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Prolonged hypervolemic hemodilution decreases functional capillary density of ileal mucosa in pigs revealed by sidestream dark-field imaging*

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Abstract: Objective: Hemodilution changes the physical properties of blood by reducing its hematocrit and blood viscosity. We tested whether prolonged hypervolemic hemodilution (HHD) impairs functional capillary density (FCD) of ileal mucosa in healthy mechanically-ventilated pigs and if there is any correlation between changes in FCD of ileal and sublingual mucosas during HHD. Methods: Sixteen domestic female pigs were anesthetized, mechanically-ventilated, and randomly assigned to the HHD (20 ml/(kg·h) Hartmann's solution for 3 h) or fluid restrictive (5 ml/(kg·h) Hartmann's solution for 3 h) group. Microcirculations of sublingual and ileal mucosas via ileostomy were visualized using sidestream dark-field (SDF) imaging at baseline conditions (t=0 h) and at selected time intervals of fluid therapy (t=1, 2, and 3 h). Results: A significant decrease of ileal FCD (285 (278–292) cm/cm^2) in the HHD group was observed after the third hour of HHD when compared to the baseline (360 (350–370) cm/cm^2) (P<0.01). This trend was not observed in the restrictive group, where the ileal mucosa FCD was significantly higher after the third hour of fluid therapy as compared to the HHD group (P<0.01). No correlation between microhemodynamic parameters obtained from sublingual and ileal mucosas was found throughout the study. Conclusions: Prolonged HHD established by crystalloid solution significantly decreased ileal villus FCD when compared to restrictive fluid regimen. An inappropriate degree of HHD can be harmful during uncomplicated abdominal surgery.

Key words: Hypervolemic hemodilution, Intestinal microcirculation, Sidestream dark-field imaging
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1 Introduction

Acute normovolemic hemodilution (ANH) involving withdrawal of whole blood with parallel infusion of fluids maintaining normovolemia was introduced into clinical practice in the 1970s (Messmer *et al*., 1972; Laks *et al*., 1973) and was accepted as an appropriate alternative to reduce the need for allogeneic transfusion during surgery (Bryson *et al*., 1998). Recent meta-analysis concerning ANH in 42 trials has shown only modest benefits from preoperative ANH mainly due to methodological differences and the lack of properly designed controlled trials (Kreimeier and Messmer, 1996; Segal *et al*., 2004). Some previous clinical trials have used acute preoperative volume expansion without any blood removal and established this relatively new technique as hypervolemic hemodilution (HHD) (Trouwborst *et al*., 1990a; 1990b; Mielke *et al*., 1997). Very little is known about the efficacy and microhemodynamic effects of HHD compared to ANH. Some reports even have suggested replacing ANH with HHD, which is easier and more timesaving to perform (Entholzner *et al*., 1992; Mielke *et al*., 1997). Previous mathematical

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analysis has shown almost identical postoperative hematocrits in the ANH and HHD groups for blood loss <40% of blood volume (Singbartl *et al*., 1999).

Macrohemodynamic alterations associated with hemodilution have been well-described previously (van der Linden *et al*., 1994; Robotham, 2004); however, the effects of ANH and especially HHD on microcirculation and their clinical significance remain subjects of intensive experimental investigation. Hemodilution changes the physical properties of blood by reducing its hematocrit and thus decreasing the oxygen-carrying capacity of blood and blood viscosity (Tsai *et al*., 1998), which is determined mainly by the concentration of red blood cells (RBCs). Microcirculatory blood flow is governed by perfusion pressure, vessel radius, vessel length, and blood viscosity. Immediate perfusion pressure and vasomotor reactivity of the microcirculation are controlled by autoregulation, which is directly dependent on neural and metabolic factors (Eckmann *et al*., 2000). Restoration of blood viscosity during hemodilution is important, because it maintains functional capillary density (FCD) (Cabrales *et al*., 2006), which is defined as the number of RBCs-perfused capillaries per unit surface of the field of view at the microscopic level. FCD is an indicator of tissue perfusion and the homogeneity of tissue oxygenation (Tsai *et al*., 1995; Groner *et al*., 1999). Recent studies have indicated microvascular function and tissue survival impairment under the terms of decreased blood viscosity by progressive hemodilution which could be explained by microvascular blood flow maldistribution, rather than by a deficit in oxygen delivery (Tsai *et al*., 1998; Cabrales *et al*., 2006). Another aspect of adequate organ perfusion is the tissue oedema formation due to increased vascular permeability or excessive fluid loading which may lead to impaired oxygen diffusion and cellular hypoxia resulting from intercapillary area expansion that consequently limits gas diffusion (Leach and Treacher, 2002).

