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EXPERIMENTAL

Vascular Occlusion in a Porcine Flap Model: Effects on Blood Cell Concentration and Oxygenation

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Background: Venous congestion in skin flaps is difficult to detect. This study evaluated the ability of tissue viability imaging (TiVi) to measure changes in the concentration of red blood cells (CRBC), oxygenation, and heterogeneity during vascular provocations in a porcine fasciocutaneous flap model.
Methods: In 5 pigs, cranial gluteal artery perforator flaps were raised (8 flaps in 5 pigs). The arterial and venous blood flow was monitored with ultrasonic flow probes. CRBC, tissue oxygenation, and heterogeneity in the skin were monitored with TiVi during baseline, 50% and 100% venous occlusion, recovery, 100% arterial occlusion and final recovery, thereby simulating venous and arterial occlusion of a free fasciocutaneous flap. A laser Doppler probe was used as a reference for microvascular perfusion in the flap.
Results: During partial and complete venous occlusion, increases in CRBC were seen in different regions of the flap. They were more pronounced in the distal part. During complete arterial occlusion, CRBC decreased in all but the most distal parts of the flap. There were also increases in tissue oxygenation and heterogeneity during venous occlusion.
Conclusions: TiVi measures regional changes in CRBC in the skin of the flap during arterial and venous occlusion, as well as an increase in oxygenated hemoglobin during venous occlusion that may be the result of reduced metabolism and impaired delivery of oxygen to the tissue. TiVi may provide a promising method for measuring flap viability because it is hand-held, easy to-use, and provides spatial information on venous congestion. *(Plast Reconstr Surg Glob Open 2017;5:e1531; doi: 10.1097/GOX.0000000000001531; Published online 17 November 2017.)*

INTRODUCTION

Free microvascular flaps are regularly used when skin and tissue defects are reconstructed after trauma or cancer surgery. Failure rates are low, between 1 and 10%,[1–3] but total or partial flap failure means considerable morbidity and salvage surgery.[4] Early detection of vascular compromise and subsequent surgical intervention is well correlated to flap survival[4–7] and monitoring is therefore indicated.

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Magnetic resonance imaging or computed tomography angiography is used for preoperative planning, but these techniques cannot be used perioperatively. During surgery, flaps can be monitored visually (change of color, capillary refill time) or with technical devices[8] such as laser Doppler flowmetry (LDF),[9] near-infrared spectroscopy,[10,11] or laser speckle contrast imaging (LSCI).[12,13] LDF is often considered a golden standard because it can reliably and sensitively detect arterial occlusion. LDF has however been found less sensitive in detecting a compromised venous outflow.[14] Also, a limitation of LDF is that it monitors the tissue at a single point, which may be unrepresentative for flap viability as a whole.

Ideally, a technique for measuring flap viability should accurately and sensitively detect venous and arterial compromise in the entire flap. Also, as a thrombosis often starts as a partial occlusion and progresses over time, it should be able to detect a partial occlusion. Furthermore, it should be easy to use, because early detection and surgical intervention can save a thrombosed flap.[5,15] Even with a patent pedicle, blood flow must be enough to keep the

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entire flap properly vascularized to survive and heal in. During flap surgery, the surgeon must use his experience when planning flap size and shape and consider if the supplying vessels will be sufficient for the entire flap. This is commonly done by visual inspection when the flap has been raised. A congested, cold appearance in the periphery, abnormal capillary refill, or absence of dermal edge bleeding indicates insufficient blood flow and may require reduction of the flap.

Tissue viability imaging (TiVi) is a technique based on digital photography combined with spectral image analysis. That provides images of skin red blood cell concentration (C_{RBC}).[16] Recently, the technique has been further developed to measure tissue oxygenation.

