Original Article

Euromediterranean Biomedical Journal
for young doctors
(formerly: Capsula Eburnea)

VENOUS THROMBOSIS IN FREE FLAPS: A STUDY OF THE PHENOMENOLOGY, HISTORY AND CLINICAL SIGNS IN AN EXPERIMENTAL MODEL OF RATS.

Luigi Montesano, Bartolo Corradino, Gabriele Giunta,
Alessio Mariolo, Salvatore D'Arpa.

SUMMARY

The introduction of free flaps with microsurgical vascular anastomosis has made reconstructions, that would have been considered impossible forty years ago, possible. The limitations of this technique are mainly due to necrosis of the transplanted tissue caused by blockage of the vessels of the flap's vascular pedicle due to the formation of thrombi at the level of the surgical anastomosis. Thrombosis is handled by removing the thrombus in the shortest possible time, thus allowing restoration of the blood flow. Currently clinical observation is the best way to evaluate the survival of a flap but, being subjective, it is dependent on the observer's experience. The purpose of this study is to identify the first sure sign of venous thrombosis in a murine model of complete venous occlusion of the pedicle of an inguinal flap, postoperatively and after the restoration of blood flow following a period of stasis; so as to simulate the monitoring of re-thrombosis after recanalization of a thrombosed anastomosis.

Introduction

Reconstructive microsurgery is currently the most refined, reliable and versatile technique available to reconstructive plastic surgeons. The use of micro-vascular free flaps, with the possibility of transferring virtually any tissue type, and the increased success rate (over 95%), has made reconstructions, that would have been considered impossible forty years ago, quite achievable. This has also enabled radical surgery to push its boundaries even further. In oncological surgery, for example, many surgical removals which were technically possible, especially in the cervico-cephalic, were limited before the invention of reconstructive microsurgery, due to an inability to repair substance loss or to do so with an acceptable morpho-functional outcome. Failures in reconstructive microsurgery are due to necrosis of the transplanted tissue caused by blockage of the vessels of the flap's vascular pedicle due to the formation of blood clots at the anastomosis level [1]. At this point in fact, there is a solution of the continuous vessel which leads to a vascular intimal exposure that, together with the presence of foreign material (the stitches), causes clots to form here. Thrombosis is handled by removing the occlusion and restoring the flap's blood flow. The only limitation on whether or not the free flaps can be saved lies in the time between the thrombotic clotting of the vessel and restoration of the blood flow [1]. During the period of ischemia, be it of the artery, vein, or a mix

Address of the authors
Dipartimento di Discipline Chirurgiche, Oncologiche e Stomatologiche, University of Palermo

Send correspondence to*: Salvatore D'Arpa, salvatore.darpa@unipa.it*

Received*: May 2nd, 2013 —* ***Revised****: May 28th, 2013 —* ***Accepted****: June 15th, 2013*

© EUROMEDITERRANEAN BIOMEDICAL JOURNAL 2013, 8(18):91-98. DOI: 10.3269/1970-5492.2013.8.18 Available on-line at: http://www.embj.org

