Cardiovascular and Haemostatic Changes in a Rabbit Microsphere Model of Pulmonary Thrombosis

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Summary

In order to further understand the pathogenesis of disseminated intravascular coagulation (DIC), to diagnose the condition in the early stage and to provide effective therapeutic intervention, valid and clinical relevant animal models are needed. Microvascular pulmonary thrombosis is one of the hallmarks in DIC. In the search for markers of microvascular pulmonary thrombosis in DIC, the present study evaluated local pulmonary effects of microvascular pulmonary thrombosis caused by polystyrene microspheres, generally assumed to be inert. Simultaneously the haemostatic system was evaluated to investigate whether microspheres are truly inert in a rabbit model of microvascular pulmonary thrombosis.

Cardiovascular and haematological parameters were measured at three time points over a 30 minute period from rabbits administered 45 μm microspheres as a single bolus dose of 0.3, 6, 16 or 32 μl microspheres/ml blood. Injection of 0.3 μl microspheres/ml blood resulted in no changes. Injection of 6 μl microspheres/ml blood resulted in a minor increase in cardiovascular parameters whereas injection of 16 μl microspheres/ml blood resulted in significant changes of cardiovascular parameters without impact on haemostatic parameters. A lethal effect was shown after injection of 32 μl microspheres/ml blood.

With i.v. injection of microspheres we succeeded in establishing an inert mechanical model of fixed size microvascular emboli in the lung vasculature of the rabbit. We showed that selected cardiovascular parameters rapidly and dose dependently reflect obstructions in the pulmonary vasculature and therefore could be very valuable and reliable parameters in experimental models of disseminated intravascular coagulation for early detection of microvascular pulmonary thrombosis.

Introduction

Disseminated intravascular coagulation (DIC) is a thrombohaemorrhagic disorder where pathological activation of the coagulation system consumes coagulation proteins and platelets, which disrupts normal coagulation and leads to haemorrhage and profound microvascular thrombosis. (*Bick* , *2002*; *Dalainas* , *2008*; *Levi et al.*, *2009*). To further un-

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derstand the pathogenesis, it is of interest to investigate the organs and systems affected in this complex disease. Specifically the lung is a target organ at high risk of microvascular thrombosis resulting in pulmonary and cardiac dysfunction (*Katsumura and.Ohtsubo*, *1999*; *Bick*, *2002*). Pathophysiological events following microvascular pulmonary thrombosis such as increased pulmonary vascular resistance; impaired gas exchange or increased airway resistance resulting from bronchoconstriction (*Goldhaber* , *2004*) can lead to severe respiratory distress. With its vast capillary bed and position in the circulation, the lung acts as a safety net to catch emboli before they reach the brain and other tissues.

Measurement of the above mentioned pathophysiological events might help to provide markers of pulmonary microvascular thrombosis and impending complications.

In animal models of pulmonary thrombosis, microspheres have been used to establish fixed size emboli in the vasculature to further investigate properties of the pulmonary microcirculation (*Lamm et al.*, *2005*) and events affecting pulmonary physiology taking place following this mechanical obstruction (*Bottiger. et al.*, *1996*; *Dias-Junior and Tanus-Santos*, *2006*). In these models microspheres are assumed to be inert and haemostatic parameters indicating possible activation of the coagulation system by the microspheres have not been evaluated. These models consider mainly the pulmonary cardiovascular aspect of pulmonary embolism (PE) as a mere mechanical obstruction and do not relate the findings to microvascular pulmonary thrombosis such as in DIC with PE as the end outcome.

There is a constant need for further understanding the pathogenesis of DIC in order to diagnose the condition early, and thereby provide intervention resulting in more efficacious therapies. Microvascular pulmonary thrombosis is one of the hallmarks in DIC (*Katsumura. and .Ohtsubo*, *1999*; *Bick*, *2002*). In the search for early markers of microvascular pulmonary thrombosis in DIC, the present study evaluated the local pulmonary effects of microvascular pulmonary thrombosis as a symptom of systemic thrombosis as observed in DIC. Simultaneously the haemostatic system was evaluated to investigate whether microspheres are truly inert in an animal model of microvascular pulmonary thrombosis.

The rabbit was chosen as the laboratory species for this animal model of pulmonary thromboembolism because of its size and availability. As considerable amounts of blood were needed for the evaluation of early markers of microvascular pulmonary thrombosis a medium sized laboratory animal such as the rabbit was needed, smaller laboratory animals such as rodents would not suffice and larger laboratory animal species, such as the dog and the pig, were unnecessary and more expensive to use.

We hypothesized that it was possible to create a rabbit model of pulmonary thromboembolism by venous injection of polystyrene microspheres thereby investigating the consequences of mimicking mechanical fixed sized microvascular pulmonary thrombo-emboli but not affecting coagulation. We hoped to discover useful parameters for an early diagnosis of the mechanical obstructive pulmonary complications of DIC.

Materials and Methods

Animals

Twenty-eight female New Zealand white rabbits (Charles River, Sulzfeld, Germany) 13-17 weeks of age, weighing 2.5–3.5 kg were included in the study.

The rabbits were housed in colonies of 10 animals in a climate-controlled barriered facility. A standard rodent pelleted diet (Altromin 2113, Altromin GmbH, Lage, Germany) was fed *ad libitum* as well as fresh tap water. Animals were acclimatised for a period of 5 weeks in which they received preventive coccidiostatic treatment during the second and fourth week (Esbetre® Vet, Novartis, Denmark).