In the current study, we aimed to assess the ability of TiVi to detect the microvascular effects of venous occlusion by analyzing regional changes in C_{RBC} in a fasciocutaneous pedicled flap during vascular provocations. Furthermore, we assessed the heterogeneity of the C_{RBC} and changes in oxygenation. We hypothesized that we could observe changes in these measures dependent on the region of the flap, during partial venous, total venous and arterial occlusion of the microcirculation in a pedicled or free flap.

MATERIALS AND METHODS

Animals

Five Swedish Landrace pigs (mean age, 4 months; weight, 45 kg) were used. Before surgery, they received an intramuscular injection of dexmedetomidine 0.1 mg/kg (Orion Pharma, Sollentuna, Sweden), tiletamine, zolazepam 5 mg/kg (Zoletil, Reading/Virbac, Carros, France) and atropine 0.05 mg/kg (APL, Apoteket, Umeå, Sweden). Anesthesia was induced and maintained using pentobarbital sodium 8 mg/kg/h and fentanyl 0.5 μg/kg/h dissolved in Ringer's acetate and the pigs were on mechanical ventilation. Vital parameters including body temperature and blood pressure were monitored and kept at a constant level by giving Ringer's acetate and covering the animals with blankets. The animals were euthanized with a mixture of pentobarbital sodium and 70% ethanol without regaining consciousness. The use of a larger animal model enabled us to make a larger flap more similar to a human pedicled or free flap where suitable and relevant regions of interest (ROI) could be easily defined. The study conformed with the declaration of Helsinki and was approved by the regional ethics review board at Linköping University.

Surgery

With each animal, two 12 × 15 cm pedicled flaps (1 on each side) were raised based on the cranial gluteal artery perforator.[17] When measurements had been completed on 1 side, the animal was turned over and the procedure was repeated on the contralateral side. Two flaps were lost due to unintentional pedicle thrombosis. The vascular pedicle was stripped so that the only vascular supply to and from the flap was the cranial gluteal artery and 1 concomitant vein. This experimental model simulates a free flap before detachment of the vascular pedicle. An ultrasound flowmeter with 2 flow probes (MA-2PSB, Transonic Systems Inc. Ithaca, N.Y.) was attached to the artery and vein. An inflatable vascular occluder (Norfolk Medical Products Inc., Skokie, Ill.) was placed around the vein distal to the flow probe. The vascular occluder was inflated to reduce venous outflow. The experimental setup has previously been described in detail by Zötterman et al.[18]

TiVi

A commercial TiVi system (TiVi600, WheelsBridge AB, Linköping, Sweden) that consists of a digital camera with polarization filters in front of flash and lens together with a dedicated software was used to measure changes in C_{RBC}, RBC heterogeneity, and oxygenation of the skin. The system has been described previously in detail.[16] The acquired images contain information from a depth of approximately 0.5 mm below the skin surface depending on the epidermal thickness. The sampling depth of the technique in pig skin has not been validated.

Red Blood Cell Heterogeneity

The cutaneous microvasculature is highly heterogeneous, with areas that are either predominantly arteriolar or venular in composition, or devoid of all microvascular elements. The heterogeneity (H) of red blood cells in the skin was assessed in each TiVi image by calculating the SD of C_{RBC} values in ROI:

$$H = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(C_{RBC,i} - \overline{C_{RBC}}\right)^2}$$

Where $\overline{C_{RBC}}$ denotes the mean of the C_{RBC} values and N the number of pixels in the ROI.

Tissue Oxygenation

The most important dynamically changing chromophores in the skin are the oxy- and deoxyhemoglobin molecules. Changes in the concentration of oxyhemoglobin (ΔC_{OH}) and deoxyhemoglobin (ΔC_{DOH}) in the skin result in changes in local green and red pixel values. By taking into account the extinction coefficients and average path lengths in the skin tabulated in the literature, as well as the known sensitivity of the camera and spectral density of the illuminating light, the change in concentration of oxyhemoglobin (ΔC_{OH}) and deoxyhemoglobin (ΔC_{DOH}) can be calculated by the TiVi system. This is under the assumption that the tissue matrix does not change in the short-term perspective.