of both, changes in the tissue and microvessels are established that progress until they are irreversible and render any restoration of the arterial or venous flow pointless. It is essential that reconstructive surgeons intervene before such phenomena sets in. Taking into account the fact that ischemia has a time tolerance of a few hours, it is fundamental to spot early signs of thrombosis and intervene promptly. To do this, the flaps must be checked every hour for the first 72 hours, as this is the time period in which there is the greatest risk of thrombosis [1]. Arterial thrombosis, which is much rarer, usually occurs within the first 24 hours. Venous thrombosis occurs later and more frequently than arterial thrombosis. It is more devious and difficult to diagnose and causes more damage to tissue [2], thus it is more often responsible for irreversible changes that cause failure of the skin flaps.
The importance of prompt intervention is confirmed by the work of Disa et al. [3]. Following analysis of 750 free flaps, Disa et al. reported a success rate of 93.5% for “submerged” free flaps - those which are inaccessible for clinical monitoring - compared with 98.2% for exposed flaps. This statistically significant difference is tied to the time differences in the diagnosis of thrombosis as well as to differences in re-exploration times. The exposed flaps, re-explored on average within 48 hours, were saved in 77% of cases, while none of the submerged flaps, re-explored on average after more than 7 days, were saved. This is because continued monitoring of exposed flaps has enabled early identification of the clinical signs of suffering and earlier intervention compared to that of submerged flaps in which the thrombosis is revealed late following complications such as infections or diastase of the wounds.
More than thirty years after the advent of microsurgery, the timings, signs, and criteria for prompt diagnosis of thrombosis, especially venous thrombosis, are still unclear and very much tied to the surgeon’s experience. Monitoring techniques now considered standard are: color, temperature, return capillary puncture, doppler with the use of various techniques [4-14]. Most of those introduced instrumental techniques are useful for diagnosing arterial thrombosis, which is rarer and easier to identify, and are therefore of limited use.
In order to increase the success rate of free flap transplants, detailed study of the lesser explored field of venous thrombosis is necessary, so that the signs and timings and may clarified.

Materials and Methods

This study was conducted on six Sprague Dawley laboratory rats. The experiments were carried out in accordance with regulations of Legislative Decree n. 116/92, issued pursuant to Directive 86/609/EEC, and subsequent circulars of the Ministry of Health No. 17 and No. 18 of 05.05.93, 04.22.94 and no 8 no 6 of 14/05 / 2001 in relation to the use of animals for experimental and educational purposes. In order to speed up the administering of the anesthetic, the rats were immobilized between two baskets with perforated walls [15]. Two little plastic baskets are used to immobilize the rats, inside one of which the rat is placed. The two baskets are positioned one on top of the other to immobilize the rat so that it is possible to inject the medication intramuscularly. The anesthesia was administered via the muscles with Ketamin (0.3ml), Midazolam (0.3ml), Fentanyl (0.5ml) and Antropina (0.02ml). The inguinal-femoral and abdominal skin was accurately shaven (figure 1A). A 2cm^2 island was drawn on the shaven skin, corresponding to the flap to be isolated (figure 1B). The full thickness of the skin of the flap was cut (figure 1C) and the flap was totally isolated from all vascular and tissue connections except for the superficial inferior epigastric pedicle (figure 1D). The artery and the vein were isolated and separated from each other at the point at which they emerge from the femoral vessels. At this point, the vein was clamped with a single clamp, using the “milking test” to check that there was no residual venous outflow. This model represents venous thrombosis by indirectly reproducing the complete occlusion of the vein and venous outflow. Once the vein was clamped, the flap was stretched out to enable viewing, with four stitches at each corner (figure 1F). The flap was not completely sewn up, as occurs in clinics, to allow for the clamp to be released at a later time. The flap was monitored every 15 minutes for 270 minutes from the moment the clamp was positioned. The parameters checked were the color of the skin and the color of the blood from the area (figure 1G -L). Degrees were established for the color of the skin (5 degrees from rosy (R) to totally blue (TB), Table 1) and for the color of the blood (3 degrees from red (R) to blue (B), Table 1). Skin color was the first parameter observed. Observation was carried out by two independent observers who attributed a corresponding letter to the skin color according to the scale described above. The skin was then pricked with a 30g needle and the color of the blood was then assessed under an operat-

