^-$ in hypoxia abrogated this effect (% area=15.70±1.99, p<0.001). Hypoxia derived CEV non-significantly increased the maximum clot formed, shortened time to max clot, and increased time to clot lysis by turbidometry. ADP-mediated platelet aggregation was significantly elevated with PEV derived from hypoxia compared to normoxia (888.0±32.2 AU*min vs 671.5.2±28.3 AU*min, p<0.01). This was abrogated by pre-treatment of hypoxic endothelial cells with NO$_2^-$ (716.5±744.3 AU*min, p<0.001).

**Conclusions:** Hypoxia-derived PEVs and CEVs exhibit increased procoagulant activity compared to normoxia-derived EVs, which we confirm to be mediated by an imbalance of
TF/TFPI. Pre-treatment of endothelial cells with NO reduces the pro-coagulant activity of EVs via a mechanism that is Hypoxia-inducible factor 1 (HIF-1) dependent, but independent of TF/TFPI.
Abbreviations

Extracellular vesicles (EVs)
Primary Human Umbilical Vein derived Endothelial Cells (HUVEC)
Human Vascular Endothelial Cell Line (HECV)
Primary HUVEC EVs (PEVs)
Cultured HECV EVs (CEVs)
Hypoxia-inducible factor 1 (HIF-1)
Nitrate (NO$_3^-$)
Nitrite (NO$_2^-$)
Nitric oxide (NO)
Nanoparticle tracking analysis (NTA)
Sodium nitrite (NaNO$_2$)
Serum-free medium (SFM)
Time-resolved fluorescence (TRF)
Tissue factor (TF)
Tissue factor pathway inhibitor (TFPI)
Von Willebrand factor (vWF)
Introduction

Extracellular vesicles (EVs) are defined as membrane-bound, submicron particles released by multiple cell types [1]. Increased EV production has been shown in a plethora of disease states, where the bioactive cargo EVs harbour is typically reflective of the stimuli which triggered their release from the parent cell [2]. Elevated levels of EV have been found in cancer [3–5], neurodegenerative disorders [6–9], and cardiovascular disease [10–12]. Endothelial cell derived EVs possess surface markers specific to endothelial cells including CD144 and CD62E [13,14] and express tissue factor, suggesting a role in augmenting the coagulation cascade [15]. When oxygen supply is limited to a particular tissue region, such as in instances of acute hypoxia or ischaemia during trans-ischaemic attack, myocardial infarction, or stroke, the number of EVs released from endothelial cells increases significantly [16,17] which then re-enter the normal circulation. Under these conditions, increased stability of hypoxia inducible factor (HIF) 1-α when $O_2$ is below 2% is responsible for the transcription of various genes involved in angiogenesis and inflammation [18,19], and as shown by our group and others, takes a central role in inducing increased endothelial derived- EVs [19,20]. These EVs exhibit markedly altered RNA and protein composition, although their role in inducing a downstream pro-coagulative state remains uncertain [21].

Hypoxia exposure of endothelial cells can reduce the expression of thrombomodulin and tissue factor pathway inhibitor (TFPI) and increase plasminogen activator inhibitor-1 expression, resulting in accelerated thrombin activity and stability of fibrin clots [22-24]. There is also accumulating evidence that has shown EVs derived from hypoxic conditions have increased pro-coagulant properties, including elevated tissue factor (TF) activity, thrombin generation and promotion of fibrinogenesis [25-28].

Nitrite (NO$_2^-$) is capable of eliciting vasoprotective effects via its chemical reduction to nitric oxide (NO) or other NO$_x$ species, which is greatly augmented in hypoxia [29]. Endogenous NO$_2^-$ regulates hypoxic vasodilation via its reduction to NO, matching blood flow and $O_2$ supply to the metabolic demand under hypoxic conditions [30]. Exogenous NO$_2^-$ (applied via dietary nitrate supplement) reduces mean arterial blood pressure by ~5-10mmHg and can reduce patient derived platelet aggregation [31,32]. NO itself is known to modulate the cellular response to hypoxia by preventing the stabilization of HIF-1α via an increase in prolyl hydroxylase-mediated degradation [33]. Its role in modulating endothelial derived EV is less clear. Impaired endogenous NO production in primary endothelial cells (HUVECs) has
been shown to increase EV formation [34]. Furthermore, we have previously shown that NO\(^2\) derived NO is capable of reducing hypoxia-mediated EV production in endothelial cells in a HIF-1\(\alpha\) dependant manner [20]. Although the effect of NO\(^2\) on vascular homeostasis is already established [35-37], its influence on the pro-thrombotic function of hypoxia-derived EVs has not been evaluated.

The aim of this study was to firstly assess the coagulation capacity of endothelial-derived EVs generated under hypoxic conditions and identify the pathways involved, and secondly, investigate whether pre-treatment of endothelial cells with inorganic NO\(^2\) was able to modulate the pro-coagulant capacity of EV produced.
Methods

Cell Culture

The human vascular endothelial cell line (HECV) is a clone line previously purchased from Interlab (Milan, Italy) and routinely used in our laboratory. Cells were maintained in 90% Dulbecco’s Modified Eagle Medium (DMEM) (PAA Laboratories, UK) supplemented with 10% (v/v) foetal bovine serum (FBS) (PAA Laboratories, UK) and 1% streptomycin/penicillin (Invitrogen, UK). Cells were incubated at 37°C in 5% CO₂. At approximately 90% confluence cells underwent passage using Trypsin-EDTA (Invitrogen, UK) and exhibited a “cobblestone”-like appearance typical of endothelial cells. HECVs were exposed to hypoxia using an InVivo2 hypoxic workstation 400 (Baker Ruskinn, UK) and maintained at 1% O₂ for 24 hours. The oxygen concentration was monitored using an i-CO₂N₂ gas mixing system (Baker Ruskinn, UK). 1% O₂ was chosen specifically because it is equivalent to a pO₂ ~7.6mmHg and in vitro model of ischaemia, as we and others have utilised extensively [20]. Sodium nitrite (NaNO₂) was administered simultaneous to hypoxic exposure, or in normoxia as a control, to endothelial cells at a final concentration of 30µM, which is a supra-physiological dose based upon the previously established maximal inhibition effect of NO₂⁻ on EV production [20].

HUVECs were isolated directly from umbilical cords, as previously described, with minor modifications [38]. Human umbilical cords were obtained from the Delivery Suite, Cardiff and Vale University Health Board Trust. Ethical approval was obtained from a Research Ethics Committee (REC) (REC reference: 14/NW/1459). Umbilical cords were cut to approximately 15 cm, avoiding any damage from clamps used during birth. Cords were washed with 0.9% saline to remove excess blood and clots. The vein was located and washed through with saline until the solution ran clear. One end of the cord was clamped, and collagenase (1 mg/mL) was infused into the umbilical vein, before incubation at room temperature for 30 minutes. The collagenase solution was removed and placed into a centrifuge tube, gently squeezing the cord to ensure complete cell detachment. The resulting solution was then centrifuged at 300 x g for 5 minutes to pellet HUVECs. HUVECs were resuspended in complete M199 medium (Gibco, ThermoFisher Scientific, UK), before being plated onto cell culture plates or flasks pre-coated with 1% gelatin. Cells were left to adhere overnight, before medium was changed after 12-18 hours in order to remove erythrocyte contamination. HUVECs were counted manually using a
HUVECs were exposed to hypoxia using an InVivo2 hypoxic workstation and NaNO₂ administered as for HECV (above).