Laser Doppler Flowmetry

A laser Doppler perfusion monitoring unit (PeriFlux System 5000, Perimed AB, Järfälla, Sweden) with thermostatic Laser Doppler probes (Probe 457; Perimed AB) was used to measure perfusion and temperature in the skin. The bandwidth of the system is 15 kHz. Before the start of the study, the system was calibrated according to the guidelines of the manufacturer. The probe used in

the current study has a fiber separation of 0.25 mm and collects perfusion data at a depth of about 0.5 mm.

Experimental Protocol

Experiments took place in a windowless room and ceiling lights were switched off. During the experiment, the TiVi camera was positioned on a camera mount 30 cm above the flap surface and acquired images at a rate of 1 image per minute. The laser Doppler flowmeter recorded tissue perfusion continuously. Arterial and venous flow was registered every 5 minutes by the ultrasonic probes. Vital parameters including flap temperature, blood pressure, heart rate, and body temperature were registered every 5 minutes. The experimental protocol consisted of a 30-minute baseline followed by 30 minutes of 50% venous occlusion and 30 minutes of 100% venous occlusion. This was followed by 40 minutes of recovery, 30 minutes of total arterial occlusion, and finally 30 minutes of recovery. The experimental protocol is shown in Figure 1.

Data Analysis and Statistics

TiVi images were processed using the system's analysis software (Wheelsbridge AB, Linköping, Sweden). In each image, ROI as seen in Figure 2 were selected. The ROIs were chosen in relation to their proximity to the vascular pedicle. ROI 1 being the closest and ROI 8 the farthest from the pedicle. For each image and each ROI, the average C_{RBC}, heterogeneity, and changes in oxy- and deoxyhemoglobin were calculated. The heterogeneity for the whole flap was calculated as the mean value in ROI 1–8.

D'Agostino & Pearson omnibus normality tests indicated that all data were normally distributed. Because of this and to maximize the statistical power of the analyses, parametric tests were used. Two-way analyses of variance (ANOVA) for repeated measures were used to analyze changes in RBC concentration and oxygenation during the experiment in different positions in the flap (ROI 1–8 or proximal compared with distal). A one-way repeated-measures ANOVA was used to analyze changes in heterogeneity during the experiment. Data from the last 5 minutes of each phase were used for these analyses. With all ANOVA, Dunnett's multiple comparisons tests were used to test for significant differences from baseline. All data in text and tables are presented as mean ± SD. Statistical calculations were done using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, San Diego, Calif., "www.graphpad.com"). For all analyses, probabilities of less than 0.05 were accepted as significant.

RESULTS

There were no changes in heart rate or temperature of the flap during the experiment. Blood pressure decreased gradually from 122/78 to 105/62 during the course of the experiment, likely as a result of prolonged anesthesia (Table 1).