The study was approved by the Danish Animal Experiments Inspectorate, the Ministry of Justice.

In vitro analyses

One female New Zealand white rabbit was pre-anaesthetized with Diazepam (Stesolid®, Alpharma, Oslo, Norway) 0.4 mg kg^{-1} i.v in a marginal ear vein, then fully anaesthetized with pentobarbital sodium 5% in sterile water (Nomeco, Copenhagen, Denmark). Xylocain®, 10 mg/ml (Astra Zeneca, Albertslund, Denmark), was injected in the neck and a catheter was placed in the left carotid artery for blood sampling. Approximately 70-80 ml blood was sampled and citrated (10%, [0.13M]) for further thromboelastographic (TEG) analysis.

Thromboelastography (TEG)

TEG is a global haemostatic profile test allowing rapid assessment of all stages of coagulation and fibrinolysis in whole blood. Parameters describing

the whole blood clotting stages are attained by measuring the forces acting on a pin in the rotating TEG cup (*Mallett and Cox*, *1992*).

Blood was aliquoted in 2 ml tubes (Safe-lock tubes, Eppendorf, Hamburg, Germany) and volumes of polystyrene microspheres in aqueous suspension (Duke Scientific Corporation, Palo Alto, CA, USA) with a diameter of 45 μm were added randomized to the samples within 20 minutes of blood sampling. Samples were inverted slowly at room temperature until loading on the TEG after 60 minutes, TEG cups were heated to 37 °C. 340 μl of sample was added to the TEG cup preloaded with 20 μl 11 mM $CaCl_2$. A schematic overview of microsphere volumes added to the samples is displayed in Table 1.

TEG parameters reported are clotting time (R (min)) reflecting the time from the blood is placed in the TEG analyzer until initial fibrin formation and angle (deg) reflecting the rapidity of fibrin build-up and cross-linking (*Wiinberg. et al.*, *2005*).

Zeneca, Albertslund, Denmark), was injected in the neck, and a catheter (Polyethylene tubing PE200, BD, Broendby, Denmark) was placed in the left carotid artery for blood sampling and measurement of blood pressure (Physiological pressure transducer, ADinstruments Ltd., Oxfordshire, UK). The catheter was kept open by slow infusion of saline (6.6 ml/hour). The right jugular vein was catheterized and the catheter advanced to the right ventricle for measurement of right ventricular blood pressure (Pressure catheter SPC-320, 2F polyurethane, connected to a micro-tip BP foundation system, ADinstruments Ltd., Oxfordshire, UK). Core temperature was maintained at 38°C using a homeothermic heating blanket with a rectal temperature probe (Homeothermic blanket system, Harvard Apparatus, Holliston, Massachusetts, USA).

Experimental design

Polystyrene microspheres in aqueous suspension

Table 1. Design of TEG *in vitro* analysis of whole blood samples spiked with microspheres.

Microsphere volume	Control (0 μl microspheres/ml blood)	32 μl microspheres/ ml blod	16 μl microspheres/ ml blod
n	8	4	4

In vivo studies

Animal preparation and instrumentation

Bodyweights were recorded and rabbits were anaesthetized as previously described. Pentobarbital sodium 5% in sterile water was supplemented as needed. Furthermore an ear vein was catheterized (Venflon 22GA, BD, Broendby, Denmark) for injection of microspheres. Xylocain®, 10 mg/ml (Astra

(Duke Scientific Corporation, Palo Alto, CA, USA) with a diameter of 45 μm were used. The suspension of microspheres was gently inverted and immersed in an ultrasonic bath for 15 seconds to prevent aggregation of microspheres. Microspheres were dosed according to blood volume (0.3, 6, 16 and 32 μl microspheres/ml blood) and by adding saline the total injection volume was the same (32μl/mL blood) for all animals (Table 2). For calculation of blood

Table 2. *In vivo* study design.

Volume of microspheres (μl)	Volume of saline (μl)	Total injection volume (μl)	N
0.3 μl/ml blood	31,7 μl/ml blood	32 μl/ml blood	8
6 μl/ml blood	26 μl/ml blood	32 μl/ml blood	8
16 μl/ml blood	16 μl/ml blood	32 μl/ml blood	8
32 μl/ml blood	0	32 μl/ml blood	3

volume, a mean of 56 ml/kg (*Diehl et al.*, *2001*) was used. Rabbits were allocated randomly to the four treatment groups. Eight rabbits were included in each dosing group except for the high dose group (32 µl microspheres/ml blood, n = 3) (Table 2). Blood samples were collected following a 10 minute baseline period (-5 min) and at 5 and 30 minutes af-

diluents supplied with the kit. Protein concentrations were determined from the optical density of each sample compared to a standard curve.