Sidestream dark-field (SDF) imaging as a successor of orthogonal polarization spectral (OPS) imaging is a new improved optical method for the visualization of mucosal and organ surface microcirculation based on the interaction between green light and oxy-/deoxy-hemoglobin of flowing RBCs. Principles and validation studies including low hematocrit states have been published and reviewed in detail previously (Harris *et al*., 2002; Cerny *et al*., 2007).

The primary goal of the present study was to evaluate the microhemodynamic effects of prolonged HHD on sublingual and ileal mucosal microcirculations visualized by SDF technology in anesthetized mechanically-ventilated pigs. We hypothesized that prolonged HHD can significantly reduce FCD of investigated tissues. The secondary goal was to assess if there is any correlation between changes in FCD of ileal and sublingual mucosas during HHD.

2 Materials and methods

All experimental procedures were performed after the University Ethical Board approval in accordance with Czech legislation on the protection of animals. Sixteen domestic female pigs (36–44 kg) were used in this study. Animals were fasted for 24 h before the experiment with free access to water. Anesthesia and surgical procedures were performed as described in detail previously (Mehta *et al*., 1999; Tugtekin *et al*., 2001; Martikainen *et al*., 2003; Pittner *et al*., 2003). Briefly, for the experiment, the anaesthesia was induced with intramuscular (IM) injection of ketamine 20 mg/kg, azaperone 4 mg/kg, and atropine 0.05 mg/kg. The ear vein was cannulated, and the animals were intubated and mechanically ventilated with a tidal volume of 10–15 ml/kg at fraction of inspired oxygen (FiO_2) 40% using a 900C Servo ventilator (Siemens-Elema, Sweden). Anesthesia and analgesia were maintained with continuous intravenous (IV) infusion of midazolame 0.3 mg/(kg·h) and fentanyl 30 μg/(kg·h) during the surgical manipulation, after which fentanyl dosage was reduced to 5 μg/(kg·h). Neuromuscular blockade was achieved with continuous IV infusion of pancuronium bromide 0.2 mg/(kg·h).

The right femoral artery was cannulated for blood sampling and blood pressure monitoring, right jugular vein was punctured for pulmonary artery catheter and central venous catheter (pressure transducers Gabarith PMSET 1DT-XX, Becton Dickenson, Singapore; monitoring system Datex-Ohmeda S/5, Instrumentarium Corp., Helsinki, Finland). Probes for electrocardiogram, oxygen saturation, and rectal temperature were inserted. A lower midline laparotomy was performed and the loop of the terminal

ileum was visualized and cautiously exteriorized. A small ileum segment, 20 cm proximal to ileocaecal valve, was opened along its antimesenteric border and the edges fixed at the skin with the mucosal surface facing upward to build a double-lumen ileostomy. Cystostomy with urinary catheter placement was built from the same abdominal incision. The abdomen was closed and the ileal stoma covered by a thin plastic wrap to minimize dehydration and heat loss (Harris *et al*., 1998).

Experimental protocol of HHD was established as follows. A stabilizing period of 1 h with a basal fluid intake of 10 ml/(kg·h) followed after instrumentation. HHD was established using Hartmann's solution at the rate of 20 ml/(kg·h) for 3 h (HHD group, n=8), while fluid intake in restrictive group (n=8) was 5 ml/(kg·h). Macrohemodynamic parameters were monitored and recorded throughout the study, microhemodynamic measurements and recording procedures at sublingual and ileal regions as well as haematocrit measurements were performed at baseline conditions (t=0 h) and at selected time intervals of fluid therapy (t=1, 2, and 3 h).