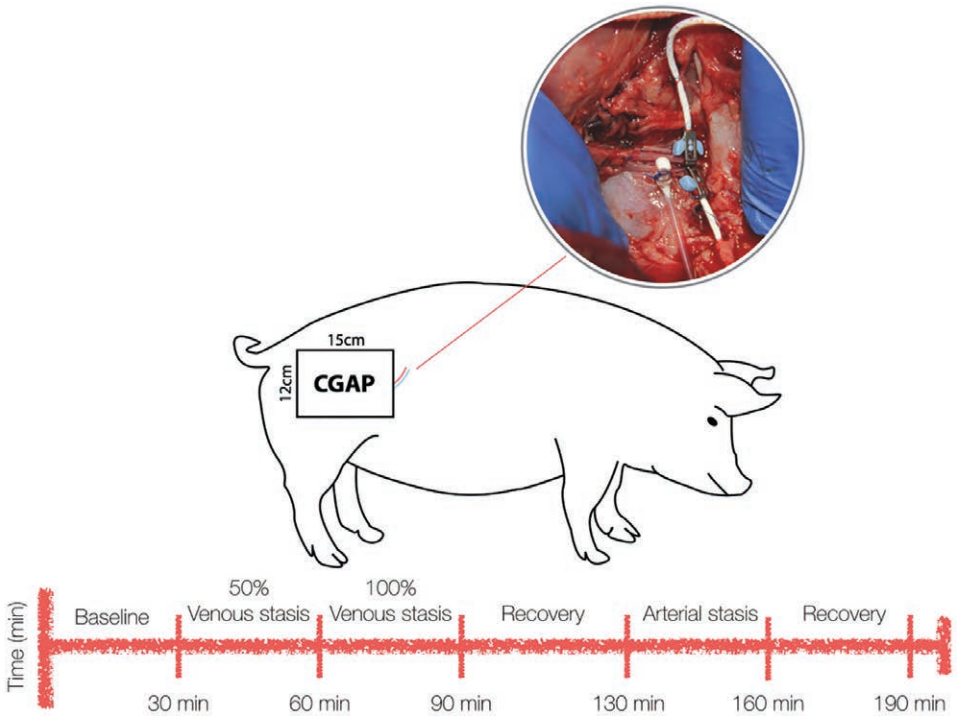

Fig. 1. Experimental model and study protocol. The experimental protocol consisted of a 30-minute baseline followed by 30 minutes of 50% venous occlusion and 30 minutes of 100% venous occlusion. This was followed by 40 minutes of recovery, 30 minutes of total arterial occlusion, and finally, 30 minutes of recovery. Partial (50%) venous occlusion was achieved by inflating the vascular occluder and monitoring the level of occlusion with the flowmeters. Complete occlusion was done with microvascular clamps.

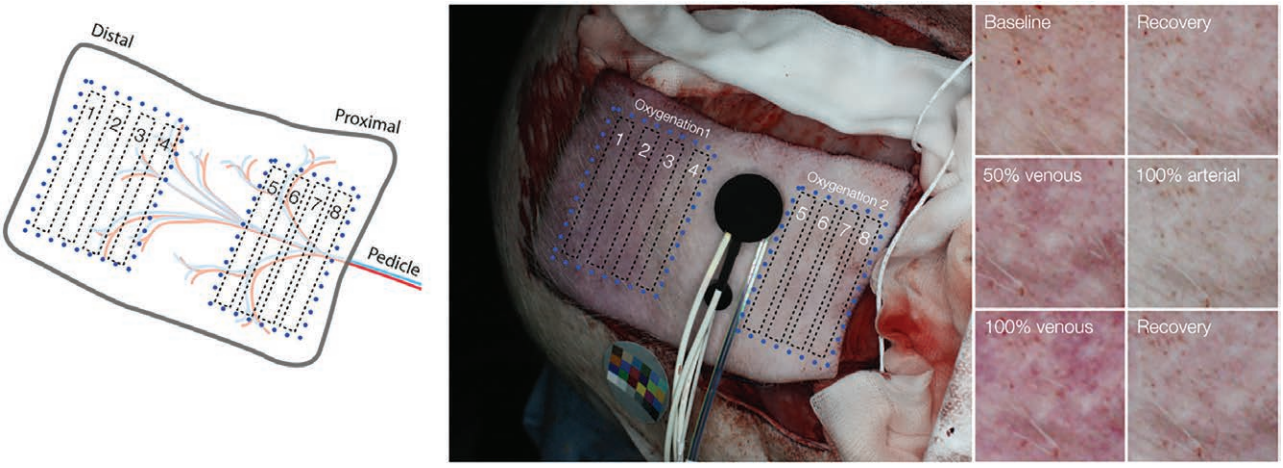

Fig. 2. The schematic drawing of the flap (A) shows the ROI in relation to the vascular tree (B). Analysis of different regions of the flap during venous and arterial occlusion. Eight 0.8 × 4 cm rectangular ROI for RBC concentration measurements[1–8] are depicted as black dashed boxes. For tissue oxygenation measurements, larger regions were needed to obtain reliable data and therefore two 4 × 4 cm ROI (Oxygenation 1 and 2) are depicted as green (proximal part) and yellow (distal part) dotted boxes. A custom LDF probe was placed in the central portion of the flap for reference perfusion values and temperature (Table 1). A color chart was also placed close to the flap for calibration of the TiVi images. Panels A–F (right) display differences in perfusion heterogeneity in the different phases of the experiment.

