

Macromolecular capillary leakage is involved in the onset of anaphylactic hypotension

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Summary Statement: During anaphylaxis, interstitial capillary leakage occurs early after shock onset. The inflammation-induced microcirculatory changes with subsequent intravascular fluid transfer into the interstitial space might be involved in the onset of inaugural anaphylactic hypotension.

Abstract

Background: The role of the hypovolemic component secondary to the microcirculatory changes in the onset of inaugural anaphylactic hypotension remains debated. We investigated the microcirculatory permeability in a model of anaphylactic shock using a fluorescence confocal microscopy imaging system.

Methods: Ovalbumin-sensitized anesthetized Brown Norway rats were randomly allocated into two groups: control and anaphylaxis, respectively induced by intravenous saline or ovalbumin at time 0 (T0). The mesentery was surgically exposed. Macromolecular fluorescein isothiocyanate-dextran (FITC-dextran) was intravenously injected at T0-5min allowing *in vivo* visualization of the mesenteric microvascular network by fluorescence microscopy. After a short period of stabilization of the contrast agent concentration, a 5-sec movie was recorded to obtain baseline signal intensity (SI). Following T0, 5-sec movies were recorded every 30 sec for 30 min. Capillary leakage of FITC-dextran was assessed in interstitium and compared between groups.

Results: Following anaphylactic shock onset, an early, progressive and global SI increase over time was detected in the interstitium. Mean index leakage differed between control and anaphylaxis (respectively 20 ± 11 vs. $170 \pm 127\%$; $p = 0.002$), starting at 2 min. after shock onset and progressively increasing. Index leakage correlated to the drop in arterial blood pressure until T0+10 min. ($r: -0.75$; $p < 0.01$).

Conclusions: During anaphylaxis, interstitial capillary leakage occurs within minutes after shock onset. Compared to control conditions, the mesenteric microcirculation showed at least 8-fold-increased macromolecular capillary leakage. The inflammation-induced microcirculatory changes with subsequent intravascular fluid transfer into the interstitial space might be involved in the onset of inaugural hypotension during anaphylactic shock.

Introduction

Anaphylactic shock has a distributive profile in which vascular dysfunction results in inadequate regional oxygen delivery ¹. Clinical guidelines for anaphylaxis recommend epinephrine as first-line therapy to counteract the vasoplegic component because of its beneficial effects mediated by α_1 - and β_1 -adrenergic receptors activation ²⁻⁴. However, the role of the hypovolemic component secondary to the inflammation-induced microcirculatory changes with subsequent intravascular fluid transfer into the interstitial space remains debated, although it may participate into inaugural anaphylactic arterial hypotension. The investigation of the microcirculatory function during anaphylaxis is therefore essential as its role is central for adequate tissue oxygenation and consequently organ function ⁵.

The *in situ*, real-time changes in capillary permeability can be evaluated by fibered confocal fluorescence microscopy (FCFM), by means of microscopic dynamic contrast-enhanced (DCE) imaging technique. FCFM allowed to assess changes in microvascular permeability in a limited number of experimental studies such as histamine-induced macromolecular leakage ⁶, sepsis ⁷⁻⁸, normal mesentery and xenografted tumors ⁹. A few experimental works showed that allergic activation of mast cells resulted in increased vascular permeability as well as leukocyte adhesion and platelet aggregation in the microcirculation, thereby amplifying the inflammatory response ¹⁰. However, microcirculatory changes during ongoing anaphylaxis have not been assessed using FCFM. Nevertheless, the volume loss was quantified in a few anaphylactic patients ¹¹⁻¹². In these patients, changes in vascular permeability might permit a 50% transfer of the intravascular fluid into the interstitial space, within 15 min. following anaphylactic shock onset ¹¹.

Based on these studies, we hypothesized that the microcirculation could be relevant diagnostic and therapeutic targets for treatment of anaphylaxis. Accordingly, important questions need to be addressed. Does capillary leakage really occur in the course of

anaphylactic shock? And if so, in which time frame does it occur? Is this capillary leakage quantifiable? How is the kinetic profile of this capillary leakage? In order to answer these questions, we used a DCE acquisition using FCFM in an anesthetized ovalbumin-induced Brown Norway rat model of anaphylactic shock to assess the microcirculatory changes during ongoing anaphylaxis.

Materials and Methods

Animals and sensitization protocol

We used ten week-old male Brown Norway rats (Janvier, Le Genest-St-Isle, France). The animals were managed in accordance with the American Physiological Society Institutional Guidelines and position of the American Heart Association on Research Animal Use. Animal care and use was performed by qualified individuals and supervised by a veterinarian, and all facilities and transportation complied with current legal requirements. The study was approved by the Animal Ethics Advisory Committee of the University Paris Descartes. Rats were sensitized by grade VI chicken egg albumin (Sigma-Aldrich, Saint-Quentin Fallavier, France) at day 0 (D₀), D₄ and D₁₄, as previously described^{1,13-14}.

Instrumentation

The Cell-viZio™ device (Mauna Kea Technologies, Paris, France) is a fibered FCFM allowing *in vivo*, real-time fluorescence microscopy. Tissues are illuminated by a laser source at a defined wavelength. Fluorescent molecules, either spontaneously present, or injected as a contrast agent, absorb this light and re-emit a new fluorescent light at a longer wavelength. A 1.5-mm-diameter flexible probe consisting of a bundle of 30,000 optical fibers is placed in contact with the organ of interest, with a spatial resolution of 1.5 μm² in-plane, and a section thickness of approximately 15 μm. The laser unit provides an excitation wavelength of 488 nm and emission band wavelengths between 500 and 650 nm. The fluorescence signal is

collected by the same fibers as those used for illumination, and video data are recorded at a temporal resolution of 12 frames.sec⁻¹. In the present study, we used fluorescein isothiocyanate-dextran (FITC-dextran), which is a macromolecular fluorescent contrast agent with a molecular weight of 70 kDa (Sigma-Aldrich, Saint-Quentin Fallavier, France), and respective excitation and emission wavelengths of 488 nm and 520 nm, compatible with our imaging system.