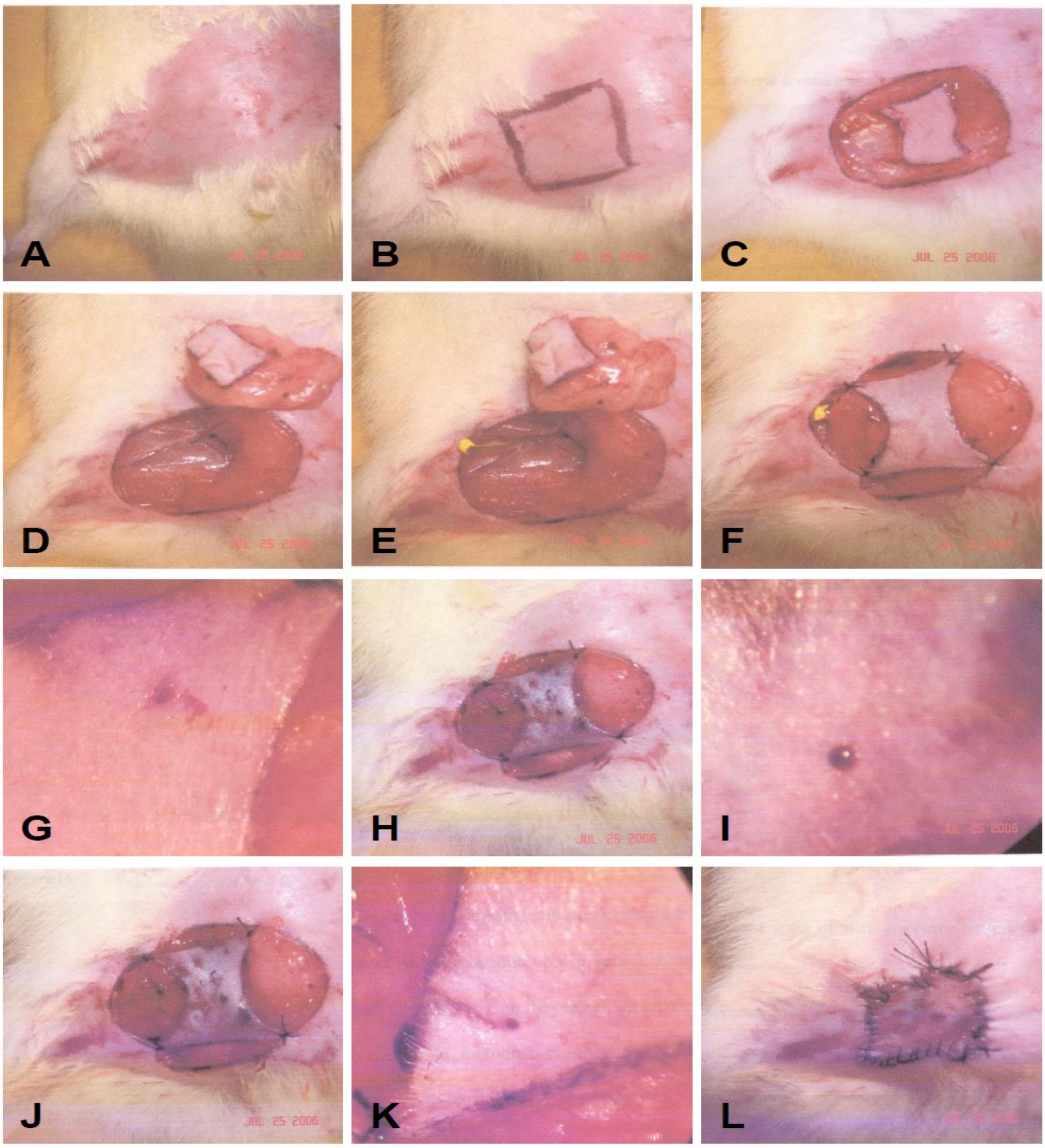

Figure 1: A. Before the intervention, the rat's inguinal-femoral and abdominal skin is shaven; **B.** A $2cm^2$ flap is drawn on the skin; **C.** The margins of the flap are cut. **D.** The flap is completely isolated to the point where it is only attached at the vascular pedicle. **E.** The artery and vain are separated and the vein is clamped. **F.** The flap at T0 is anchored at the corners. **G.** The surface of the flap at T0 after being pricked, photographed under the microscope. Rosy-colored blood seeps out. **H.** More than 80% of the surface of the skin flap is mottled (Stage B++). **I.** "Blue" blood shown under a microscope. **J.** On release of the clamp, the skin color remains mottled. **K.** On release of the clamp, the blood goes back to being red. **L.** On sealing the flap, the color remains mottled, albeit to a lesser degree than in H.