RBC Concentration

The absolute mean (SD) C_{RBC} in different regions and during the different phases of the experiment are given in Table 2.

During all phases, mean C_{RBC} was lowest in the central regions of the flap and increased successively toward the peripheral parts, both proximal and distal. During 50% venous occlusion, increases in C_{RBC} were observed within minutes (Fig. 3). There was a significant increase in all regions ($P = 0.01$ in ROI 8; all other regions $P < 0.001$). The largest changes in C_{RBC} during venous occlusion were, however, observed in the distal parts of the flap (ROI 1–3). In central and proximal regions (ROI 4–8), the changes were still significant compared with baseline. During 100% occlusion, C_{RBC} increased further compared with baseline in all regions ($P < 0.001$), but the changes were largest in the distal part of the flap.

During recovery after 100% venous occlusion, C_{RBC} in all regions returned to baseline level. With no significant differences from baseline ($P > 0.12$). During arterial occlusion, there was a significant reduction in C_{RBC} in all regions except region 2 ($P = 0.07$), and the largest decreases were observed in the proximal regions. Finally, during recovery after arterial occlusion C_{RBC} returned to a level not significantly different from baseline, although a tendency was seen toward lower C_{RBC} particularly in proximal regions.

Table 1. Overview of Physiological Parameters

	BP (mm Hg)	HR (min^{-1})	T (°C)	ABF (ml/min)	VBF (ml/min)	SkBF (PU)
Baseline	122/78 (10/10)	95 (6)	39 (0)	13.4 (0.8)	13.4 (0.7)	36.6 (17.3)
50% venous occlusion	119/76 (8/10)	95 (6)	39 (0)	9.4 (0.7)	5.2 (0.4)	31.3 (15.7)
100% venous occlusion	112/74 (8/16)	95 (8)	40 (1)	5.0 (0.7)	0.0 (0.0)	16.7 (12.8)*†
Recovery 1	112/68 (10/15)	95 (5)	40 (1)	14.7 (0.6	13.0 (0.5)	36.1 (17.9)
100% arterial occlusion	108/64 (8/13)*†	91 (15)	40 (0)	0.0 (0.2)	0.2 (0.2)	8.5 (4.0)**†
Recovery 2	105/64 (1/5)*†	92 (10)	40 (0)	15.7 (0.7)	14.8 (1.0)	35.9 (16.2)

*$P < 0.05$; **$P < 0.01$; compared with baseline.
†Significant change compared with baseline.
ABF, arterial blood flow; BP, blood pressure; HR, heart rate; SkBF, skin blood flow; T, body temperature; VBF, venous blood flow.

Table 2. Mean (SD) RBC Concentration (AU) during Venous and Arterial Occlusion in Different Regions of the Flap (N = 8)

	RBC concentration (AU)								Heterogeneity
	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5	ROI 6	ROI 7	ROI 8	All
Baseline	140 (31)	123 (29)	116 (27)	110 (23)	118 (35)	125 (31)	135 (26)	142 (26)	89 (26)
50% venous occlusion	184 (30)*	171 (29)*	153 (22)*	146 (18)*	144 (24)*	152 (18)*	158 (18)*	158 (21)**	115 (30)**
100% venous occlusion	201 (56)*	189 (50)*	172 (44)*	162 (43)*	158 (47)*	162 (39)*	167 (38)*	174 (34)*	133 (42)**
Recovery 1	144 (56)	134 (51)	116 (41)	110 (39)	110 (51)	119 (45)	131 (39)	139 (37)	117 (48)
100% arterial occlusion	123 (45)***	111 (43)	97 (35)***	90 (32)***	89 (34)*	100 (37)*	110 (38)*	116 (36)*	96 (42)
Recovery 2	129 (52)	119 (49)	107 (41)	101 (39)	106 (52)	114 (49)	123 (49)	129 (54)	82 (22)

*$P < 0.001$ compared with baseline; **$P < 0.05$; ***$P < 0.01$.