Animal preparation and fluorescence microscopy imaging

The surgical procedure was performed under general anesthesia on day 21 (D₂₁) using 60 mg.kg⁻¹ intra-peritoneal sodium pentobarbital (Sigma-Aldrich, Saint-Quentin Fallavier, France) and maintained with intravenous additional doses (2 mg.kg⁻¹) when required. Rectal temperature was maintained at 38 ± 0.5°C by intermittent warming with a heating blanket (Harvard Apparatus, Les Ulis, France). A fluid-filled polyethylene catheter (internal diameter: 0.58 mm, external diameter: 0.96 mm) (Harvard Apparatus, Les Ulis, France) was inserted in the right common carotid artery for mean arterial blood pressure (MAP) and heart rate monitoring. Another fluid-filled catheter was inserted in the left external jugular vein for administration of drugs and fluid maintenance (10 ml.kg⁻¹.h⁻¹) with 9 ‰ sodium chloride injection USP (Baxter, Maurepas, France). Tracheotomy was performed and the lungs were mechanically ventilated with 100% oxygen using a Harvard Rodent respirator model 683 (Harvard Apparatus, Les Ulis, France) (rate: 60 c.min⁻¹, tidal volume: 1 ml). MAP and heart rate were recorded using a strain gauge catheter transduction coupled to a computer via Emka hardware and IOX Software (EMKA Technologies, Paris, France) for continuous digital recording. Hemodynamic values were allowed to stabilize for 30 min (stabilization period). The mesentery was surgically exposed through a mid-abdominal incision for *in vivo* visualization. All exposed tissue was covered with warmed and saline-soaked gauze to maintain tissue temperature and avoid tissue dehydration. The mesentery was placed on a

homemade platform to minimize respiratory motion. The optical probe of the fluorescence microscope was placed in contact of the mesentery and stabilized, to ensure a steady image during and after FITC-dextran intravenous injection (150 mg/kg diluted in 200 μ l of 9 ‰ saline, administered over 30 sec) allowing *in vivo* visualization of the mesenteric microvascular network⁹. After a period of 5 minutes allowing for stabilization of the contrast agent concentration, a 5-sec movie was recorded to obtain baseline signal intensity (SI) in capillaries and interstitium. The laser source was turned off between each movie to minimize tissue heating and contrast agent bleaching (*i.e.* irreversible fluorescence loss when the contrast agent is submitted to high energy and prolonged illumination).

Randomization of animals

Animals were randomly allocated into two groups: control or anaphylaxis (n = 6 in each group), with 9 ‰ saline (500 μ l) or ovalbumin (1 mg diluted in 500 μ l of saline) respectively, injected intravenously over one minute. Time 0 (T0) corresponded to the beginning of ovalbumin or saline injection. Pre-anaphylactic shock or control values of MAP and heart rate were recorded just before (T0-5 min/baseline) and during ovalbumin or saline injection (T0). Hemodynamic variables were then continuously measured until T0+30 min (Fig. 1).

***In vivo* microvascular permeability measurement using fluorescence microscopy**

In both groups, baseline images of the mesenteric microcirculation were recorded at T0-5 min. Starting at T0, 5-sec movies were then recorded every 30 sec for 30 min. (T0+30 min). Following the end of experiments, rats were killed by exsanguination. Visual examination of the movies was first performed, to evaluate the absence or presence of contrast agent leakage into the interstitium as well as its spatial distribution (homogeneous or heterogeneous). Raw amplitude movies were transferred to a workstation for processing using a MATLAB-based (version 7.2 Mathworks, Natick, MA) custom-made software

(PhysioD3D)¹⁵, allowing measurements of fluorescent SI over time reflecting the quantity of contrast agent in the tissue. A region of interest, including both vessels and interstitium, was drawn on the whole image (90195-92070 pixels) and automatically propagated to all the images in the relevant movies. The region of interest propagation yielded a SI curve over time reflecting changes of contrast agent quantity in tissues during the experiment. SI was averaged over the first five images of each movie to improve the signal-to-noise ratio and was divided by the SI in the image at T0-5 (SI₀), yielding normalized SI (SI/SI₀) curves over time for each animal. The shape of the SI curves was analyzed for SI increase in total tissue reflecting capillary leakage from the vessels into the interstitium. Two parameters were calculated for quantification of capillary leakage: i) the delay between T0 and the beginning of capillary leakage also called minimum time-to-leak (min); ii) the magnitude of contrast agent leakage was assessed as the ratio of perivascular intensity (Ip) to intravascular intensity (Ii) and expressed as follows: index leakage (%) = $\Sigma [(Ip1/Ii1) + (Ip2/Ii2) + \dots + (Ipn/Iin)] \times 100/n$; where n is the number of measured portions in each rat^{8,16}. Thus, the SI within three different capillaries and contiguous interstitial areas was averaged at each of the following time points: T0-5 min, T0, and every minute until T0+10 min.