**EV isolation**

HUVECs and HECVs were grown to confluence and incubated under their specified conditions in serum-free media (SFM) for 24 hours prior to EV isolation. Cell-conditioned culture medium was then removed and subjected to a differential ultracentrifugation method, as described previously [39]. Briefly, cell media (typically 30mLs) was centrifuged at 300 x g for 5 minutes to remove any detached cells in suspension. This supernatant was taken and subjected to a second centrifugation at 15,000 x g for 15 minutes at 4°C to pellet any cellular debris or apoptotic bodies. This supernatant was then isolated and subjected to a third and final ultracentrifugation at 100,000 x g for 60 minutes at 4°C in order to pellet EVs (typically ~100µL). This pellet was then resuspended in 750µL 1x PBS, which had been filtered with a 0.22 µm filter (Millex®, Merck Millipore, Ireland).

**Nanoparticle Tracking Analysis**

The concentration of PEVs and CEVs was determined using nanoparticle tracking analysis (NTA, NanoSight LM10 system, UK) as we have described previously [40]. NTA is a laser illuminated microscopic technique equipped with a 642 nm laser and a high sensitivity digital camera system (OrcaFlash2.8, Hamamatsu, NanoSight Ltd) that determines the Brownian motion of individual nanoparticles in real-time to assess size and concentration. Sixty-second videos were recorded, and particle movement was analysed using NTA software (version 2.3). Shutter speed was fixed at 30.01 ms and camera gain to 500. Camera sensitivity and detection threshold were typically (11–14) and (4–6), respectively. PEV and CEV samples were diluted in EV-free sterile water (Fresenius Kabi, Runcorn, UK) and the EV concentration was normalised prior to application to in vitro functional assays.

**Characterisation of EVs**

Time-resolved fluorescence coupled with a modified ELISA technique was used to assess both the surface protein and content of the isolated PEVs derived from various conditions, as described previously [40]. PEVs were loaded onto a high protein binding 96-well plate (Greiner Bio-One, Germany) overnight at 4°C, before non-specific sites were blocked with 1% BSA (R&D Systems) for two hours. PEVs were permeabilised using a RIPA lysis buffer (Santa Cruz, CA, USA) to allow analysis of intra-vesicular proteins. PEVs were incubated
overnight with mouse anti-human antibodies for typical EV markers CD9, ALIX, and TSG101, and for typical endothelial markers CD144 (VE-Cadherin), Von Willebrand factor (vWF), tissue factor (TF), and TFPI, (all Abcam, Cambridge, UK) overnight at 4°C. Markers were detected using a biotinylated anti-mouse IgG secondary antibody (PerkinElmer, Buckinghamshire, UK) and a streptavidin:europium conjugate (PerkinElmer, Buckinghamshire, UK) and measured by time-resolved fluorescence (delay time: 400 µs, measurement window: 400 µs) using a BMG Labtech CLARIOstar.

Fluorescent Fibrin Polymer Formation

The Alexa Fluor 594 human fibrinogen conjugate (Thermo Fisher Scientific, USA), with absorption and emission maxima of 592 nm and 618 nm respectively, was used to visualise the formation of fibrin-based polymers in real time, adapted from a method previously described [41] and reviewed [42,43]. Thawed human plasma was introduced to fluorescent fibrinogen at a final concentration of 5% and endothelial CEVs at a concentration of 1x10^9 EVs/mL in 100µL consecutively. Simultaneously, a Hanks Based Salt-Solution (HBSS) containing 5U/mL thrombin (Merck, USA) and 200mM calcium chloride (CaCl\textsubscript{2}) (Beckman Coulter, UK) was added to the plasma containing CEVs and fluorescent fibrinogen, for a final concentration of 0.5U/mL thrombin and 20mM CaCl\textsubscript{2} in 100µL. The introduction of HBSS to plasma represented time point 0. A high concentration of thrombin catalyses the conversion of fibrinogen to fibrin. 20µL of this mixture was placed on a glass microscope slide and covered with a coverslip. Fibrin polymer formation was monitored over time at 100x magnification on a Nikon-80i microscope (Nikon, Japan) and captured using Volocity-64x software (a typical time-course is shown in supplementary material – Figure 1). Maximal clot formation was reflected from images captured and measured at 15 minutes, where beyond 15 minutes, only a slight increase in fluorescence occurs [41]. Controls were lacking the addition of CEVs. Results were analysed on Adobe Photoshop (Adobe Inc., California, USA) from 16 individual points of interest on each single repetition, where ‘fibrin percentage area’ represents a pixel-by-pixel coverage of the image by fibrin polymers versus total pixels, where 14.42 pixels represents 1µm. The number of fibrin polymers intersecting at a junction was determined by the number of strands per junction and is a measure of fibrin clot formation.

Turbidometry
Turbidometry was employed to analyse clot formation and lysis. The assay was originally developed by Professor Ajjan’s group at Leeds University Hospital [44,45] and modified as follows. EVs from cells subjected to normoxic and hypoxic conditions (+/- nitrite), respectively, were added to human plasma (a pooled standard that had originated from healthy control subjects) at a concentration of $1 \times 10^9$ EVs/mL in 100µL before the addition of thrombin and calcium at a final concentration of 0.03U/mL thrombin, 7.5mM CaCl$_2$, and 83.2ng/mL tissue plasminogen activator (tPA) in a total assay volume of 150µL. Samples were prepared in triplicate. Absorbance readings at 340 nm were recorded using a CLARIOstar Plus 96 well plate reader (BMG Labtech, England) at 37°C for up to 60 min. From the absorbance profile over time, the maximal clot formed was measured from the peak absorbance, the rate of clot formation calculated from baseline to peak absorbance over time, rate of clot lysis calculated from the peak to baseline return over time, and time to 50% lysis. These were compared to a paired control consisting of plasma with no EVs added.

Figure 1. Typical coagulation trace using thrombin as the activator and tPA as the lysis initiator in platelet free plasma without the addition of EVs. A = the delay in clot formation (seconds). B = the maximum optical density (au), the maximum strength of the clot. C = clot formation, from which the time taken for clot formation to reach its maximum point can be calculated (minutes). D = Clot lysis, the rate of clot lysis can be calculated (minutes). The graph demonstrates clot formation and lysis is complete within 1 hour.