Haemostatic parameters
Five ml blood was collected from the carotid artery at each time-point into a syringe and transferred
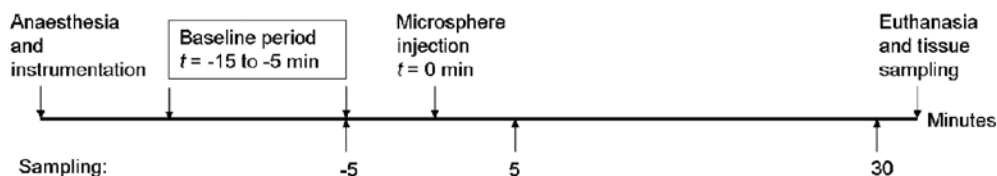
Figure 1. Time line. Animals were left resting for 10 minutes with catheters and ECG electrodes in place. Microspheres were injected at time 0 minutes. Measurements were taken at the time points -5, 5 and 30 minutes. Animals were sacrificed after the last recording.

ter injection of microspheres (Figure 1).
Cardiovascular parameters
Systemic blood pressure and right ventricular blood pressure was determined. An electrocardiogram (ECG) was obtained by subcutaneous placement of 3 needle electrodes (knee, left hind leg, right and left axillae). Heart rate was determined from the electrocardiographic measurements. The cardiovascular parameters were recorded continuously throughout the study with an ADinstruments Powerlab (ADinstruments Ltd., Oxfordshire, UK) connected to a computer. Measurements reported are systolic peak averages of 30 heart beats immediately prior to the blood sampling time points at -5, 5 and 30 minutes after injection of microspheres.

Furthermore blood gas levels were measured at each time point using heparinised capillaries for immediate analysis (GEM Premiere 3000, ILS, Alleroed, Denmark).

Cardiac troponin I (cTnI), a biomarker of cardiomyocyte damage, was determined by enzyme-linked immunosorbent assays (Life Diagnostics, West Chester, PA). Briefly serum samples were thawed on the day of analysis and diluted appropriately with

to EDTA coated, citrate (10%, [0.13M]) and plain tubes immediately. The tubes were inverted gently 10 times in order to ensure sufficient mixing. Due to the need of distributing small volumes of blood to the different tubes this blood sampling procedure was chosen to prevent activation of the blood.

Complete blood counts were determined from EDTA stabilized whole blood using an automatic counter (Medonic CA 620 (Boule Nordic, Kastrup, Denmark). Serum samples and citrated plasma samples were obtained by centrifugation at 4000g for 5 min. Plasma and serum were stored at -80 °C until analysis.

Plasma fibrinogen, prothrombin time (PT) and activated thromboplastin time (aPTT) were determined using a coagulation assay (ACL 10000, ILS Laboratories, Alleroed, Denmark).

Plasma thrombin-antithrombin (TAT) and plasma fibrinogen/fibrin degradation product (FDP) levels were determined by the Hemostasis Reference Laboratory, Henderson Research Centre, Ontario, Canada. In brief, fibrinogen/FDPs were measured by ELISA using a binding and a detecting antibody. TAT was also measured by ELISA, but with two

different binding antibodies cross reacting with rabbit thrombin and rabbit anti-thrombin respectively (*Pelzer et al.*, *1988*). The reference range for the TAT analysis on rabbit plasma is 304-904 pM and for the FDP analysis 29.0-77.8 mg/L.

Pathological examination

The animals were sacrificed by exsanguination, by cutting the abdominal aorta and caudal vena cava, under full anaesthesia 30 min after administration of microspheres. Kidneys, heart, brain and lung were harvested and fixed in 10% formalin (VWR – Bie & Berntsen, Herlev, Denmark). Formalin was infused into the bronchial tree before placing the lung in formalin. The tissues were trimmed, dehydrated and embedded in paraffin. The organs were cut at a nominal thickness of 4 µm and stained with haematoxylin eosin (HE) for morphology overview and phosphotungstic acid haematoxylin (PTAH) for fibrin detection. All sections were evaluated by light microscopy. Pathological changes such as thrombi and fibrin formation, local inflammation or haemorrhage were qualitatively described.

Statistical analysis

Graph Pad Prism 5® for Windows (GraphPad Software, Inc., San Diego, CA, USA) was used for the graphic presentation of the results, descriptive statistics and one way ANOVA analysis. Mixed linear model analysis and repeated measures analysis were performed using SAS® (version 9.1; SAS Institute Inc., Cary, NC, USA). Statistical significance was established at $p < 0.05$. Data are reported as means ± SD unless otherwise noted.

Results

Thromboelastographic in vitro analyses

After adding 32 µl microspheres/ml blood clotting time (R) was significantly shortened to 8.28 ± 0.75 min versus 10.48 ± 1.44 min in controls; $p < 0.05$. The angle (α) was increased significantly (62.58 ± 2.36 deg) versus controls (55.10 ± 5.75 deg); $p < 0.05$. Adding 16 µl microspheres/ml blood did not change clotting time or angle significantly (Figure 2).