Statistical analysis

Data are expressed as mean \pm SD or mean \pm SD [95% confidence interval: CI_{95%}]. Intra and between groups comparisons of hemodynamic variables were performed using repeated measures one-way and two-way analysis of variance (Instat, Graphpad, San Diego CA). When a significant interaction was detected by analysis of variance, selected paired comparisons were performed using the Fisher exact test. Between-group comparison of minimum time-to-leak and index leakage at each time point was performed using the rank sum test. Intra group comparison was performed at each time point compared to baseline (T0-5 min.) using the signed rank test. Individual values of index leakage were plotted against

individual values of mean arterial blood pressure for each time point (T0-5 min, T0 and every minute until T0+10 min) and were correlated using a Pearson correlation test. All p values were two-tailed and a p value < 0.05 was considered significant.

Results

Twelve ovalbumin- or saline-treated Brown Norway rats (weight on the day of, but before shock induction: 302 ± 6 g) were studied (6 in each group).

***In vivo* hemodynamic measurements**

The MAP values did not significantly differ between the two groups during the pre-shock (T0-5 min/baseline) and T0 time points (Fig. 2). MAP values remained unchanged in the control group throughout the entire study. In anaphylactic rats, MAP values showed a continuous decrease over time when compared to baseline, starting at T0+1 min ($p < 0.001$). At T0+5 min and T0+30 min, mean MAP values showed a decrease of 60% and 70% compared to baseline ($p < 0.001$), significantly lower ($p < 0.001$) when compared to control rats. The between-group difference ($p < 0.001$) appeared at T0+1 min and persisted thereafter.

Heart rate measurements

Heart rate remained unchanged in both groups from T0-5 min through T0+5 min. Heart rate values remained unchanged in the control group throughout the entire study. In anaphylactic rats, a significant ($p < 0.05$) decrease was observed by T0+10 min compared with baseline heart rate, and this decrease persisted for the remainder of the *in vivo* recording, as previously reported¹⁷ (*data not shown*).

***In vivo* microvascular permeability measurement using fluorescence microscopy**

In the control group, FITC-dextran remained in the capillaries during the entire study period (Fig. 3A and 3B). SI curve showed an early decrease probably due to initial bleaching of the contrast agent without subsequent increase over time (Fig. 4). In anaphylactic rats, a

progressive fluorescence increase in the interstitium was observed over time (Fig. 3C and 3D). The SI curve also showed the initial decrease, followed by a constant and progressive global increase reflecting the increase in fluorescence in the interstitium (Fig. 4). In four rats, a heterogeneous distribution of the contrast agent extravasation was observed, with interstitial areas of high fluorescence enhancement and others where no leak of contrast agent into the interstitium could be seen. In anaphylactic rats, increase of SI in the interstitium did not allow differentiation of interstitium from capillaries after 10 min.

In the controls rats, mean index leakage values did not significantly differ from baseline at each time point. In anaphylactic rats, mean index leakage showed a significant difference from baseline ($p = 0.03$), with a mean minimum time-to-leak of 5 ± 3 min [CI_{95%}: 4-12 min] after shock onset. Mean index leakage calculated at each time point differed between control and anaphylactic rats starting at T0 + 2 min. (respectively 20 ± 11 vs. $170 \pm 127\%$; $p = 0.002$) and progressively increasing (Fig. 5) up-to 25-fold during the time of observation. A good correlation was found between individual values of index leakage and arterial blood pressure in both groups ($r: -0.75$, $p < 0.01$) (Fig. 6).

Discussion

The main findings of this study were: i) interstitial capillary leakage occurred a few minutes after anaphylactic shock onset; ii) this macromolecular capillary leakage showed up to 25-fold increase when compared to control conditions and; iii) this leakage correlated to the drop in arterial blood pressure during the early stage of anaphylaxis. These results have both mechanistic and therapeutic implications.

The experimental model and the study design were chosen in order to reproduce the clinical conditions experienced by patients under anesthesia when anaphylactic shock occurs. We therefore deliberately decided to perform our study in rats experiencing true *in vivo*

anaphylaxis and not in a model that attempts to mimic acute inflammation-like conditions using histamine as previously shown ¹⁸. However, extrapolation of our results to humans should be cautious. We selected FITC-dextran 70 KDa as its molecular weight is close to that of serum albumin, therefore mimicking physiological macromolecules. Like albumin, FITC dextran remains in the vascular compartment under normal conditions ⁹. In cases of increased permeability, the macromolecule leaks and accumulates in the interstitium ⁶. Real-time changes in capillary permeability underlying anaphylactic shock were visualized by means of FCFM that provides *in vivo* microvascular observations in order to evaluate the magnitude of macromolecular leakage as an indicator of endothelial alterations.

In the present study, macromolecular capillary leakage started within the first two minutes after anaphylactic shock onset. This macromolecular leakage was progressive, after shock onset with an up to 25-fold increase during the first 10 min. of observation, and was followed by a further global capillary leakage in the mesenteric compartment during the remaining study period. A possible explanation of our findings could have been that excitation of FITC-dextran *per se* influences capillary permeability ¹⁹. However under control conditions, macromolecular leakage did not develop over time suggesting that the responses were specific to anaphylaxis and not to the illumination itself. Interestingly, the minimum time-to-leak and the onset of cardiovascular disturbances occurred within the same time frame, *i.e.* 1-2 min, after shock onset suggesting a relationship between the two phenomena. Accordingly, the magnitude of the macromolecular capillary leakage was highly correlated to the decrease in arterial blood pressure. Thus, it was consistently low in controls, and progressively increased with the arterial blood pressure drop in anaphylactic rats, starting 2 min after shock onset and persisting until 10 min after. Past the first 10 min of observation, massive extravasation did not allow further analysis of SI. These findings are consistent with previous clinical reports where the component resulting from plasma losses, 10 and 15 min