Platelet aggregation
Whole blood (300µL) of healthy volunteers collected into a hirudin BD Vacutainer® was diluted 1:1 with EVs in PBS (final concentration: 2x10⁸/mL) and preheated to 37°C in a single use test cell for 3 minutes for Multiplate® platelet aggregometry. Samples were continuously homogenised using a Teflon coated stirring bar at 1000 rpm. Platelet activation was then initiated by the addition of either ADP (20µL, final concentration 6.5µM) or TRAP (Thrombin receptor activating peptide, 20µL, final concentration 32µM) (Roche Diagnostics Ltd, Switzerland). An example of ADP and TRAP aggregation curves is shown in Figure 2.

An increase in electrical impedance was recorded for 6 minutes, with timing based on Multiplate® test parameters that are required to assess velocity of aggregation, maximum aggregation, and area under the curve (AUC), and expressed as arbitrary aggregation units.

<table>
<thead>
<tr>
<th>Area under the curve</th>
<th>Area under the curve</th>
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<tbody>
<tr>
<td>705 AU•min. (569 - 1130)</td>
<td>1121 AU•min. (836 - 1280)</td>
</tr>
<tr>
<td>Aggregation:</td>
<td>Aggregation:</td>
</tr>
<tr>
<td>FU0: 130.3 AU</td>
<td>RUC: 195.6 AU</td>
</tr>
<tr>
<td>Velocity:</td>
<td>Velocity:</td>
</tr>
<tr>
<td>FU0: 16.6 AU/min.</td>
<td>RUC: 27.3 AU/min.</td>
</tr>
<tr>
<td>CC=0.399, DIF=1.063%</td>
<td>CC=0.398, DIF=2.876%</td>
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</table>

**Figure 2. Representative aggregation curves for ADP and TRAP.** Whole blood was incubated, before platelets were stimulated with either ADP (left) or TRAP (right). Aggregation units calculated as area under the curve after 6 minutes (impedance:time).

**Fractal Dimension and Clot Formation Time**

The haemorheological gel point technique has been previously described [46,47]. Briefly, blood was loaded into a double concentric measuring geometry on a controlled stress rheometer, AR-G2 (TA Instruments, New Castle, DE, USA). Small amplitude oscillatory
shear measurements were performed at test frequencies (2 Hz, 0.93 Hz, 0.43 Hz and 0.2 Hz) with a peak stress amplitude of 0.03 Pa. This allowed the phase angle (δ) (the difference between the applied stress and measured strain) to be determined. The method detects the Gel Point (GP) by measuring the difference in δ at the varying frequencies with respect to time. GP represents the formation of the incipient blood clot; the first point at which the fluid transitions to a solid. GP allows quantification of how the fibrin clot is organised using fractal analysis, to determine the clot’s fractal dimension ($d_f$), which was normalised to the PBS control. In addition, measurement of the GP provides a measure of clot formation time ($T_{GP}$) which is the time taken to form the clot.

Blood from healthy volunteers was taken into 3.2% sodium citrate vacutainers®, aliquoted into 7mL vials and incubated at 37°C with PEVs (final concentration $2 \times 10^8$/mL) for 15 minutes. Citrated blood samples were then re-calcified by adding 334µL 0.2M calcium chloride, before being immediately loaded into the rheometer. The process from recalcifying the blood to initialization of the measurement was performed in less than 60 seconds. Samples were allowed to run for sufficient time to reach the GP, never exceeding more than 10 minutes.

**Thrombin generation assay**

Thrombin activity was assessed using the Thrombin activity assay kit (Abcam, Cambridge, United Kingdom). The assay was undertaken as per the manufacturer’s instructions. Briefly, a set of thrombin standards was prepared, before plasma isolated from the blood of healthy volunteers was diluted and added to the microplate. PEVs were added to plasma samples (final concentration $2 \times 10^8$/mL) and incubated for 1 hour at 37°C, before a reaction mix (containing the fluorogenic thrombin substrate and buffer) was added to both plasma and standard wells. Fluorescence was measured at Ex/Em = 350/450 nm every 3 minutes for 60 minutes at 37°C. Following completion of this incubation, two time points were chosen within the linear portion of the time course to calculate the change in fluorescence and thus Thrombin activity. This change in fluorescence was then compared to a standard curve in order to calculate ng of Thrombin, allowing thrombin activity to be expressed as ng/mL.

**Scanning electron microscopy**

Scanning electron microscopy was used to visualise fibrin clots and the influence of PEVs in whole blood. Fibrin clots were formed at 37°C for 2 hours (due to correlations between
gelation time and final clot structure) by adding CaCl₂ (20mM). Clots were washed 3 times
with sodium cacodylate buffer (0.2M), before being fixed in 2% glutaraldehyde in PBS (v/v)
for 1 hour at room temperature. Clots were subjected to dehydration through graded ethanol
(30-100%), before being fixed using hexamethyldisiazane. Finally, the sample was splutter
coated with gold, and imaged using a Hitachi Ultra-high resolution FE-SEM S-4800.

**Statistics**

Data were analysed using GraphPad Prism (version 5.0; GraphPad Software Inc., San Diego, USA). D'Agostino's K-squared test was used to check data for normality. Multiple
comparison of means were undertaken using one-way ANOVA followed by a Tukey’s
multiple pairwise comparisons test. Results are expressed as mean ± standard error (SE)
unless stated. A p-value of < 0.05 was regarded as statistically significant throughout. All
experimental runs are paired with relevant controls. The authors acknowledge that as a
consequence this can lead to results that can engender substantial variability within the
methodology and the controls, however, this also ensures experimental repeats are considered
in the context of negative and positive controls.
Results

Effect of hypoxia on EV concentration, size, and size distribution

As Table 1 indicates, hypoxia exposure (1% O₂ – EV\textsubscript{hypo}) significantly increased CEV production in comparison to CEVs in normoxia (21% O₂ - EV\textsuperscript{norm}) (EV\textsubscript{hypo}: 1825 ± 72 EVs/cell vs EV\textsuperscript{norm}: 117 ± 9 EVs/cell, \(p < 0.001\), respectively). CEV production was significantly reduced following the introduction of nitrite to cells prior to incubation in hypoxia (EV\textsubscript{hypo+N}: 842 ± 91, \(p < 0.001\)). However, neither hypoxic conditions, nor the introduction of nitrite, affected EV mean size (EV\textsubscript{hypo}: 221 ± 6 nm, EV\textsuperscript{norm}: 203 ± 4 nm, EV\textsubscript{hypo+N}: 211 ± 7 nm, EV\textsuperscript{norm+N}: 206 ± 3, \(p > 0.05\)).

CEV size distribution was assessed (according to 50 nm bin size), showing EV\textsubscript{hypo} exhibited an elevated concentration of CEVs at bin size ranges 101-300 nm, and 351-400 nm. The introduction of nitrite to cells incubated in hypoxia reduced the concentration of CEVs versus cells exposed to hypoxia alone at bin size ranges 151-200 nm, 251-300 nm, and 351-400 nm respectively. See Figure 3 for the depiction of CEV size distribution.