A standardized protocol for SDF imaging procedure has been used as described previously (Turek *et al*., 2008). To minimize artificial pressure and movement artifacts, the SDF imaging probe (MicroScan Video Microscope, Microvision Medical Inc., Amsterdam, the Netherlands) was attached to a custom made (Arrow International CR, a.s., Czech Republic) flexible arm with special adapter allowing micromovement of the SDF probe in various axes according to inclination of the flexible arm (Fig. 1). In an effort to objectify the SDF imaging of microcirculation as much as possible, the following methodology was established. Once a sector for imaging was selected, the SDF imaging probe was placed just 1 mm above the target tissue using a flexible arm. Then the arm was fixed and the probe covered with plastic lens was moved towards tissue by the adapter for micromovement. Immediately after the first contact with the investigated organ, the focus ring of the probe was used to bring the proper layer into focus to create a sharp and high contrast image appropriate for later off-line analysis. All SDF imaging data of the microcirculation were digitally recorded. SDF images were obtained from at least three different areas within the site of interest as recommended recently (Boerma *et al*., 2005). Each area was recorded for a duration of 2 min and saved.

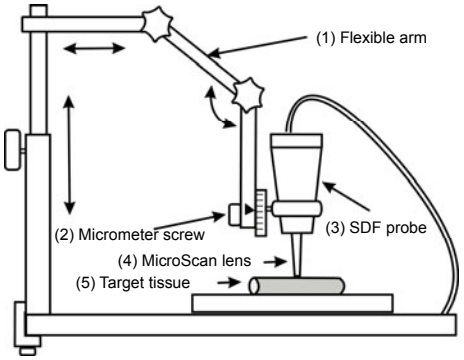

Fig. 1 Scheme of videomicroscopy setting
Flexible arm allows horizontal, vertical, and rotating movements of adapter with micrometer screw fixating sidestream dark-field (SDF) imaging probe equipped with MicroScan lens intended for contact with target tissue (Turek *et al*., 2008)

For each measurement, video files were randomly coded and analyzed off-line using AVA V1.0 software (AMC, University of Amsterdam, the Netherlands) by an observer blinded as to the file order. Sublingual and ileal mucosal microcirculations were assessed at each measurement time interval according to the protocol described below. Off-line selection of the most stable clips with clear images for final analysis was performed. The scheme for off-line analysis was established with regard to different characters and structurizations of sublingual and ileal mucosal microcirculations; current recommendations for assessment of microcirculation in sublingual and intestinal mucosas were endorsed (Nakajima *et al*., 2001; de Backer *et al*., 2007).

The following parameters were analyzed off-line from the sublingual mucosa:

1. FCD (cm/cm^2 or μm/μm^2) defined as the length of RBCs-perfused capillaries per observation area. After proper calibration antecedent to analysis, FCD was calculated as ratio of total vessel length to image area using AVA V1.0 software.

2. Proportion of continuously perfused vessel (PPV; %) calculated as: $(n_t-n_n-n_i)/n_t\times100\%$, where n_t is total number of vessels, n_n is the number of vessels without flow, and n_i is the number of vessels with intermittent flow.

Small vessels, especially capillaries, were separated from large vessels using a 20-μm cut-off for

vessel diameter (de Backer *et al*., 2007).

The following parameters were analyzed off-line from intestinal (ileal) mucosa:

1. FCD (cm/cm^2 or μm/μm^2) defined as the length of RBCs-perfused villus capillaries per observation area.

2. Villus density (VD; %) expressed by the fraction of homogenously perfused villi versus total number of villi during each observation as a semiquantitative parameter (Nakajima *et al*., 2001).

The systemic hemodynamic data and fluid intake volumes are presented as mean±standard deviation (SD), and the microcirculatory non-normally distributed data are presented as mean (95% confidence interval (CI) of mean). Baseline and consecutive data were compared with analysis of variance (ANOVA) and Kruskal-Wallis test for repeated measurements, unpaired Student's *t*-test was used for differences between the treatment groups. A *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed with the use of SIGMASTAT 2.0 (Jandel Scientific, San Rafael, CA, USA).