after anaphylactic shock onset has been estimated to be respectively as high as 35% and 73% of the circulating blood volume¹¹⁻¹². Others have reported the occurrence of bradycardia also called “*paradoxical bradycardia*” despite severe hypotension in as many as 10% of patients experiencing perioperative anaphylaxis³. This “*paradoxical bradycardia*” allows the ventricles to fill before they start contracting again despite a massive hypovolemia²⁰⁻²¹. Consequently, the occurrence of bradycardia during the early course of anaphylaxis might reflect the ongoing marked macromolecular capillary leakage with subsequent hypovolemia. We therefore suggest that the inflammation-induced microcirculatory changes with subsequent intravascular fluid transfer into the interstitial space might be involved in the onset of anaphylactic hypotension. Finally in most (66%) anaphylactic rats, the progressive enhancement in the interstitium had a heterogeneous spatial distribution, with zones seeming to leak more intensively than others. This may reflect differential changes in capillary permeability according to microvessels similar to spatial heterogeneity of capillary perfusion and decrease in functional capillary density as reported during sepsis²².

One of the major objectives of this study was to more closely examine the contribution of the microvascular alterations elicited by anaphylactic shock. Anaphylaxis is an acute inflammatory IgE-mediated response to a foreign antigen due to massive release of inflammatory preformed mediators from sensitized mast cells and basophils such as histamine and *de novo* synthesized mediators²¹. Our study does not elucidate the mechanisms of capillary leakage occurring during anaphylaxis. Previous studies showed transient increased venular permeability in the rat mesenteric microcirculation beginning 1 or 2 min after histamine suffusion with peaks between 5 and 15 min after and return to control levels at 20-30 min²³ that were attributable to venular junctional gaps *via* activation of endothelial H₁-receptors²⁴. Other mechanisms might however also be involved²⁴. Histamine also leads to increased nitric oxide (NO) production from endothelial cells¹⁸ while endothelial NO

synthase (eNOS) increases microvascular permeability to macromolecules in response to inflammatory agents ²⁵. Thus, eNOS has been suggested to be the primary vasodilator during anaphylactic shock ²⁶, suggesting that histamine might induce an increase in microvascular permeability to macromolecules *via* NO ²⁷ at an early stage of anaphylaxis. Further experimental studies are required to validate these hypotheses.

There are some limitations to our study. Optical imaging signal intensity is the result of a combination of complex phenomena including reflection, absorption and fluorescence of photons. Despite turning off the laser between acquisitions, an important SI decrease was observed in the first minute due to photobleaching of the contrast agent. This signal loss was so intense that it precluded detection of any other optical phenomenon, and it is therefore possible that capillary leakage of macromolecules occurred before the minimum time-to-leak we observed but was undetectable. The main limit of the complex relationship between SI and concentration of contrast agent is that it does not allow absolute quantification of volume nor rate of macromolecular leakage. In the present study, macromolecular leakage was qualitatively analyzed by SI curves over time and semi-quantitatively using the index leakage. It may be noted that the index leakage is not null in baseline conditions (approximately 20% before saline or albumin injection), due to the presence of spontaneous signal in the interstitium despite the absence of contrast agent ²⁸.

The present study suggests therapeutic implications. Since capillary leakage occurs immediately after anaphylactic shock onset and correlates to the drop in arterial blood pressure, these findings may contribute to the decreased preload and the onset of inaugural hypotension. The exact requirement for fluid therapy remains unknown during anaphylaxis and has not been investigated. If the use of crystalloids followed by colloids is usually recommended by the current clinical guidelines ²⁻⁴, some authors however estimate that *there is no evidence that one is better than the other (ie. crystalloids vs colloids)* ³.

In conclusion, we provide information on microcirculatory changes during ongoing anaphylaxis and demonstrate that in this experimental model, increased capillary leakage occurs early during anaphylactic shock. The inflammation-induced microcirculatory changes with subsequent intravascular fluid transfer into the interstitial space (hypovolemic component) might be involved in the onset of anaphylactic hypotension. Further work is needed to study the mechanisms of capillary leakage during anaphylaxis.