<table>
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<th>Normoxia</th>
<th>Hypoxia</th>
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<tr>
<td></td>
<td>Without NO\textsubscript{2}</td>
<td>With NO\textsubscript{2}</td>
</tr>
<tr>
<td>CEV concentration (EVs/cell)</td>
<td>117 ± 9</td>
<td>123 ± 11</td>
</tr>
<tr>
<td>CEV size (nm)</td>
<td>203 ± 4</td>
<td>206 ± 3</td>
</tr>
</tbody>
</table>

Table 1. The effect of hypoxia on CEV concentration and size. CEVs were measured using nanoparticle tracking analysis. ** represents \(p < 0.001\) compared to normoxia. ††† represents \(p < 0.001\) compared to hypoxia without NO\textsubscript{2}. Results reflect N=5.
**Figure 3.** The effect of hypoxia and nitrite on CEV size distribution. CEVs were measured using nanoparticle tracking analysis. Assessed in 50 nm bin sizes, results reflect N=5. EV<sub>norm</sub> = normoxic EV, EV<sub>norm+N</sub> = normoxic EV + nitrite, EV<sub>hypo</sub> = hypoxic EV, EV<sub>hypo+N</sub> = hypoxic EV + nitrite. ***, ** and * reflect p < 0.001, 0.01 and 0.05, respectively.
Characterisation of EVs

HECV are a cell line and as such do not express all the characteristics of primary endothelial cells. We therefore chose to assess the influence of hypoxia and nitrite using primary HUVEC. Like HECVs, hypoxia significantly increases the PEV yield from HUVEC (PEVs/Cell = 1227 ± 50 vs 361 ± 20, \( p < 0.001 \)). Table 2 illustrates that TFPI expression was significantly reduced in \( \text{EV}^{\text{hypo}} \) compared to \( \text{EV}^{\text{norm}} \) (RFU = 9799 ± 2353 vs 19723 ± 2698, \( p < 0.05 \), respectively). The simultaneous addition of \( \text{NO}_2^- \) to hypoxia-treated HUVECs had no impact on the TFPI expression of the PEVs produced (\( \text{EV}^{\text{hypo+N}} \)) (RFU = 9799 ± 2353). TF expression was significantly elevated in \( \text{EV}^{\text{hypo}} \) and \( \text{EV}^{\text{hypo+N}} \) compared to pre-treatment of HUVECs incubated in normoxia with \( \text{NO}_2^- \) (\( \text{EV}^{\text{norm+N}} \)) (\( \text{EV}^{\text{hypo}} \): RFU = 7666 ± 1698, \( \text{EV}^{\text{hypo+N}} \): 9505 ± 1260 vs \( \text{EV}^{\text{norm+N}} \): 4239 ± 605, \( p < 0.001 \), respectively). \( \text{EV}^{\text{hypo+N}} \) also expressed significantly higher TF than \( \text{EV}^{\text{norm}} \) (9505 ± 1260 vs 5958 ± 1644, \( p < 0.001 \)). \( \text{EV}^{\text{norm+N}} \) also had no effect on the expression of PEV vWF, TF or TFPI. \( \text{EV}^{\text{norm}} \) and \( \text{EV}^{\text{hypo}} \) exhibited similar levels of the endothelial marker CD144 and the EV associated markers CD9, ALIX, and TSG101 (see Supplementary Data – Figure 2).

<table>
<thead>
<tr>
<th></th>
<th>( \text{EV}^{\text{norm}} )</th>
<th>( \text{EV}^{\text{hypo}} )</th>
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<tbody>
<tr>
<td></td>
<td>Without ( \text{NO}_2^- )</td>
<td>With ( \text{NO}_2^- )</td>
</tr>
<tr>
<td>vWF</td>
<td>11389 ± 1765</td>
<td>12534 ± 3688</td>
</tr>
<tr>
<td>TF</td>
<td>5958 ± 1644</td>
<td>4239 ± 605</td>
</tr>
<tr>
<td>TFPI</td>
<td>19723 ± 2698</td>
<td>19323 ± 2053</td>
</tr>
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</table>

Table 2. Level of coagulation proteins expressed on PEVs. Results are relative fluorescent units (RFU). * represents \( p < 0.001 \) compared to normoxia with \( \text{NO}_2^- \). † represents \( p < 0.001 \) compared to normoxia without \( \text{NO}_2^- \). * represents \( p < 0.05 \) compared to normoxia without \( \text{NO}_2^- \). † represents \( p < 0.05 \) compared to normoxia with \( \text{NO}_2^- \). Results reflect N=4.
Fluorescent fibrin polymer formation

As displayed by Figures 4A and 4C, fibrin polymer formation was significantly increased following the addition of EV\textsuperscript{norm} to plasma in comparison to control (plasma alone - no CEVs added) (% area = 36.36 ± 0.72 vs. 16.66 ± 1.17, \( p < 0.001 \), respectively). Similarly, the addition of EV\textsuperscript{hypo} significantly increased the fibrin % area compared to control (% area = 46.98 ± 0.97, \( p < 0.001 \)). The influence of EV\textsuperscript{hypo} was significantly greater than EV\textsuperscript{norm} (\( p < 0.01 \)). Given TFPI regulates the action of the extrinsic coagulation cascade and EV\textsuperscript{hypo} expressed more TF and less TFPI than EV\textsuperscript{Norm}, we investigated if the addition of exogenous TFPI restored fibrin polymerization to the level observed with addition of EV\textsuperscript{norm}. The increased fibrin % area seen following the addition of EV\textsuperscript{hypo} was fully attenuated following the addition of exogenous TFPI in combination with hypoxia-derived EVs (EV\textsuperscript{hypo+TFPI}) (19.91 ± 4.91, \( p < 0.001 \)). The addition of TFPI in combination with normoxia-derived EVs had no effect on the fibrin % area (% area = 26.60 ± 0.59, \( p > 0.05 \)). Following the addition of EV\textsuperscript{hypo+N} to plasma, fibrin % area was significantly reduced in comparison to EV\textsuperscript{hypo} (% area = 15.70 ± 1.99 vs 35.97 ± 3.28, respectively, \( p < 0.001 \)). EV\textsuperscript{hypo+N} returned levels to baseline, comparable to EV\textsuperscript{norm}. There was no significant difference between EV\textsuperscript{norm+N} and EV\textsuperscript{norm} (% area = 19.06 ± 2.34 vs 18.59 ± 2.02, respectively, \( p > 0.05 \)). Supplementary Data - Figure 3 illustrates typical formation of fibrin polymers with the addition of normoxia- and hypoxia-derived EVs in the presence and absence of nitrite.