3 Results

A total of 16 female pigs were used in this study. Baseline values for the systemic hemodynamic parameters of the restrictive and HHD groups were not significantly different (Table 1). Systolic blood pressure and cardiac output increased significantly after the third hour of fluid therapy (t=3 h) in the HHD group (P=0.02 and P=0.03, respectively) when compared to baseline. The values of systolic (P=0.02) and diastolic blood pressures (P<0.01), central venous pressure (P=0.02), pulmonary artery occlusion pressure (P=0.03), and cardiac output (P=0.03) were significantly higher in the HHD group after the third hour of HHD when compared to the restrictive group. Mean total fluid intake was (3300±175) ml in the HHD group and (985±115) ml in the restrictive group. There was a statistically significant decrease of haematocrit in the HHD group at t=3 h [(22±2.9)%] versus baseline [(32±3.1)%] (P<0.01).

Clear high contrast and stable images were successfully obtained from sublingual and ileal mucosas when using fixation by flexible arm for SDF probe as described above. The baseline (t=0 h) values of FCD from sublingual mucosa were 224 (220–229) cm/cm^2 in the HHD group and 216 (213–219) cm/cm^2 in the restrictive group, without significant differences (P=0.272) between groups. The baseline values of FCD from the surface of ileal mucosa were 360 (350–370) cm/cm^2 in the HHD group and 352 (347–357) cm/cm^2 in the restrictive group, and no statistically significant differences between groups (P=0.252) were detected. A significant increase of ileal mucosa FCD both in the HHD [380 (370–390) cm/cm^2] and restrictive [372 (366–378) cm/cm^2] groups was found after the second hour of fluid therapy (t=2 h) when compared to baseline (P<0.01). A significant reduction of ileal mucosa FCD [285 (278–292) cm/cm^2] was detected in the HHD group after the third hour of HHD (t=3 h) when compared to baseline (P<0.01) and to timepoint t=2 h (25% fall in FCD, P<0.01). This trend was not observed in the restrictive group, where the ileal mucosa FCD at t=3 h was significantly higher as compared to the HHD group (P<0.01). Impaired villi perfusion in the HHD group at t=3 h was also detected semiquantitatively (P=0.01) when using VD [82% (78%–85%)] versus baseline [96% (95%–97%)]. Analyses of FCD and PPV of sublingual mucosa have shown no significant differences between the groups during fluid therapy throughout the study. No correlation between microhemodynamic parameters obtained from sublingual and ileal mucosas under the terms of HHD was identified. Time trends of ileal and sublingual microcirculatory parameters both in the HHD and restrictive groups are summarized (Table 2).

4 Discussion

The presented experimental study focused on the effects of hypervolemia with a model of HHD in pigs at microcirculatory level. The main findings were that prolonged HHD established by crystalloid solution significantly decreased FCD of ileal mucosa visualized by SDF technology when assessed both quantitatively and semiquantitatively. Decrease of FCD by 25% in the HHD group was considered to be both statistically and clinically significant. The initial increase of ileal mucosa FCD both in the HHD and restrictive groups indicated that adequate initial fluid