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Figures

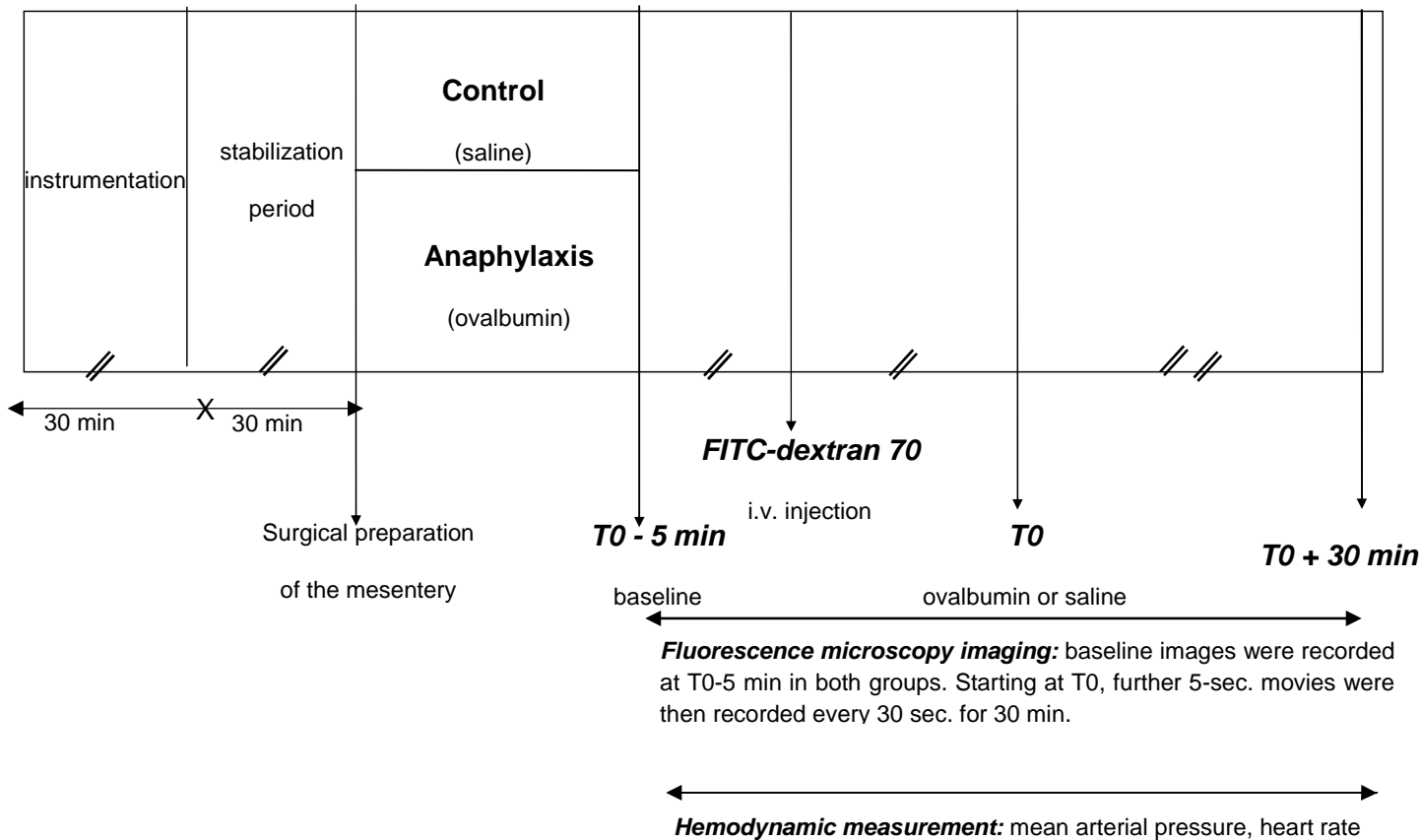


Figure 1: Experimental design of measurements and animal allocation: control (saline) and anaphylaxis (ovalbumin) groups ($n = 6$ rats in each group). Time 0 (T_0) corresponds to the injection of saline (control) or ovalbumin (anaphylaxis). Pre-anaphylactic or control values of MAP and heart rate were recorded after allocation of the 2 groups of rats and before anaphylaxis induction or saline injection ($T_0 - 5 \text{ min}$ /baseline). After a short period of stabilization of the contrast agent concentration, a 5-sec movie was recorded to obtain baseline signal intensity (SI) in tissues followed by further 5-sec movies for 30 min ($T_0 + 30 \text{ min}$).