Figures 4B and 4D compare the number of fibrin strands at each junction. This is a measure of the mechanical strength and hence its resistance to breakdown [48]. The addition of EV\textsuperscript{norm} to plasma significantly reduced strands per junction compared to control plasma (\( spj = 2.93 ± 0.05 \) vs 3.18 ± 0.02, respectively, \( p < 0.05 \)). Following the addition of EV\textsuperscript{norm+N}, strands per junction were reduced further compared to EV\textsuperscript{norm} alone (\( spj = 2.71 ± 0.03 \) vs 2.93 ± 0.05, respectively, \( p < 0.001 \)). This effect was mirrored by the addition of EV\textsuperscript{hypo+N} compared to EV\textsuperscript{hypo} (\( spj = 2.79 ± 0.04 \) vs 3.24 ± 0.04, respectively, \( p < 0.001 \)).
Figure 4. The effect of nitrite and/or TFPI treated CEVs on the contribution to clot formation. CEVs were incubated with platelet poor plasma before clot formation was initiated with a combination of calcium and thrombin. Images were then captured after 15 minutes. A. % Area (EV with NO$_2^-$). B. Strands Per Junction (EV with NO$_2^-$). C. % Area (EV with TFPI). D. Strands Per Junction (EV with TFPI). EV$^{norm}$ = normoxic EV, EV$^{norm+N}$ = normoxic EV + nitrite, EV$^{hypo}$ = hypoxic EV, EV$^{hypo+N}$ = hypoxic EV + nitrite. Control – Filtered PBS. EVs at concentration 1 x 10$^9$ EVs. Results represent [N=6]. ***, ** and * reflect p < 0.001, 0.01 and 0.05, respectively.
Turbidometry

The maximum optical density (MaxOD), reflecting total clot formed, was greater following addition of CEVs produced under hypoxia compared to normoxia, however this did not reach statistical significance (EV\text{norm}: 0.437 \pm 0.009 AU, EV\text{norm+N}: 0.446 \pm 0.015 AU, EV\text{hypo}: 0.472 \pm 0.007 AU, EV\text{hypo+N}: 0.459 \pm 0.022 AU, Control: 0.434 \pm 0.004 AU, respectively, \( p > 0.05 \)) (Figure 5A).

The time taken for optical density to reach 50% of its MaxOD reflects the rate of clot formation. No significant differences were observed between CEV samples (EV\text{norm}: 6.66 \pm 0.338 mins, EV\text{norm+N}: 6.628 \pm 0.417 mins, EV\text{hypo}: 6.086 \pm 0.482 mins, EV\text{hypo+N}: 5.978 \pm 0.298 mins, Control: 6.89 \pm 0.227 mins, respectively, \( p > 0.05 \)) (Figure 5B). The time taken for the optical density to return to 50% of its MaxOD reflects lysis and is a surrogate for the strength of the clot. In the presence of EV\text{hypo} the time to 50% lysis was greater than EV\text{norm} however this did not reach statistical significance (EV\text{norm}: 8.84 \pm 0.471 mins, EV\text{norm+N}: 8.4 \pm 0.4 mins, EV\text{hypo}: 11.4 \pm 1.421 mins, EV\text{hypo+N}: 10.12 \pm 0.913 mins, Control: 8 \pm 0.544 mins, \( p > 0.05 \)) (Figure 5C). Similarly, the rate of lysis in the presence of EVs +/- nitrite did not show significant differences (EV\text{norm}: -0.030 \pm 0.003 AU/min, EV\text{norm+N}: -0.034 \pm 0.004 AU/min, EV\text{hypo}: -0.022 \pm 0.004 AU/min, EV\text{hypo+N}: -0.028 \pm 0.003 AU/min, Control: -0.033 \pm 0.003 AU/min, \( p > 0.05 \)) (Figure 5D).
Figure 5. The effect of CEVs on thrombin and calcium induced turbidometry. CEVs were incubated with platelet poor plasma before clot formation was initiated with a combination of calcium and thrombin. A. MaxOD B. Time to 50% clotting C. Time to 50% lysis D. Average rate of lysis. EVnorm = normoxic EV, EVnorm+N = normoxic EV + nitrite, EVhypo = hypoxic EV, EVhypo+N = hypoxic EV + nitrite. Control – Filtered PBS. Results reflect [N=5].
Platelet activity

Platelet aggregation in response to ADP in the presence of PEV was significantly increased following whole blood incubation with EV\textsuperscript{hypo} compared to control (888.0 ± 32.2 AU*min vs 647.2 ± 38.1 AU*min respectively, $p < 0.01$) (Figure 6A). EV\textsuperscript{hypo} also demonstrated an increase in platelet activity following stimulation by ADP, in comparison to EV\textsuperscript{norm} (671.5 ± 28.3 AU*min, $p < 0.01$) and EV\textsuperscript{norm+N} (616.0 ± 44.9 AU*min, $p < 0.001$). EV\textsuperscript{hypo+N} significantly reduced platelet aggregation in response to ADP compared to EV\textsuperscript{hypo} to levels similar to EV\textsuperscript{norm} (716.5 ± 44.3 AU*min vs 888.0 ± 32.2 AU*min respectively, $p < 0.001$). EV\textsuperscript{norm} and EV\textsuperscript{norm+N} had no effect on platelet activity stimulated via ADP in comparison to control (EV\textsuperscript{norm}: 671.5 ± 28.3 AU*min, EV\textsuperscript{norm+N}: 616.0 ± 44.9 AU*min, control: 647.2 ± 38.1 AU*min, $p > 0.05$).

Following stimulation with TRAP in the presence of PEV, EV\textsuperscript{hypo} increased platelet aggregation in comparison to EV\textsuperscript{norm} (922.2 ± 30.1 AU*min vs 641.2 ± 25.2 AU*min, $p < 0.05$) (Figure 6B). EV\textsuperscript{hypo+N} significantly reduced platelet aggregation compared to EV\textsuperscript{hypo} (650.3 ± 45.4 AU*min vs 922.2 ± 30.1 AU*min respectively, $p < 0.05$). EV\textsuperscript{norm+N} had no effect on platelet aggregation compared to EV\textsuperscript{norm} (612.3 ± 115.2 AU*min vs 641.2 ± 25.2 AU*min respectively, $p > 0.05$).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{The effect of PEVs on ADP- and TRAP- mediated platelet aggregation. PEVs were incubated with whole blood, before platelets were stimulated with either ADP (A) or TRAP (B). Aggregation units calculated as area under the curve after 6 minutes (impedance:time). EV\textsuperscript{norm} = normoxic EV, EV\textsuperscript{norm+N} = normoxic EV + nitrite, EV\textsuperscript{hypo} = hypoxic EV, EV\textsuperscript{hypo+N} = hypoxic EV + nitrite. Control – Filtered PBS. Results represent [N=6]. ***, ** and * reflect $p < 0.001$ and 0.01 and 0.05, respectively.}
\end{figure}
Fractal Dimension and Clot Formation Time