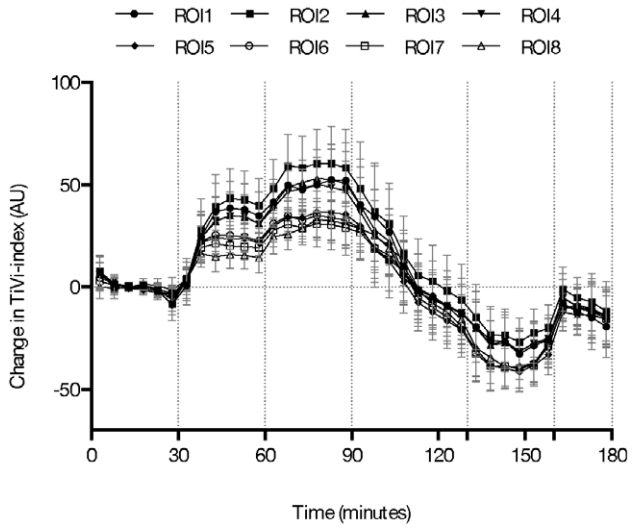

Fig. 3. Changes in RBC concentration during the different phases of the experiment. Each line represents a mean and SD of all flaps. The ROI are the same as shown in Figure 2.

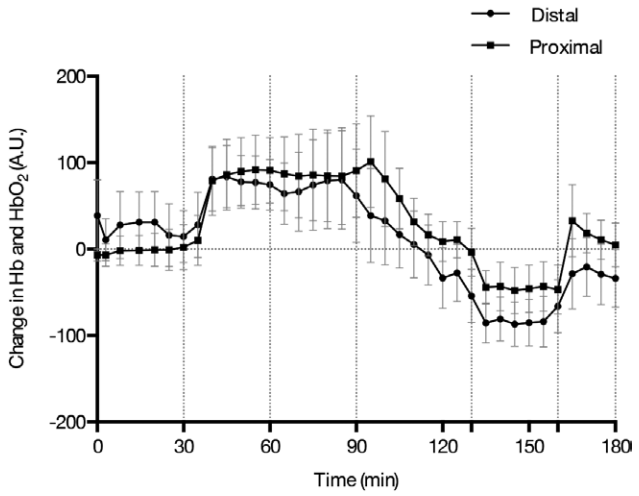

Fig. 5. Oxygenation of red blood cells. Mean and SD of distal and proximal part of flap, respectively.

RBC Heterogeneity

The heterogeneity of RBC in the skin increased during 50% venous occlusion ($P = 0.03$) and further during 100% venous occlusion ($P = 0.04$; Table 2; Fig. 4). During recovery from venous occlusion, a gradual decrease in heterogeneity was observed, and this decrease continued during 100% arterial occlusion. Heterogeneity was not significantly different from baseline during both recovery phases nor during 100% arterial occlusion.