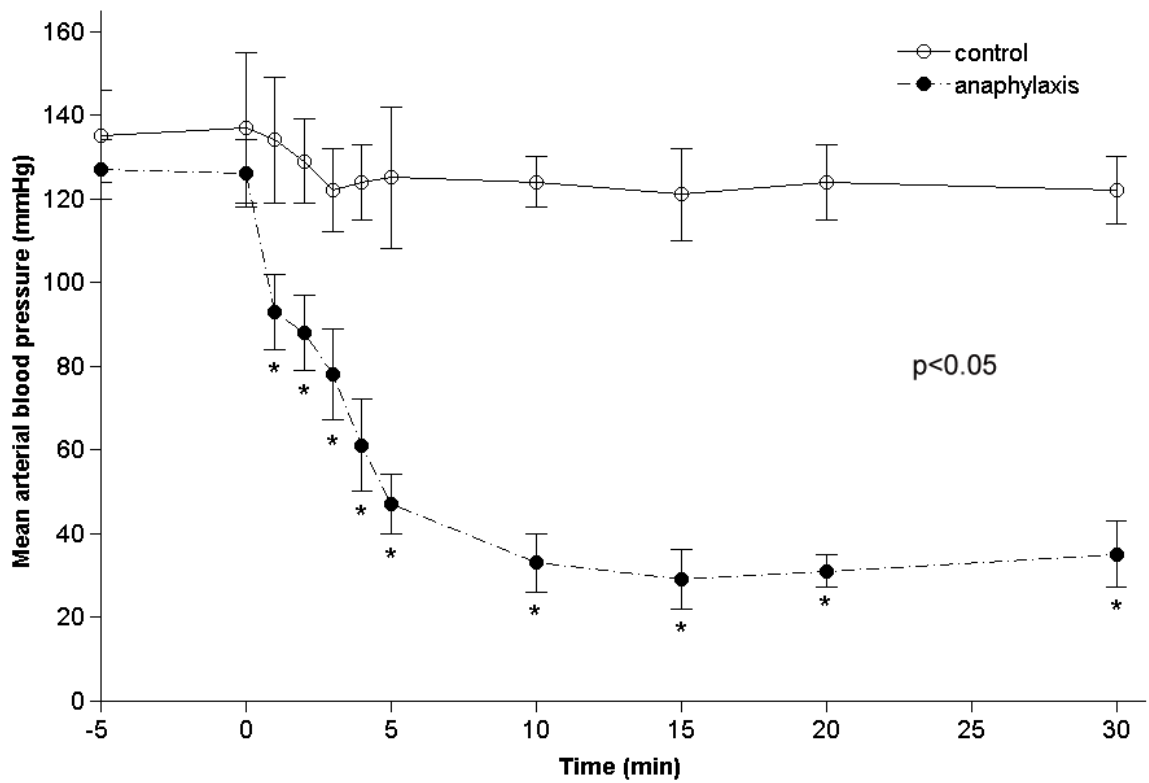
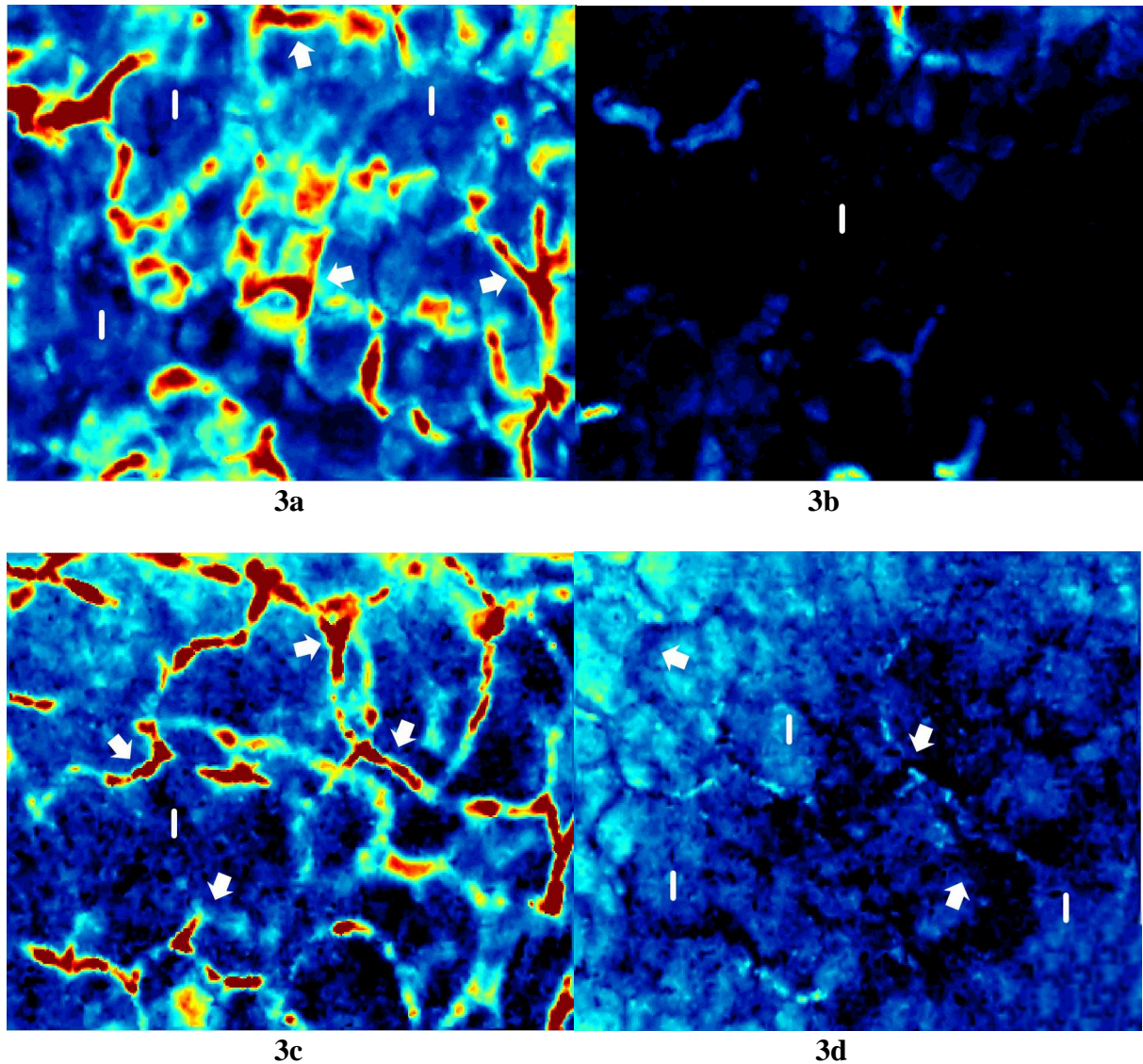


Figure 2: Mean arterial blood pressure (MAP, mmHg) profiles in control and anaphylaxis groups (n = 6 rats in each group). Values are expressed as mean \pm SD. Time 0 (T0) corresponds to the injection of ovalbumin (anaphylaxis) or saline (control). * Intra-group difference: $p < 0.05$ vs T0 - 5 min (baseline). P value refers to global between group difference.



Figures 3: Colour maps of signal intensity in control rats at T0 (Fig. 3a) and 10 min. (T0 + 10 min.) after saline injection (Fig. 3b), and in anaphylactic rats at T0 (Fig. 3c) and 10 min. (T0 + 10 min.) after ovalbumin injection (Fig. 3d). Signal intensity (SI) was represented using a color scale ranging from black (void of SI) to blue (low SI) and red (high SI).

In control rats, FITC-dextran remained in the capillaries during the whole time of observation. At T0, SI is high in capillaries (red) and low in interstitium (black) (Fig. 3a). No SI could be observed in the interstitium, even at 10 min. Thus, global SI decreases progressively in the image, due to bleaching (Fig. 3b).

In anaphylactic rats, FITC-dextran remained in the capillaries before ovalbumin injection (T0); SI is high in capillaries (red) and low in interstitium (black) (Fig. 3c). Following ovalbumin injection (T0), an early and progressive fluorescence increase in the interstitium was detected. Thus, 10 min. after ovalbumin injection (T0 + 10 min.) (Fig. 3d), the interstitium showed a higher SI (blue), while capillaries appeared black, due to bleaching.