To corroborate the findings of the plasma fibrinogen assay in a whole blood assay, fractal dimension ($d_f$) and clot formation time ($T_{GP}$) were measured in the presence and absence of PEV produced under various conditions. $d_f$ was significantly elevated following the addition of EV$^{\text{hypo}}$ to whole blood, in comparison to control ($d_f = 1.03 \pm 0.0067$ vs $1.00 \pm 0.00$, respectively, $p < 0.001$) (Figure 7A). EV$^{\text{hypo}}$ also significantly increased $d_f$ in whole blood in comparison to EV$^{\text{norm}}$ and EV$^{\text{norm}+N}$ ($d_f =$ EV$^{\text{hypo}}$: $1.030 \pm 0.0067$, EV$^{\text{norm}}$: $1.012 \pm 0.0029$, EV$^{\text{norm}+N}$: $1.007 \pm 0.0027$, $p < 0.05$ and $p < 0.01$ respectively). Interestingly, EV$^{\text{hypo}+N}$ significantly reduced $d_f$ in comparison to EV$^{\text{hypo}}$ alone, to similar levels seen with EV$^{\text{norm}}$ ($d_f = 1.005 \pm 0.0017$ vs $1.012 \pm 0.0029$ respectively, $p < 0.001$). $d_f$ was not significantly different between EV$^{\text{norm}}$ and control ($d_f = 1.012 \pm 0.0030$ vs $1.00 \pm 0.00$ respectively, $p > 0.05$).

$T_{GP}$ was significantly reduced by EV$^{\text{norm}}$ in comparison to control ($T_{GP} = 221.4 \pm 19.71$ seconds vs $273.4 \pm 12.31$ seconds respectively, $p < 0.05$) (Figure 7B). EV$^{\text{norm}+N}$ increased $T_{GP}$ in comparison to EV$^{\text{norm}}$ ($T_{GP} = 278.6 \pm 7.78$ secs vs $221.4 \pm 19.71$ secs respectively, $p < 0.05$). EV$^{\text{hypo}}$ significantly reduced $T_{GP}$ in comparison to control ($T_{GP} = 181.6 \pm 8.98$ seconds vs $273.4 \pm 12.31$ seconds respectively, $p < 0.05$). Interestingly, EV$^{\text{hypo}}$ did not significantly alter $T_{GP}$ in comparison to EV$^{\text{norm}}$ ($T_{GP} = 181.6 \pm 8.98$ seconds vs $221.4 \pm 19.71$ seconds respectively, $p > 0.05$). EV$^{\text{hypo}+N}$ appeared to reverse the effect of EV$^{\text{hypo}}$ on $T_{GP}$, with EV$^{\text{hypo}+N}$ restoring $T_{GP}$ to a similar level as the control ($T_{GP} = \text{EV}^{\text{hypo}+N}$: $250.8 \pm 4.2$ seconds vs EV$^{\text{hypo}}$: $181.6 \pm 8.99$ seconds, $p < 0.01$).
Figure 7. The effect of hypoxia and nitrite treated PEVs on fractal dimension ($d_f$) and clot formation time ($T_{GP}$). A. EVs were added to whole blood and incubated for 15 minutes form, in seconds. Results are normalised to an EV-free PBS control, and represent mean ± standard error. $EV^{\text{norm}} = \text{normoxic EV}$, $EV^{\text{norm}+N} = \text{normoxic EV + nitrite}$, $EV^{\text{hypo}} = \text{hypoxic EV}$, $EV^{\text{hypo}+N} = \text{hypoxic EV + nitrite}$. Control – EV-free filtered PBS. B. $T_{GP}$ represents the time taken for a blood clot to EVs. $d_f$ results are normalised to the EV-free control. Results represent [N=5]. ***, ** and * reflect $p < 0.001$, $0.01$ and $0.05$, respectively.
**Thrombin activity**

Thrombin activity was measured in the plasma of healthy volunteers and was not affected by the presence of EV\textsuperscript{norm} in comparison to control (128.9 ± 4.2 ng/mL vs 114.6 ± 15.6 ng/mL respectively, \( p > 0.05 \)) (Figure 8). EV\textsuperscript{norm+N} did not alter thrombin activity compared to EV\textsuperscript{norm} (EV\textsuperscript{norm+N}: 131.6 ± 9.5 ng/mL vs EV\textsuperscript{norm}: 128.9 ± 4.2 ng/mL, \( p > 0.05 \)). EV\textsuperscript{hypo} significantly increased thrombin activity in comparison to both EV\textsuperscript{norm} and EV\textsuperscript{norm+N} (EV\textsuperscript{hypo}: 187.1 ± 14.8 ng/mL, EV\textsuperscript{norm}: 128.9 ± 4.2 ng/mL, EV\textsuperscript{norm+N}: 131.6 ± 9.5 ng/mL, \( p < 0.05 \)). EV\textsuperscript{hypo+N} did not significantly reduce thrombin activity in comparison to EV\textsuperscript{hypo} (190.7 ± 18.1 ng/mL vs 187.1 ± 14.8 ng/mL, respectively). EV\textsuperscript{hypo+N} significantly increased thrombin activity in comparison to EV\textsuperscript{norm} and EV\textsuperscript{norm+N} (EV\textsuperscript{hypo+N}: 190.7 ± 18.1 ng/mL, EV\textsuperscript{norm}: 128.9 ± 4.2 ng/mL, EV\textsuperscript{norm+N}: 131.6 ± 9.5 ng/mL, \( p < 0.05 \)).

**Figure 8. The effect of PEVs on thrombin activity.** A. Thrombin generation was measured in the plasma of healthy volunteers incubated with EVs over the course of 60 minutes. B. Thrombin activity was then calculated, using a standard curve of known thrombin concentration. EV\textsuperscript{norm} = normoxic EV, EV\textsuperscript{norm+N} = normoxic EV + nitrite, EV\textsuperscript{hypo} = hypoxic EV, EV\textsuperscript{hypo+N} = hypoxic EV + nitrite. Control – Filtered PBS. Results reflect \([N=8]\) ** and * reflect \( p < 0.05 \) and 0.01, respectively.
Electron microscopy

Scanning electron microscopy highlighted structural differences in fibrin clots produced following whole blood incubation with PEV isolated from various conditions (Figure 9). The addition of PEV appeared to lead to direct incorporation into the fibrin clot, with multiple sub-micron particles visibly attached to the fibres, largely absent in the control (no EV added). Qualitatively, clots formed in the presence of EV\(^{\text{hypo}}\) and EV\(^{\text{hypo+N}}\) appear denser and more compact. EV\(^{\text{norm+N}}\) appeared to reduce the density of the clot in comparison to EV\(^{\text{norm}}\), a finding consistent with the quantitative fibrin clot formation assay and fractal dimension results.