Table 1 Blood gas values, hemodynamics, and hematocrit during the study

Parameter	Value				*P* (*t*=3 h vs. baseline)
	Baseline	*t*=1 h	*t*=2 h	*t*=3 h	
Restrictive group					
PaO_2 (mmHg)	153±12	161±19	149±16	145±14	0.25
$PaCO_2$ (mmHg)	42±4	40±4	43±5	44±6	0.50
Arterial pH	7.38±0.04	7.35±0.03	7.32±0.02	7.35±0.06	0.06
Heart rate (beat/min)	115±20	99±14	102±15	110±14	0.09
SBP (mmHg)	105±10	112±12	115±13	110±143	0.08
DBP (mmHg)	65±8	68±6	70±8	68±5	0.08
CVP (mmHg)	5.1±1.3	6.1±2.0	6.3±1.9	6.0±2.3	0.10
CO (ml/min)	2550±280	2620±305	2650±256	2610±279	0.30
mPAP (mmHg)	20.6±3.1	22.5±2.9	21.0±3.5	21.5±3.2	0.75
PAOP (mmHg)	4.5±0.7	4.2±0.5	4.7±0.8	4.8±0.8	0.64
Core temp. (°C)	37.5±0.4	37.4±0.5	37.2±0.5	37.6±0.4	0.80
Htc (%)	33±2.3	32±3.6	29±2.9	29±3.2	0.06
Lactate (mmol/L)	0.85±0.15	1.20±0.10	1.25±0.15	1.30±0.20	0.02
HHD group					
PaO_2 (mmHg)	155±16	165±18	131±15	115±12*	0.02
$PaCO_2$ (mmHg)	40±4	42±5	39±2	43±3	0.50
Arterial pH	7.33±0.05	7.30±0.06	7.29±0.04	7.28±0.06*	0.04
Heart rate (beat/min)	106±17	105±20	115±21	117±21	0.03
SBP (mmHg)	108±11	115±10	129±12	138±15*	0.02
DBP (mmHg)	63±9	70±8	76±10	86±10*	<0.01
CVP (mmHg)	5.6±1.5	6.4±2.0	7.9±2.3	9.2±2.9*	0.02
CO (ml/min)	2460±259	2730±285	2855±297	2953±270*	0.03
mPAP (mmHg)	21.4±4.4	22.0±4.0	20.4±3.5	22.9±3.0	0.68
PAOP (mmHg)	4.2±1.0	4.4±0.8	8.2±0.9*	10.5±0.7*	0.03
Core temp. (°C)	37.6±0.5	37.4±0.7	37.8±0.6	37.3±0.6	0.75
Htc (%)	32±3.1	31±2.2	27±2.5	22±2.9*	<0.01
Lactate (mmol/L)	0.75±0.12	1.10±0.15	1.05±0.10	1.10±0.20	0.10

PaO_2: arterial oxygen partial pressure; $PaCO_2$: arterial carbon dioxide partial pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; CVP: central venous pressure; CO: cardiac output; mPAP: mean pulmonary arterial pressure; PAOP: pulmonary artery occlusion pressure; Core temp.: core temperature; Htc: hematocrit; HHD: hypervolemic hemodilution. Data are presented as mean±SD. All *P* values are from ANOVA for repeated measurements. * Significance for the HHD group when compared to the restrictive group: $P<0.05$ (unpaired Student's *t*-test)

Table 2 Time trends of ileal and sublingual microcirculatory parameters during the study

Time of fluid therapy	Restrictive group				HHD group			
	Ileal mucosa		Sublingual mucosa		Ileal mucosa		Sublingual mucosa	
	FCD (cm/cm^2)	VD (%)	FCD (cm/cm^2)	PPV (%)	FCD (cm/cm^2)	VD (%)	FCD (cm/cm^2)	PPV (%)
Baseline	352 (347–357)	95 (93–97)	216 (213–219)	93 (91–95)	360 (350–370)	96 (95–97)	224 (220–229)	94 (93–95)
t=1 h	358 (352–364)	95 (94–96)	220 (217–223)	95 (93.5–96.5)	372 (363–381)	97 (95–99)	228 (224–231)	95 (94–96)
t=2 h	372 (366–378)*	98 (96.5–99.5)	224 (220–228)	96 (94–98)	380 (370–390)*	98 (97–99)	225 (222–228)	96 (95–97)
t=3 h	370 (365–375)*	98 (97–99)	220 (216–224)	96 (94.5–97.5)	285 (278–292)*†	82 (78–85)*†	221 (219–223)	94 (92.5–95.5)

HHD: hypervolemic hemodilution; FCD: functional capillary density; VD: villus density; PPV: proportion of continuously perfused vessel. Data are presented as means (95% CI of mean). * Significantly ($P<0.01$) different from baseline (ANOVA and Kruskal-Wallis test for repeated measurements); † Significantly ($P<0.01$) different from the restrictive group at corresponding time point (unpaired Student's *t*-test)

therapy avoiding hypovolemia is necessary to compensate both splanchnic circulatory response to surgical stress and vasodilatatory effects of anesthetic drugs. The changes of FCD described above for ileal mucosa were not observable within sublingual mucosa both in the HHD and restrictive groups.