In vivo results

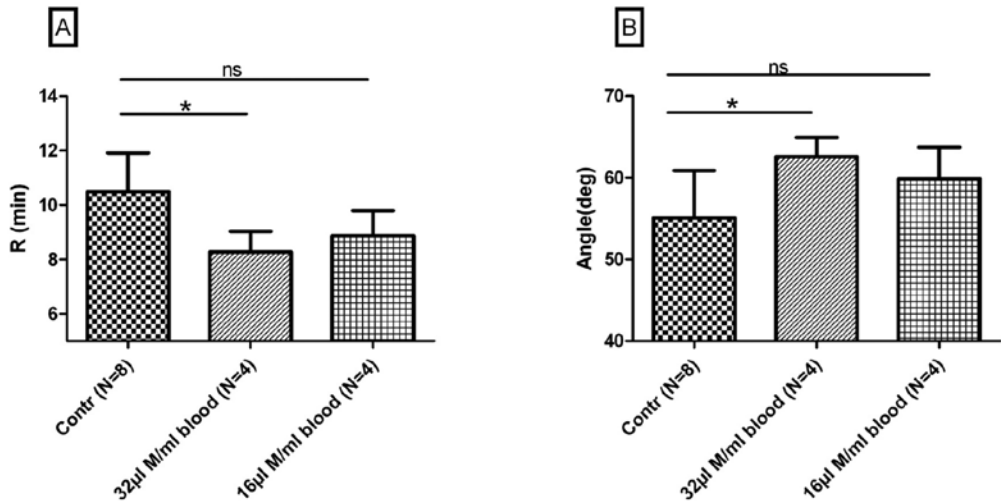There was no difference in weight of the animals in the groups (data not shown).

Figure 2. Thromboelastographic results**.** 2A: clotting time (R) 32 µl microspheres/ml blood significantly shortened clotting time compared to controls ($p < 0.05$). 2B: Angle in degrees. Adding 32 µl microspheres/ml blood significantly increased the angle compared to controls ($p < 0.05$). Data are displayed as mean +SD.

Only 3 animals were included in the group receiving 32 µl microspheres/ml blood because animals died during the injection of microspheres. For the same reason, no data at 30 minutes after injection of microspheres were recorded for this group. 3 of the 8 rabbits receiving 16 µl microspheres/ml blood died of the injection.

Cardiovascular parameters
At baseline, animals had a mean heart rate of 205 ±15 bpm, an oxygen saturation of arterial blood of 86.8 ±4.1%, a peak systolic blood pressure of 100.9 ±4.1 mmHg, a maximal right ventricular blood pressure of 15.5 ±0.9 mmHg and an ST-height on the ECG of 0.011 ±0.011 mV (total mean for all groups ± SD at time point -5 minutes). No statistical differences were found between baseline parameters for the different groups.

Blood pressure
A dose dependent drop in systolic blood pressure was observed at 5 minutes following injection of microspheres (Figure 3). For the group receiving 16 µl microspheres/ml blood the systolic blood pressure dropped to 56.56 ±29.75 mmHg at 5 minutes compared to the baseline value of 106.67 ±4.63 mmHg ($p<0.0001$) and remained significantly decreased at

30 minutes (81.58 ±9.18 mmHg) ($p<0.001$) compared to baseline values. For the group receiving 32 µl microspheres/ml blood, the systolic blood pressure decreased until death of the animals (data are not included in Figure). The groups receiving 0.3 and 6 µl microspheres/ml blood showed no changes in systolic blood pressure.

Right ventricular blood pressure
A dose dependent effect on right ventricular blood pressure following administration of microspheres was observed (Figure 4). Administration of 16 µl microspheres/ml blood induced a significant increase in maximal right ventricular pressure (RVPmax) after 5 minutes (27.34 ±9.66 mmHg; $p<0.0001$) and 30 minutes (25.78 ±8.64 mmHg; $p<0.01$) compared to baseline values (16.69 ±4.24 mmHg). Rabbits dosed with 6 and 32 µl microspheres/ml blood exhibited an increase in RVPmax at 5 minutes, though the increase was only statistically significant in the 6 µl microspheres/ml blood group (19.33 ± 8.76 mmHg versus 15.09 ±6.26 mmHg at baseline; $p<0.05$). The measurements for the group receiving 32 µl microspheres/ml blood at time point 5 minutes were influenced by the fact that the animals were dying and data are not included in the figure.
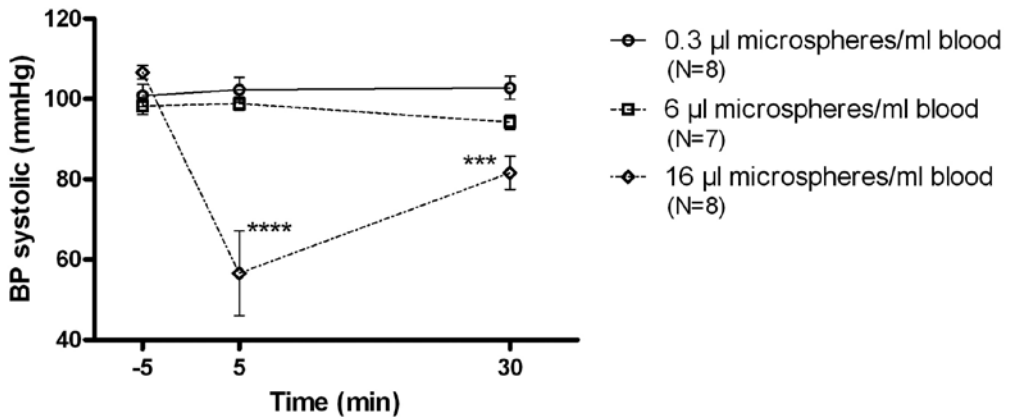

Figure 3. Change in systolic blood pressure (BP). Data displayed as mean (± SD). *** p< 0.001; **** p<0.0001 compared to respective baseline values.