**Figure 9. Typical scanning electron micrographs of fibrin clots.** PEV appear to be incorporated within the fibrin network. Control – Filtered PBS added to whole blood. The magnification bar is 5 µm for all images, shown in 500 nm increments.
Discussion

This study demonstrates that endothelial cell-derived EV production is markedly increased under hypoxic conditions, and these EV exhibit pro-coagulant characteristics and function under normal conditions in comparison to endothelial EVs derived from normoxia. The mechanism appears to be mediated by an imbalance in TF/TFPI content of hypoxia-derived EVs. Pre-incubation of hypoxic HUVECs with inorganic NO$_2^-$ abrogated the ability of the PEVs to exert their pro-coagulant effect measured in three separate model systems of clot formation.

The mechanical properties of blood clots are essential for homeostasis and the prevention of blood loss. However, clots composed of compact fibrin strands are more resistant to lysis, and can predispose individuals to arterial thrombotic events [48–52]. Indeed, alterations in clot structure have been implicated in various thrombotic diseases, including ischemic stroke [48,51], heart failure [52] and coronary artery disease [53,54]. The structural composition of the clot defines its fibrinolytic properties, with a compact, tight fibrin network reducing the penetration of fibrinolytic components. Conversely, a loose structure with thick fibres is more susceptible to lysis due to high plasmin penetration of fibrinolytic components [55, 56].

EVs derived from platelets and endothelial cells form an intrinsic part of clot formation and stability. Consistent with this, our data in three separate model systems shows endothelial EVs generated under hypoxic conditions exhibit increased pro-coagulative capacity when tested under normal conditions. Significant increases in fibrin polymerisation were induced on addition of EV$^{\text{hypo}}$ to plasma. This effect was abrogated when plasma was incubated with EV$^{\text{hypo+N}}$, confirming that NO$_2^-$ is capable of modulating the character of EVs produced under hypoxic conditions. EV$^{\text{norm}}$ and EV$^{\text{norm+N}}$ had no effect on fibrin polymerisation in comparison to control. Platelet aggregation in whole blood was also elevated in response to the agonists ADP and TRAP when pre-incubated with EV$^{\text{hypo}}$, whilst addition of NO$_2^-$ to cells alleviated this effect, regardless of the agonist used for stimulation. Thus, NO$_2^-$ appears to protect against the generation of pro coagulant, hypoxic CEVs. This was further confirmed using fractal dimension ($d_f$) and clot formation time ($T_{GP}$) in whole blood. A significant relationship has
previously been observed between the SEM of fibrin clots and \( d_f \), whereby a higher \( d_f \) was associated with highly dense clots formed of smaller fibrin fibres [57,58].

We also show that EV\textsuperscript{hypo} elevated thrombin production significantly. However, NO\textsuperscript{2-} pre-treatment of cells had no effect on thrombin generating capacity of EV\textsuperscript{hypo}. On examination of the biogenic cargo of the PEVs, NO\textsuperscript{2-} had no effect on levels of vWF, TF, thrombomodulin or TFPI, suggesting the NO\textsuperscript{2-} effect is independent of the presence of these mediators of the coagulation cascade.

Qualitatively, scanning electron microscopy revealed EV\textsuperscript{hypo} promoted the formation of a more compact, denser clot in comparison to EV\textsuperscript{norm}, which may imply greater resistance to subsequent fibrinolysis. This is consistent with our findings of adding EV\textsuperscript{hypo} in our fluorescent fibrin assay and \( d_f \) analysis. Previous work by Weisel et al has shown that platelet-derived EVs attach to fibrin and incorporate themselves within the clot network [59]. Consistent with this, we observed that EV treatment leads to the incorporation of spherical particles 200-500 nm in size into the fibrin clot that are largely absent in the controls.

Prior to clot activation we observed only diffuse fluorescence (fibrinogen) which became visible as fibrin strands only when incorporated into the fibrin polymer network stimulated by thrombin (see Supplementary Figure 3). Interestingly, even in the absence of hypoxia, pre-incubation of endothelial cells with NO\textsuperscript{2-} resulted in CEVs that significantly reduced the number of fibrin strands per junction, compared to controls, implying NO\textsuperscript{2-} can reduce the pro-coagulant effect of normoxic CEV. NO\textsuperscript{2-} at a concentration similar to that which was used in our own study [59-63] is thought to elicit its vasoprotective effects via reduction to “NOx” species, a pathway that is significantly enhanced during hypoxia. In vivo, there is certainly evidence that NO\textsuperscript{2-} may also elicit its effects via hypoxia-independent mechanisms involving the formation of a nitrosothiol (RSNO) [64,65]. Feelisch et al [66] have recently shown that NO\textsuperscript{2-} induces delayed and long-lasting blood pressure lowering effects even in normoxia via an NO-independent mechanism involving redox intermediates. Importantly, in the present studies NO\textsuperscript{2-} (30\( \mu \)M) was added to cells in culture and the resulting EVs harvested via multiple ultracentrifugation steps. To confirm little carry over of NO\textsuperscript{2-} into our functional assays, ozone-based chemiluminescence detection of NO\textsuperscript{2-} [67] showed levels typically <100nM. Furthermore, addition of NO\textsuperscript{2-} at 30\( \mu \)M directly to plasma without CEV had no effect on fibrin strand formation. In order to test for differential metabolism of NO\textsuperscript{2-} by endothelial cells in
normoxia compared to hypoxia, we measured nitrite and nitrate by ozone based chemiluminescence and observed a relatively small 2.82µM and 1.61µM decrease in NO$_2^-$, respectively (N=3). This indicates overall reduced nitrite metabolism under hypoxic conditions. The authors are careful not to over interpret this result, since we had predicted the overall nitrite/nitrate concentration would remain unchanged. This was based on the fact production of NO from NO$_2^-$ under hypoxic conditions will ultimately lead to products that are relatively unstable and will ultimately re-cycle back to nitrite and nitrate that are detected by ozone-based chemiluminescence in the cell culture medium. However, in our experience studies undertaken to recover nitrite/nitrate from cell culture based experiments are fraught with difficulty including the sampling time point and the stability of multiple products formed. The importance of NO$_2^-$ reduction and involvement of NO in control of EV generation by endothelial cells under hypoxic conditions has previously been confirmed [20]. Approximately 60% of NO$_2^-$ reduction is attributable to xanthine oxidoreductase activity at 1% O$_2$, as shown by co-treatment with Allopurinol (100µM). Furthermore, the importance of nitrite reduction to NO in hypoxia in the context of inhibiting the stability of HIF-1α and induction of EV production (as opposed to a direct effect of nitrite on HIF-1) is also evident from our previous finding that addition of nitrite to cells co-treated with the hypoxia mimetic agent Desferrioxamine (100µM) under normoxic conditions so as to induce HIF had no effect on typical “hypoxia” mediated increases in EV.