I: interstitium. The arrows show some selected capillaries.

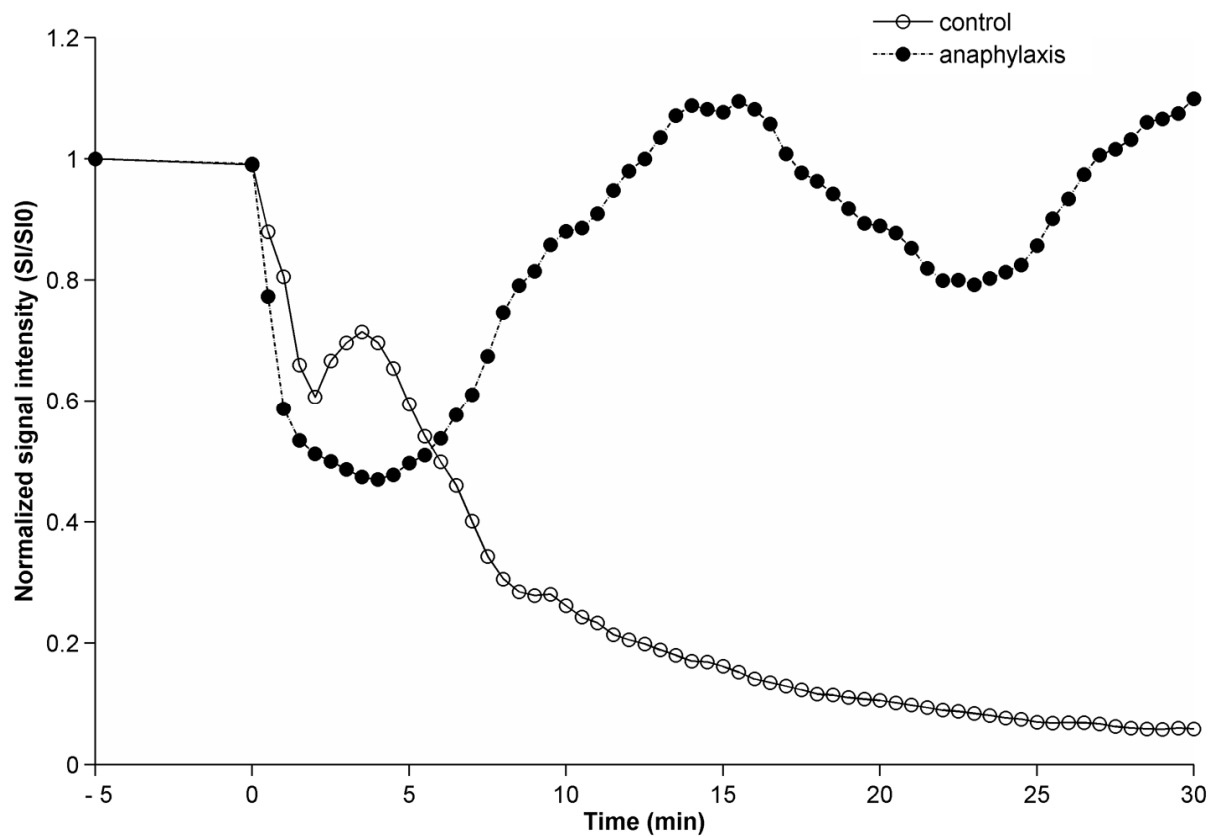


Figure 4: Measurements of fluorescence intensity in the mesenteric microcirculation of control and anaphylactic rats ($n = 6$ in each group). In both groups, normalized signal intensity (SI/S_0) curve showed an early decrease due to initial bleaching of the contrast agent (FITC-dextran 70). In control rats, no subsequent SI increase was observed during the remaining study period, reflecting the absence of fluorescence increase in the interstitium. In contrast, in anaphylactic rats, the initial SI decrease was followed by a constant and progressive global SI increase reflecting the increase in fluorescence by leakage of the macromolecular contrast agent in the interstitium.