The controversy of liberal versus restricted therapy is often being discussed and reviewed (Holte *et al*., 2002). The presented study indirectly supports previous theory of postoperative gut dysfunction (Lobo *et al*., 2002), suggesting that prolonged intraoperative infusions of crystalloids can lead to the development of intestinal oedema (Prien *et al*., 1990). Interstitial oedema of the gut is a possible mechanism which can explain findings in this study, but the tissue wet to dry weight ratio was not determined to confirm this theory explicitly. Recent studies in pigs demonstrated that a high fluid regimen alone does not increase intestinal tissue oxygen tension in healthy, perianastomotic, or intra-anastomotic colon tissue (Kimberger *et al*., 2007). Nearly identical findings were observed in the jejunum in porcine model of different perioperative fluid managements (Hiltebrand *et al*., 2007). In humans, a high volume of infusion with Ringer's solution during major surgery decreases muscle tissue oxygen tension (Lang *et al*., 2001). Decrease in hematocrit and blood viscosity is another possible mechanism affecting microhemodynamics and blood flow distribution in intestinal microcirculatory network. Recent experimental studies have described the relationship between hematocrit decrease and vasculature constriction. Vascular resistance increased by about 20% and remained at this level up to a hematocrit decrease of 20%, clearly indicating that the vasculature constricted with lowered hematocrit (Vazquez *et al*., 2010). Additional mechanisms that potentially contribute to impairment of intestinal FCD include altered erythrocyte rheology, such as reduced erythrocyte deformability, during acute hemodilution. This alteration may contribute to redistribution of blood flow away from splanchnic organs or redirect perfusion to larger, non-nutrient conducting and shunting vessels within the splanchnic region (Schwarte *et al*., 2005).

Most of previous clinical studies using OPS imaging and SDF imaging for visualization of microcirculation focused on assessment of microcirculatory alterations of accessible sublingual mucosa, especially during sepsis (de Backer *et al*., 2002; Sakr *et al*., 2004). In the presented study, no significant changes in sublingual FCD were detected both in the HHD and restrictive groups throughout the study. This fact could be explained by minor susceptibility of sublingual mucosa to form interstitial oedema when compared to intestinal villi microcirculation. Thus, sublingual mucosa may not be sufficiently sensitive markers to detect FCD changes during HHD or fluid overload. Recent studies have suggested the possibility of organ-specific microcirculatory response to hemodilution (Gottschalk *et al*., 2005), which could be explained by different autoregulatory mechanisms in microcirculation. Microcirculatory changes during septic shock, which can be observed and well characterized in the sublingual area, are probably much more profound than those detectable in HHD conditions.

SDF imaging allows direct in vivo observation of the mucosal and organ surfaces. Despite further development and improvement of SDF technology, as compared to OPS techniques, several methodological limitations remain (Sakr *et al*., 2004). Pressure artifacts and lateral movement of the tissue make continuous microcirculatory monitoring very difficult. A custom-made fixation and flexible device for SDF probe is crucial to eliminate pressure artifacts (Lindert *et al*., 2002; Turek *et al*., 2008). Video frames for off-line software assessment with sluggish or stopped venular flow should be excluded from the analysis process as recommended previously (de Backer *et al*., 2007). A potential great benefit of the SDF technology lies in the possibility of its intraoperative use in clinical practice thanks to the non-invasive and hand-held option for the SDF probe.

5 Conclusions

In conclusion, prolonged HHD significantly decreased FCD of ileal mucosa as assessed by SDF imaging. This trend was not observed in the sublingual region. The presented data demonstrate a possible relationship between fluid overload and microcirculatory alterations in the small intestine.

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