Hypoxia has been shown to induce changes in several proteins in cells involved in coagulation via HIF-1, including upregulation of TF [68] and PAI-1 [24], and downregulation of thrombomodulin [69] and TFPI [70]. Our immunoassay results suggest that EV$^{\text{hypo}}$ reflect the composition of their parent cells; with significant reductions in TFPI levels compared to EV$^{\text{norm}}$. The addition of exogenous TFPI to the assay in combination with EV$^{\text{hypo}}$ to plasma, resulted in a significant reduction in total fibrin polymerisation compared to EV$^{\text{hypo}}$ alone, confirming EV$^{\text{hypo}}$ exhibit their pro-coagulant effect primarily through the extrinsic pathway of the coagulation cascade. Taken together with the finding that EV$^{\text{hypo}}$ also express increased levels of TF compared to EV$^{\text{norm}}$, the imbalance in TF/TFPI can result in increased TF-FVII complex formation, activation of factor Xa and ultimately, enhanced fibrin polymerization. The effect of endothelial EVs under different culture conditions on the coagulation cascade is summarised in Figure 10.
Previous studies have shown that hypoxia-derived EVs express increased TF [71-73]. In agreement with this, EV\textsuperscript{hypo} expressed increased TF in the presence and absence of nitrite. HIFs are capable of repressing transcription of genes such as TFPI by directly binding to the hormone response element (HRE) [22], as shown previously in breast cancer cells where inhibition of HIF-1\(\alpha\) reversed the effects of hypoxia on TFPI expression [70]. There is also evidence to suggest HIF may also regulate TF expression, however this is yet to be fully elucidated [73,74]. Interestingly, a previous study identified an imbalance between TF and TFPI within circulating EVs in individuals with Behçet’s Syndrome and concluded this may predispose these individuals to thrombosis [75].

![Figure 10. Summary of the influence of oxygen concentration on EV generation and EV-mediated coagulation.](image)

A. EVs isolated from cells conditioned in 21% O\textsubscript{2}. B. EVs isolated from cells conditioned in 1% O\textsubscript{2}. C. EVs isolated from cells conditioned in 1% O\textsubscript{2}, pre-conditioned with NaNO\textsubscript{2}. Green EV represent EV high in TFPI, whereas red EV represent EV with low TFPI.

On examination of the biogenic cargo of the EVs, NO\textsubscript{2}\textsuperscript{-} had no effect on levels of vWF, TF or TFPI, suggesting the inhibitory effects of NO\textsubscript{2}\textsuperscript{-} observed are independent of EV supply of these mediators of the coagulation cascade. The high level of pro-coagulant phosphatidylserine (PS) exposure on the surface of EVs can also provide a negatively charged surface for formation of the prothrombinase complex [76], mediated by the calcium-dependent floppase and scramblase
membrane bound enzymes [77]. Since NO elicits many of its effects, including modulation of platelet activation and vasodilation, via a reduction in intracellular calcium [78], it is reasonable to suggest NO\(^{-}\) could act via a similar mechanism. In addition, we have previously observed that treatment of endothelial cells with NO\(^{-}\) under hypoxic conditions abrogates the hypoxia-induced increase in EV number via its destabilising effects on HIF-1\(\alpha\). Since PS exposure on the cell membrane is a critical step in EV generation, it is reasonable to predict that the effects of NO\(^{-}\) on PS exposure might be linked to its effects on HIF-1\(\alpha\) under hypoxic conditions [20]. This remains to be confirmed.

The pathological role of endothelial EV within cardiovascular disease, specifically coagulation, is well established [79,80]. Clinically, the role of hypoxia in stimulating endothelial EV in the context of acute ischaemic stroke [81] and myocardial infarction [82, 83] is now recognised. Extrapolating the results from this study to \textit{in vivo}, modulation of the pro-thrombotic character of hypoxia derived endothelial EV by increasing NO\(^{-}\) availability may prove beneficial in patients at risk of thrombotic events. Our group have previously shown increases in plasma NO\(^{-}\) and RSNO (via an acute dietary nitrate supplement) in CAD patients can reduce circulating plasma EV, coupled with a reduction in platelet aggregation [16], studies which need replicating in large patient cohorts.

An important limitation of these studies is we chose to focus on a NO\(^{-}\) dose of 30\(\mu\)M based upon the maximal inhibition effect of NO\(^{-}\) on EV production [20]. This is significantly higher than typical baseline nitrite concentration observed in plasma. However, we are keen to point out when exogenous nitrite is given to subjects (either directly in the form of an infusion or indirectly in the form of dietary nitrate such as beetroot), plasma nitrite peak reaches low \(\mu\)M ranges of NO\(^{-}\) in healthy subjects (reviewed in [84]). Nevertheless, it is important to point out 30\(\mu\)M represents a supra-physiological level and these studies are proof of principle. A further limitation is we chose 1\% O\(_2\) as an in vitro model of tissue ischaemia. We had previously shown that HIF is expressed at O\(_2\) concentrations <2\% [20] and we have applied this model extensively to mimic in vivo hypoxia associated with ischaemia. This is consistent with the concept that hypoxia in cell culture models is considered to be below 5\% O\(_2\) [85]. It also agrees with the work of Mann \textit{et al} [86] who have studied the concept of “Physioxia” extensively and suggest between 3-13\% O\(_2\) best represents
true physiological conditions in an in vitro setting (depending on the cell type) whereas below this should be termed “Hypoxia”.

In summary, this study highlights the pro-coagulant potential of hypoxia-derived endothelial-EVs and confirms this to be mediated by an imbalance in EV TF/TFPI content. Pre-incubation of endothelial cells in hypoxia with NO₂⁻ alleviated the pro-coagulant effect of the resulting EV, however, the mechanism was independent of TF/TFPI and appears to be mediated by the de-stabilizing effect of NO (from NO₂⁻) on HIF-1α. Future studies should further elucidate the mechanisms by which NO₂⁻ modulates the biogenic cargo and subsequent function of pro-coagulant EVs.

Conflict of Interests

None declared.

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Supplemental Data

Fluorescent fibrin polymer formation

Figure 1. Image capture of fluorescently labelled fibrin polymer formation over 15 minutes. A – represents 5 minutes post clot activation using thrombin and CaCl$_2$. B – captured 10 minutes post activation. C – captured 15 minutes post activation and used to perform the analysis on. Images captured at 100x magnification. This is based on modification of a previously established assay [41].

Characterisation of EVs

EVs derived from all conditions displayed similar levels of exosomal (CD9, ALIX, TSG101) and endothelial (CD144) markers, with no significant differences observed between groups (Figure 2).

![Graph showing relative fluorescence units for different conditions](image)

Figure 2. Character of PEVs incubated in hypoxia ± nitrite. EV, exosomal and endothelial marker levels. $EV^{\text{norm}}$ = normoxic EV, $EV^{\text{norm}+N}$ = normoxic EV + nitrite, $EV^{\text{hypo}}$ = hypoxic EV, $EV^{\text{hypo}+N}$ = hypoxic EV + nitrite. Results reflect $N=4$. * reflects $p < 0.05$. 
Figure 3. Typical images captured following the formation of fibrin polymers with the addition of CEVs. Combined image overlay of activated clot with the addition of an EV subgroup. Captured 15 minutes post clot activation using a 488nm laser at 100x magnification.
References