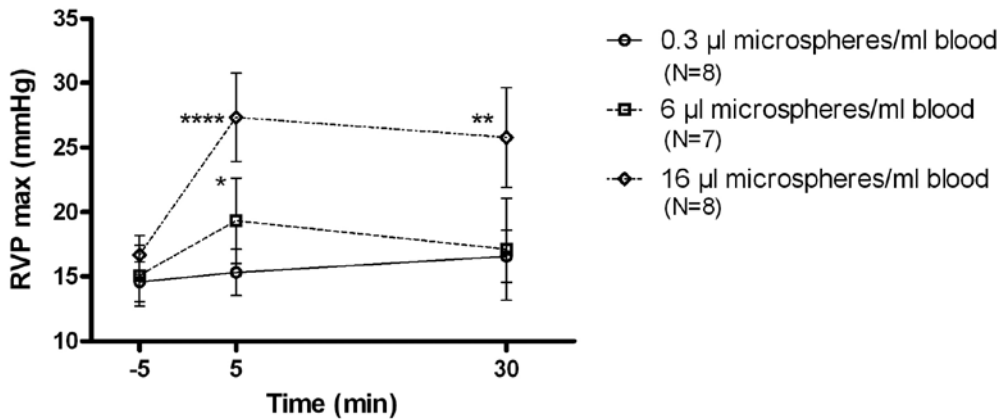**Figure 4.** Change in maximal right ventricular pressure (RVP). Data displayed as mean (± SD). * p<0.05; ** p<0.01; **** p<0.0001 compared to respective baseline values.

ECG

There was a clear tendency for rabbits dosed with 16 and 32 μl microspheres/ml blood to have a decrease in ST-height at the 5 minute time point compared to baseline values. However none of the groups showed statistically significant changes in ST-height during the experiment. In addition, none of the groups showed statistically significant changes in T-wave amplitude during the experiment (data not shown).

Heart rate and arterial oxygen saturation remained unchanged, staying within the normal range for all groups during the experiment (data not shown).

Haemostatic parameters
Platelet and white blood cell counts

The platelet count decreased for rabbits dosed with 16 and 32 μl microspheres/ml blood. For rabbits receiving 16 μl microspheres/ml, the platelet count decreased to 134 ±33 x10^9/L at 5 minutes and 125 ±30 x10^9/L at 30 minutes (166 ±63 x10^9/L at baseline; p<0.0001). For rabbits receiving 32 μl microspheres/ml blood, the platelet count decreased to 87 ±57 x10^9/L at 5 minutes (versus 156 ±29 x10^9/L at baseline; p<0.0001). No significant decreases in platelet count were observed for the animals receiving 0.3 and 6 μl microspheres/ml blood (Figure 5).

In rabbits receiving 6, 16 and 32 μl microspheres/ml blood, the WBC decreased significantly and dose-dependently compared to the respective baseline values of the groups (Figure 6). At 5 minutes after injection of microspheres WBC decreased to 3.6 ±1.2 x10^9/L from a baseline value of 4.4 ±1.2 x10^9/L (p<0.01) for animals receiving 6 μl microspheres/ml blood, for animals receiving 16 μl microspheres/ml blood WBC decreased to 3.0 ±0.9 x10^9/L from baseline values of 4.2 ±0.7 x10^9/L (p<0.0001) and for animals receiving 32 μl microspheres/ml blood WBC decreased to 1.2 ±1.1 x10^9/L from baseline values of 4.6 ±1.3 x10^9/L (p<0.0001). At 30 minutes, WBC was still significantly lower than baseline values for animals receiving 6 μl microspheres/ml blood (3.0 ±1.1 x10^9/L, p<0.0001) and 16 μl microspheres/ml blood (3.2 SD±0.6 x10^9/L, p<0.0001). No statistically significant changes in WBC were seen for animals receiving 0.3 μl microspheres/ml blood.