Tissue Oxygenation

Oxygenation in the proximal part of the flap was significantly increased from baseline during 50% and 100% venous occlusion, from 2 (69) to 91 (101) and 91 (143) AU, respectively ($P = 0.009$; Fig. 5). A nonsignificant increase was seen in the distal part, from 14 (80) to 75 (78) ($P = 0.11$) and 62 (143) AU, respectively ($P = 0.28$). During recovery after venous occlusion, oxygenation in the proximal part was restored to baseline levels, 9 (61) AU, after about 20–30 minutes, and to -33 (93) AU in the distal part. Then, after onset of 100% arterial occlusion, the oxygenation in the proximal part decreased and stabilized at -47 (75) AU within 5 minutes ($P = 0.25$) and in the distal part to -66 (81) AU ($P = 0.02$). After restoring the arterial flow, a postocclusive hyperoxygenation was observed in the proximal region of the flap, but not in the distal region, where the oxygenation gradually returned to baseline level, -2 (59) AU (proximal part) and -44 (80) AU (distal part).

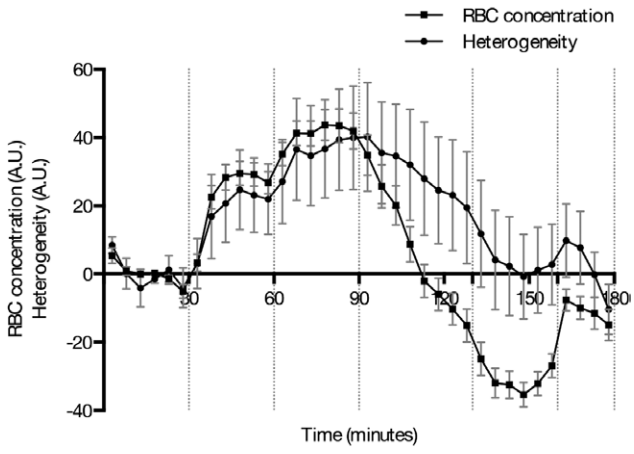

Fig. 4. RBC concentration and heterogeneity during experiments. Mean of entire flap with SD.

Perfusion

During 50% venous occlusion, a nonsignificant decrease in perfusion was observed, and during 100% venous occlusion, perfusion was significantly decreased from baseline ($P = 0.04$). During 100% arterial occlusion, perfusion was also significantly decreased from baseline ($P = 0.01$).

DISCUSSION

Venous occlusion is a serious complication of free flap surgery. Although more common than arterial occlusion, it is less explored. According to previous research, even small changes in flap appearance can correspond to a 50% venous obstruction, which is easily missed clinically.[15] Early detection of altered venous and arterial blood flow is therefore of critical value.

The main finding in this study is that TiVi can reliably detect changes in C_{RBC}, RBC heterogeneity, and tissue oxygenation during partial and full venous occlusion in the cranial gluteal artery perforator flap model, and that venous occlusion caused a paradoxical increase in tissue oxygenation.

TiVi measures red blood cell concentration and is therefore not affected by a reduction of average RBC velocity during venous stasis. On the other hand, LDF measures perfusion, which is a product of concentration and velocity of moving blood cells. During venous stasis, there is a simultaneous increase in concentration and a

decrease in velocity. This can explain why, in the current study, there was no consistent change in perfusion during 50% stasis. A decrease in perfusion was observed in 6 of 8 flaps, whereas the other 2 showed either no change or an increase in perfusion.

The changes in C_{RBC} during venous occlusion differed markedly between different areas of the flap along the distal–proximal axis. Although C_{RBC} increased in all regions of the flap, the largest increases from baseline were seen in the distal regions. In contrary, during complete arterial occlusion, the proximal parts of the flap showed the largest decrease in C_{RBC}. This indicates that the effects of a global change in blood flow to or from the flap has regional effects on skin blood concentration, which most likely depends on spatial variations in the structure of the microvascular tree and its distance from entrance of the pedicle into the flap. It is to be expected that the most distant part of the flap is more congested as it is in the periphery and the fine venules may be poorly drained. A partial or total venous occlusion makes the regional differences in concentration even more pronounced. A free or pedicled flap with a congested appearance in its periphery indicates that the distal part has an insufficient venous drainage/arterial supply and should be resected to avoid a partial necrosis. This gradient of venous congestion could, to a certain extent, be seen with the naked eye during our experiments. But TiVi could provide assistance in determining proper flap size and shape as well as monitoring spatial changes in venous drainage/arterial supply. This requires that threshold values for flap viability are determined. In a future study, using larger flaps that will include areas of very low tissue perfusion, we aim to identify such threshold values that later can be used in the clinic.