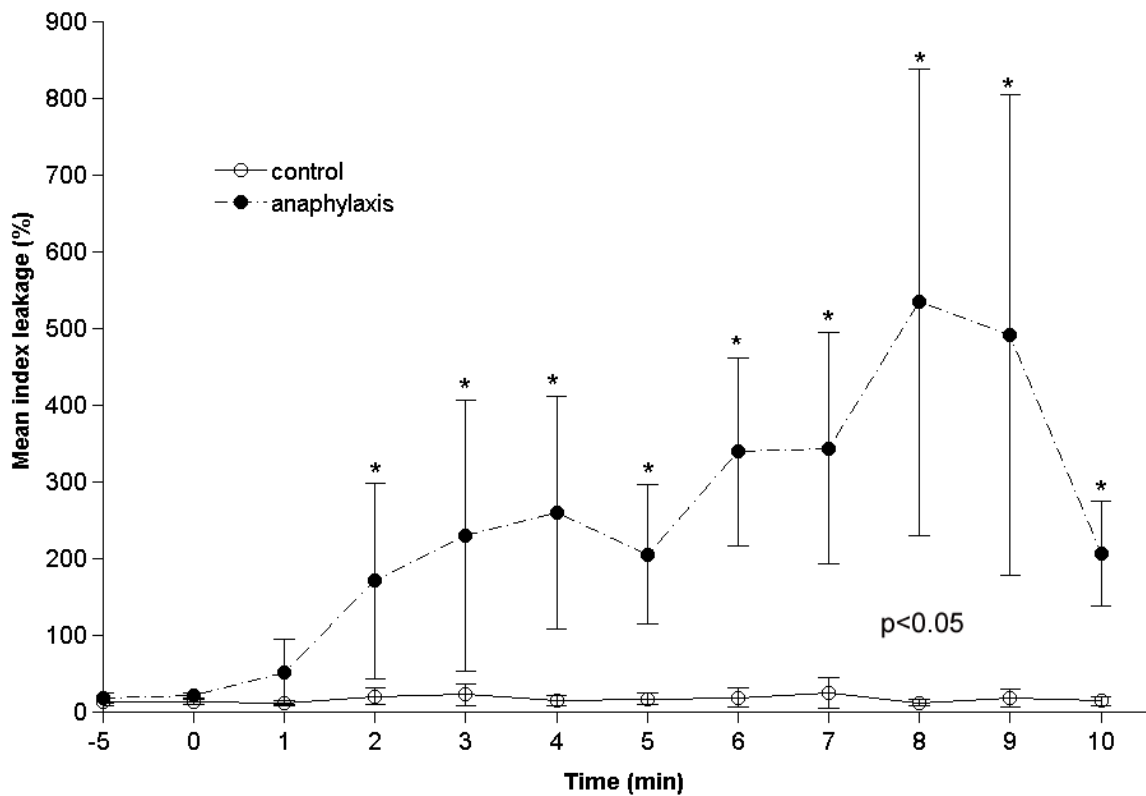


Figure 5: Time course changes of mean contrast agent leakage in the mesenteric microcirculation in control and anaphylactic groups (n = 6 rats in each group). Values are expressed as mean \pm SD. The magnitude of contrast agent leakage was assessed as the ratio of perivascular intensity (I_p) to intravascular intensity (I_i) from three different capillaries and expressed as follows: index leakage (%) = $\Sigma [(I_{p1}/I_{i1}) + (I_{p2}/I_{i2}) + (I_{p3}/I_{i3})] \times 100/3$. In control rats, mean index leakage values did not significantly differ from baseline at each time point. In anaphylactic rats, mean index leakage showed a significant difference from baseline starting at T0 + 2 min (p = 0.03) and persisting thereafter. * p < 0.05 vs T0 - 5 min. P value refers to global between group difference.

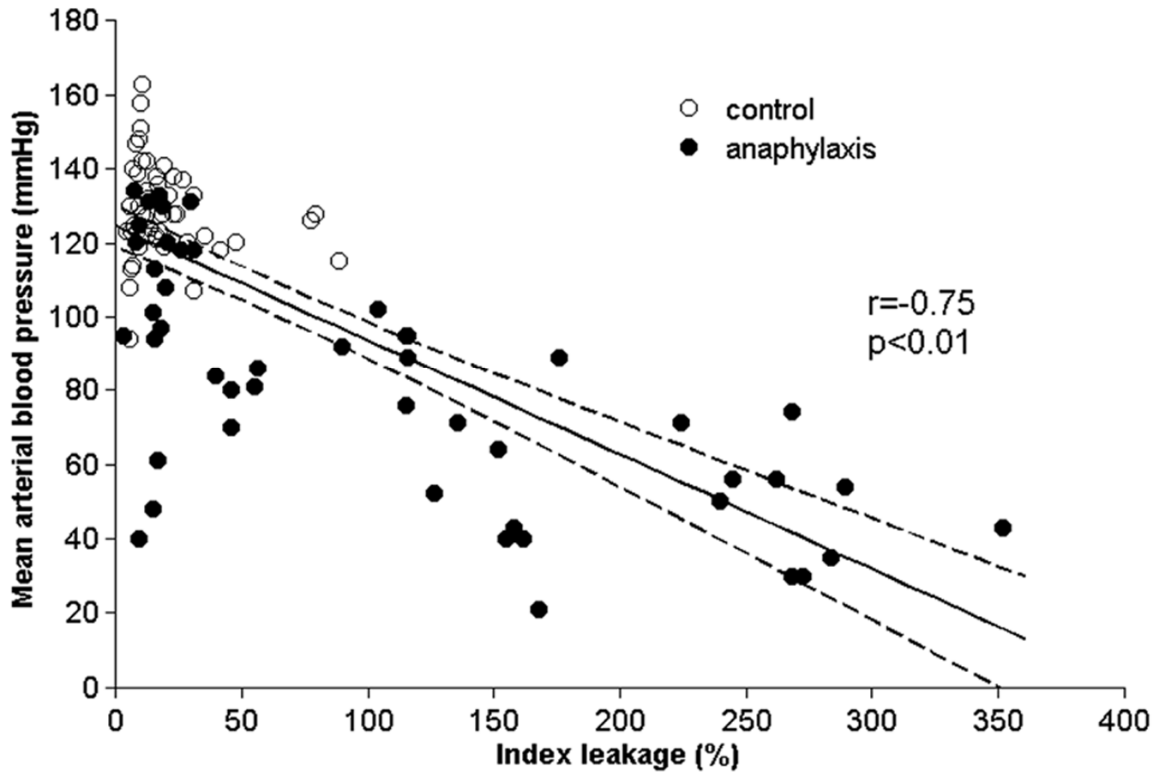


Figure 6: Individual values of index leakage (%) in control and anaphylactic groups (n = 6 rats in each group), were plotted against individual values of mean arterial blood pressure (mmHg) for each time point (T0-5 min, T0 and every minute until T0+10 min), yielding a good correlation. Continuous line represents the linear regression for the two parameters, and dotted lines its CI_{95%}.