Fibrinogen

Fibrinogen levels were dose-dependently affected by the administration of microspheres. A significant decline in the fibrinogen level was shown for the group receiving 32 μl microspheres/ml blood during the first 5 minutes after dosing. The fibrino-
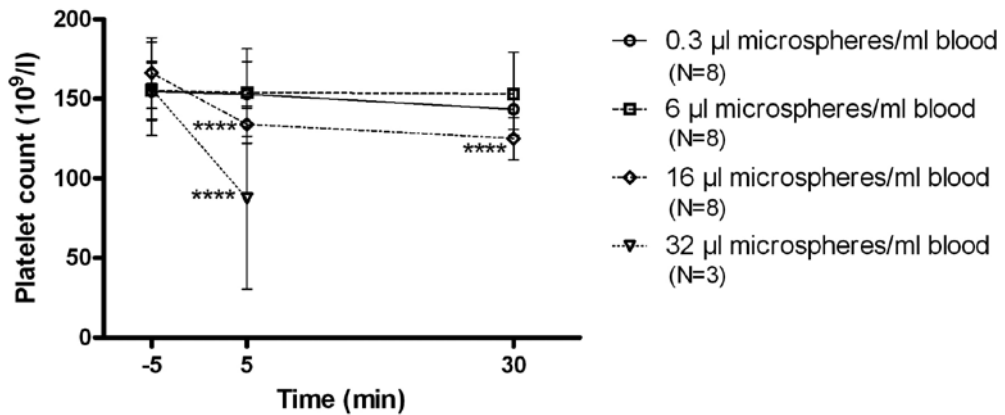
Figure 5. Change in platelet number. Data are displayed as mean (± SD). **** p<0.0001 compared to respective baseline values.
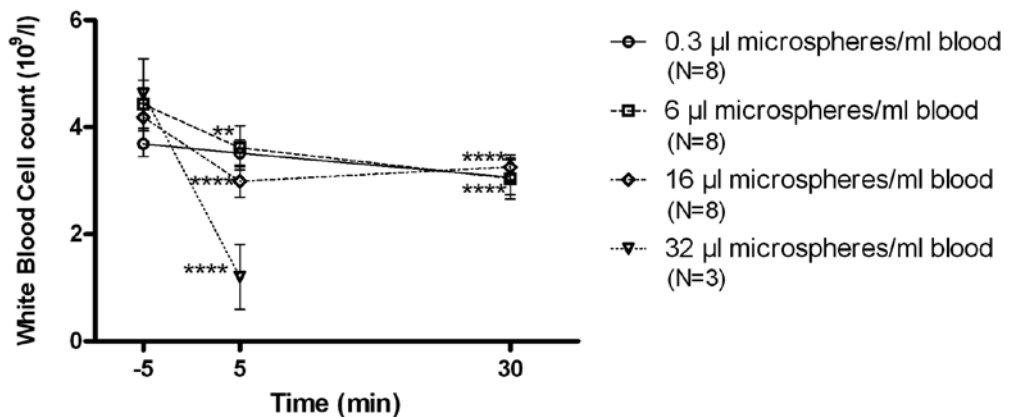

Figure 6. Change in white blood cell count. Data are displayed as mean (± SD). ** p<0.01; **** p<0.0001 compared to respective baseline values.

gen level dropped from 2.07 ±0.18 g/L at baseline to 1.80 ±0.19 g/L at 5 minutes. For rabbits receiving 16 μl microspheres/ml blood the decreases in fibrinogen levels were also significant and dropped from 2.36 ±0.47 g/L at baseline to 2.12 ±0.18 g/L at 5 minutes (p<0.01) and 2.05 ±0.19 g/L at 30 minutes (p<0.001). No statistically significant decrease in fibrinogen levels was observed in the two remaining groups (Figure 7).

Prothrombin time (PT) and activated partial thromboplastin time (aPTT)
Average PT for all groups at baseline was 9.48 ±0.25 sec. After administration of 32 μl microspheres/ml blood the PT increased significantly (1.25 sec on average) at time point 5 minutes compared to baseline values (p<0.0001). No statistically significant changes in PT were observed for any of the other groups (data not shown).
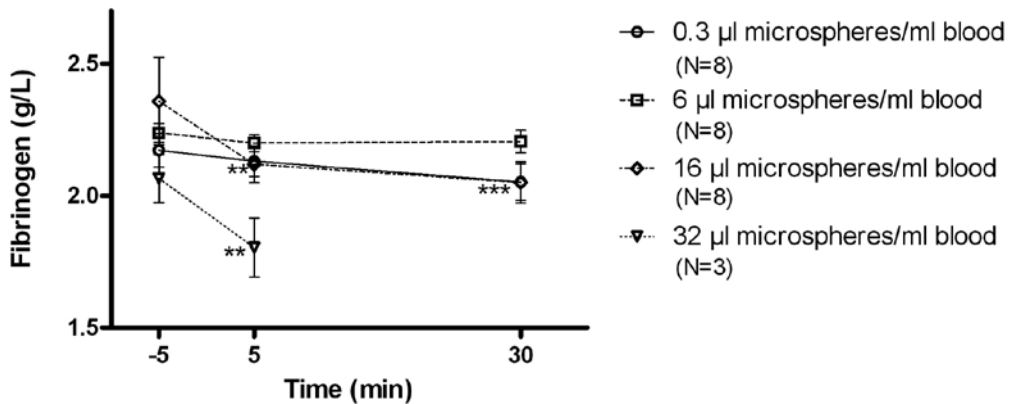
Figure 7. Fibrinogen levels. Data are displayed as mean (± SD). ** p<0.01; *** p<0.001 compared to respective baseline values.

No statistically significant changes were observed in the aPTT for any of the groups (data not shown).

Thrombin anti-Thrombin (TAT) and Fibrin degradation products (FDP)
TAT and FDP levels were within or very close to reference values for all groups at all time points with no differences between groups (data not shown).

Cardiac Troponin I
Although there was a tendency for a dose dependent increase in cardiac troponin I, the levels were close to baseline values and there were no statistically significant changes for any of the groups (data not shown).

Pathological examination
For all rabbits surviving the injection of microspheres, microspheres were found only in the lung vasculature (Figure 8A and 8B). For rabbits receiving a lethal injection (32 and for some 16 μl microspheres/ml blood) microspheres were also found in the vessels of the myocardium and in one single case in the cortex of one kidney. No fibrin formation was observed around any of the microspheres in any of the animals.

With injection of large doses of microspheres a tendency for the microspheres to lodge in the same vessels was observed (Figure 8C and 8D). No microspheres showed signs of aggregation by an increased embolus diameter (>45μm) which would have blocked larger vessels than intended.