During venous occlusion, the appearance of the flap often becomes more heterogeneous or blotchy. This can be explained by differences in areas of the dermal matrix with arterial, venous, or avascular dominance.[19] During venous occlusion, the venules in areas of venous dominance are filled and expanded, whereas the other areas become pale due to diminished arterial inflow. When we quantified this heterogeneity with TiVi, we observed that the heterogeneity indeed increased proportionally with the increase in RBC concentration during venous stasis, and that it gradually returned to baseline during recovery. With complete arterial occlusion, no further decrease in heterogeneity was seen. This indicates that no further redistribution of RBC occurs in the microvascular bed of the flap when the feeding arteries are occluded completely. Heterogeneity of RBC in the flap could therefore possibly be used as an additional measure of impending venous occlusion and for distinguishing venous from arterial occlusion.

Interestingly, an increase in oxygenated blood was seen in both the distal and the proximal part of the flap with both partial and complete venous occlusion. An explanation for this may be that, while oxygenated blood flows into the flap, the oxygen consumption in the flap decreases as it gets congested with increasing tissue edema. The tissue edema has been hypothesized to act as a direct diffusion barrier for the delivery of oxygen into the tissue. This is believed to be partly the result of an altered perfusion pattern, through an increase in arteriovenous shunting away from the affected capillary bed.[20,21] Also, it can be speculated that the oxygen extraction capacity of the tissue is reduced as a result of its congested state and that the metabolic activity of the congested tissue is downregulated by the increased release of vasoactive substances, including adenosine,[22] and serotonin[23]as well as a decreased release of nitric oxide.[24] Similar results have been reported in experimental models of vascular trauma, in which an uncoupling was suggested between tissue metabolism and altered microvascular blood flow. These effects were particularly evident after blunt trauma to the neurovascular bundle.[25] Similarly, in our model, the vascular bundle is skeletonized and the accompanying nerve transected during flap preparation. These findings together suggest an involvement of neurovascular mechanisms in the paradoxical increase in tissue oxygenation in the flap during venous occlusion.

A limitation in this study is the relatively small number of animals. The sample size was however sufficient for the establishment of a reproducible model of partial and complete venous obstruction as well as arterial obstruction. Also, the model allows for qualitative representation of flap physiology, such as C_{RBC} and tissue oxygenation, under different levels of venous and arterial obstruction where increased numbers of animals would add little value.

Another limitation of this study is that the experiments were conducted in a strictly controlled surgical environment. Before results can be translated into clinical use, more studies have to be done in patients in a postoperative setting. These studies should take into account possible confounding environmental factors including room light, tissue curvature, and patient movements.

Finally, determining when a compromised flap is no longer suitable for salvage was not within the frame of this study as it would require a much longer study period and having animals either kept awake with a raised flap or under general anesthesia under a prolonged period.

CONCLUSIONS

These results suggest that it is possible to detect early signs of partial and total venous stasis as well as arterial occlusion in flaps using a relatively simple, camera-based technique. An increase in RBC concentration, combined with an increase in tissue heterogeneity and increase in oxygenation of red blood cells, would indicate insufficient venous drainage of the flap. A decrease in RBC concentration together with decreased heterogeneity and low levels of oxygenated hemoglobin would indicate an arterial occlusion. TiVi may also be used when planning the size and shape of the flap and to identify parts of the flap with insufficient circulation which may become partially necrotic. Another possible future use is to evaluate viability when raising other types of flaps, such as rotational and propeller flaps. This may be particularly beneficial when the flap is in close proximity to a zone of injury where blunt trauma has damaged the microcirculation. Further

studies are however needed to fully appreciate the technique in these applications.

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