Discussion
When injecting different doses of polystyrene microspheres into the venous system of rabbits to establish fixed size emboli in the lung vasculature, dose dependent effects on cardiovascular parameters were observed. When administering 0.3 μl microspheres/ml blood no effects on cardiovascular or haemostatic parameters were seen. With injection of 6 μl microspheres/ml blood cardiovascular parameters were affected but no effects were observed on haemostatic parameters. At high doses of microspheres (16 and 32 μl microspheres/ml blood) a clear mechanical effect on cardiovascular parameters, and a slight activation of the coagulation system, were observed. This was seen as a decrease in platelet count, a small decrease in fibrinogen levels and a small increase in PT time for 32 μl microspheres/ml blood. The procoagulant effects were however very limited and only seen in rabbits dosed with volumes of microspheres causing increased
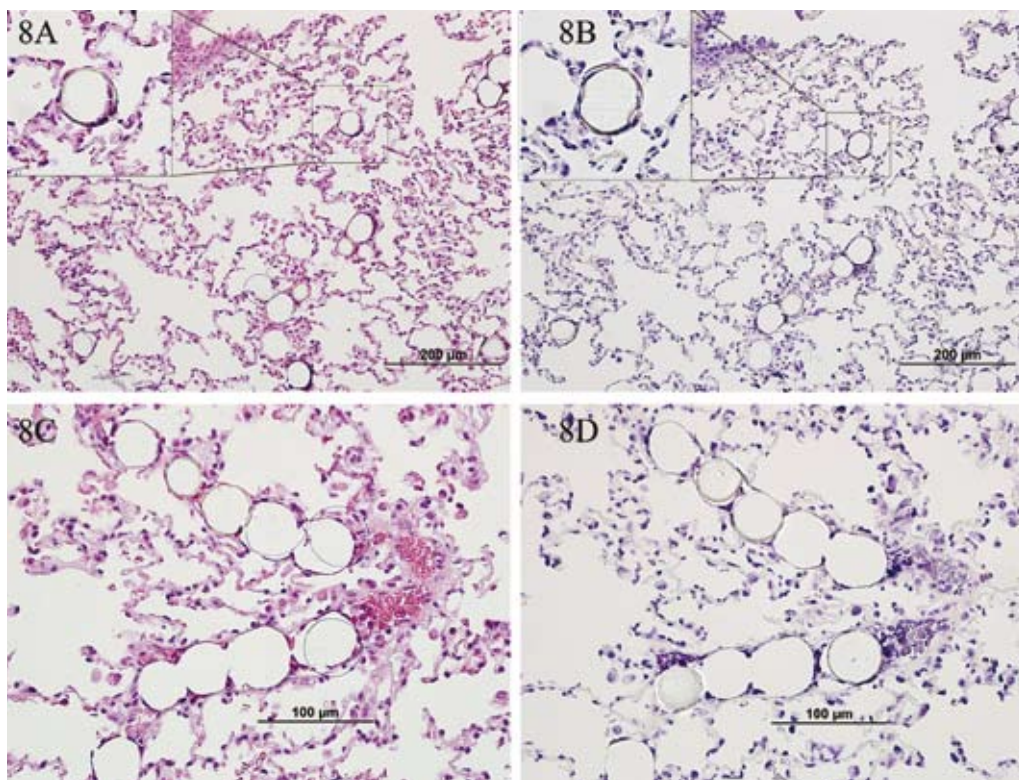
Figure 8. Lung histology. Sections are from rabbits injected with 6 μl microspheres/ml blood. 8A + 8C are stained with hematoxylin/eosin for morphology overview, 8B + 8D are stained with phosphotungstic acid hematoxylin for fibrin detection. 8A + 8B show microspheres lodged individually in the lung vasculature with zoom view of a single lodged microsphere showing no fibrin formation. 8C + 8D show microspheres lodged as a string of pearls in the same vessel, again without fibrin formation.

mortality (16 and 32 μl microspheres/ml blood). The effect of microspheres on the haemostatic system was characterized, according to the score developed by the International Society of Thrombosis and Haemostasis (ISTH) subcommittee on disseminated intravascular coagulation, by dividing DIC into two stages: non-overt DIC and overt DIC including a number of global tests of coagulation factors (*Taylor et al.*, *2001*). This standardization has been used previously in a rabbit model of non-overt DIC (*Olrik et al.*, *2009*). In the current study the minimal changes in haemostatic parameters did not reach a score as either non-overt or overt DIC, hence hae-mostatic changes are interpreted as a minor activation of the coagulation system in relation to injection of high concentrations of microspheres.

As the purpose of the current study was to discover useful parameters for early diagnosis of microvascular pulmonary thromboembolism, the microspheres used were chosen to be 45 μm in width. From previous pilot studies it was observed that 45 μm microspheres induced cardiovascular changes without affecting the ventilation-perfusion relationship as was the case for larger microspheres.

No fibrin formation was observed around the microspheres in the lung vasculature which supports the

proposal that the changes in haemostatic parameters were caused by the very high concentration of microspheres created during the injection rather than by lodged microspheres in the lung vasculature. The microsphere concentration is diluted by the blood volume as the microspheres are transported away from the injection site and the initial activation of coagulation might be caused by contact activation by the large amount of foreign surface. This theory is supported by the *in vitro* results showing no decrease in clotting time for 16 µl microspheres/ml blood and only a small decrease in clotting time for 32 µl microspheres/ml blood.

The cardiovascular parameters; ECG, right ventricular pressure and blood pressure were dose dependently affected by the microsphere injection. The tendency towards a decreased ST height for rabbits receiving 16 and 32 µl microspheres/ml blood at time point 5 minutes indicates that the decrease in blood pressure and increase in right ventricular pressure resulted in ischemic stress on the myocardium. However the myocardial ischemia was mild, and did not result in statistically significant changes in cardiac troponin I levels (*Uzuelli et al.*, *2008*).

Microspheres were found in the myocardium of rabbits receiving lethal injections of microspheres but not in rabbits surviving the injection of microspheres. It is not possible on the basis of the findings of this study to conclude whether the microspheres pass the lung capillaries ante-, peri- or post mortem but only to conclude that this phenomenon occurs with this size and dose of microspheres.

The dose dependent decrease in blood pressure is caused primarily by pulmonary vascular obstruction (*Jones et al.*, *2003*). The pathophysiological events following microvascular pulmonary thrombo-embolism caused by neurohumoral mechanisms, baroreceptor activity and protein kinases (*Goldhaber*, *2004*; *Toba. et al.*, *2010*) do without a doubt play a role in the increased vascular resistance but have not been characterized in this study. However the vasoconstriction induced by platelet-derived mediators would not be expected to play any significant role in this study, as the activation of the coagulation system was subtle and not specifically related to the pulmonary vasculature. When applying these cardiovascular parameters to an animal model of DIC we might therefore expect even higher pulmonary vascular resistance in the case of pulmonary thromboembolism caused by haemostatic activation as argued by Böttiger and co-workers (*Bottiger. et al.*, *1996*).

Another pathophysiological event expected following pulmonary thromboembolism is impairment of the gas exchange in the lung (*Altemeier et al.*, *1998*). As blood gas levels were not affected in any of the groups this was not a major feature of this acute model of microvascular pulmonary thrombosis. One plausible explanation is that the number of emboli was too low to detect any subtle changes in oxygen saturation. However this is not likely as a lethal dose of microspheres in several of the rabbits was reached.

Several authors (*Young et al.*, *1980*; *Hedenstierna et al.*, *2000*) have shown that a certain minimum embolus size is required to impair gas exchange by obstructing a vessel supplying all alveoli that can exchange gas via collateral ventilation (the functional lung unit) and that this certain minimum size is dependent on the individual species, e.g. 100-150 µm in the dog (*Young et al.*, *1980*) and about 60 µm in the pig (*Hedenstierna et al.*, *2000*). The size of the vessel perfusing a functional lung unit has, to our knowledge, not yet been determined for the rabbit but it is plausible that the size of this vessel is larger than the 45 µm obstructed by the microspheres in this study. Finally microspheres may not occlude the vessels completely as has been documented by intravital microscopy (*Lamm. et al.*, *2005*).

Animals receiving lethal doses of microspheres are believed to die because of right ventricular failure due to mechanical obstruction of the pulmonary vasculature. As a result, the RVP only increases minimally for these animals and the blood pressure decreases rapidly. As all of the rabbits receiving 32 µl microspheres/ml blood died and 3 of the 8 rabbits receiving 16 µl microspheres/ml blood died during the experiment, the 16 µl microspheres/ml

blood dose seems to be close to the maximal non-lethal dose and it is speculated that small individual differences in cardiovascular response or distribution of microspheres in the pulmonary vasculature determines whether the rabbit dies during injection or not. Previous pilot studies showed that measurements at time points beyond 30 minutes did not differ significantly from measurements at 30 minutes and to prove changes observed at 5 minutes as being acute and reversible, the 30 minute measurement was found important. As the primary interest in the current study was the immediate and acute effect of microvascular pulmonary thrombosis the time points -5, 5 and 30 minutes were chosen as optimal to get baseline, acute effect and to follow up on the acute effect. Previous pilot studies showed no difference in cardiovascular and haemostatic values for rabbits receiving 0.3 μl microspheres/ml blood and rabbits receiving saline, thus this group was included to demonstrate that the microspheres themselves are inert *in vivo* and that cardiovascular and haemostatic effects of microsphere injection are dose dependent.

In summary, injection of 0.3 μl microspheres/ml blood changed neither cardiovascular nor haemostatic parameters. Injection of 6 μl microspheres/ml blood resulted in a minor increase in cardiovascular parameters whereas injection of 16 μl microspheres/ml blood resulted in significant changes of cardiovascular parameters. Neither 6 nor 16 μl microspheres/ml blood had a significant impact on haemostatic parameters. A lethal effect was shown in connection with injection of 32 μl microspheres/ml blood. Both cardiovascular and haemostatic parameters were affected at this high dose, but the latter might be related to a high microsphere concentration during injection.

With i.v. injection of microspheres we succeeded in establishing a mechanical model of fixed size acute microvascular emboli in the lung vasculature of the rabbit and showed that cardiovascular parameters such as systemic blood pressure and right ventricular pressure rapidly and dose dependently reflect obstructions in the pulmonary vasculature. Therefore they could be valuable and reliable parameters in experimental models of disseminated intravascular coagulation for early detection of microvascular pulmonary thrombosis.

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