ing microscope (Vasconcellos ®) with the same brightness, zoom and focus, by two independent observers. The two observers assigned a corresponding letter to the color of the blood according to the scale outlined above. The values were then presented in tabular form. We find it worthy of note that both observers always agreed on the letter to be assigned in their objective responses. Given the small sample size analyzed, and the fact that these were only preliminary observations, no statistical survey was applied to the recorded results. The variables calculated were: the average time for the onset of change in skin color, the average time for the skin color to progress to a higher grade, and the average time for the onset of changes in the color

T	RAT 1 BLOOD/SKIN		RAT 2 BLOOD/SKIN		RAT 3 BLOOD/SKIN		RAT 4 BLOOD/SKIN		RAT 5 BLOOD/SKIN		RAT 6 BLOOD/SKIN	
0 (0 min)	R	R	R	R	R	R	R	R	R	R	R	R
1 (15 min)	B	R	RS	P	RS	P	RS	P	R	P	R	P
2 (30 min)	B	B	B	P	RS	P	RS	P	RS	B	RS	P
3 (45 min)	B	B+	B	B	RS	B	RS	P	RS	B+	RS	B
4 (60 min)	B	B+	B	B+	RS	B+	RS	P	B	B++	B	B
	RELEASE OF CLAMP								RELEASE OF CLAMP			
5 (75 min)	RS	B+	B	B++	RS	B+	RS	P	R	B++	B	B+
			RELEASE OF CLAMP								RELEASE OF CLAMP	
6 (90 min)	R	B+	R	B++	RS	B++	RS	B	R	B++	RS	B+
7 (105 min)	R	B+	R	B++	B	AB	RS	B+	R	B+	R	B
					RELEASE OF CLAMP							
8 (120 min)	R	B+	R	B+	RS	AB	RS	B+	R	B+	R	B
9 (135 min)	R	B+	R	B+	RS	AB	RS	B+	R	B+	R	B
10 (150 min)	R	B+	R	B+	R	AB	RS	B+	R	B+	R	B
11 (165 min)	R	B+	R	B+	R	AB	RS	B+	R	B+	R	B
12 (180 min)	R	B+	R	B+	R	AB	B	B+	R	B+	R	B
13 (195 min)	R	B+	R	B+	R	AB	B	B+	R	B+	R	B
							RELEASE OF CLAMP					
14 (210 min)	R	B	R	B+	R	AB	R	B	R	B+	R	B
15 (225 min)	R	B	R	B+	R	AB	R	B	R	B+	R	B
16 (240 min)	R	B	R	B+	R	AB	R	B	R	B+	R	B
17 (255 min)	R	B	R	B+	R	AB	R	B	R	B+	R	B
18 (270 min)	R	B	R	B+	R	AB	R	B	R	B+	R	B
Colour of blood		*R: red; RS: dark red; B: Blu*										
Colour of skin		*R: pink; B: initial bluish coloration; B+: Blue patches in less than 50% of the flap; B++: Blue patches in more than 50% of the flap; TB: all blue*										

Table 1: values assigned to the color of the blood and skin of the flap for each rat and to draw each bservation time (T).

of the blood. The results obtained have been recorded and are reported in the following section, which has been dedicated to these findings. Once significant variations in the parameters under investigation were obtained, the clamp was released. After the clamp's release, the patency of the vein was checked using the "milking test". This constitutes a model for simulating the clinical situation in which, after the re-exploration of the flap and revision of anastomosis, the venous outflow is restored. Even in this situation in a clinic the same monitoring of the flap should apply as with the first intervention. All timings of the experiment have been photographed according to standard protocol, at the same times and in the same way in order to also obtain visual documentation of the experiment for any future checks or for any revision of the results remotely and by other operators. All of the photographs were obtained using the same digital camera (Sony DSC-N1®) in standard light conditions and at a fixed distance. In order to maintain a fixed distance, rather than seek a laborious (in the context of the laboratory) measuring of the distance of the objective, a set field was photographed in macro function without a zoom. The photos of the blood resulting from the prick were taken under the microscope, with the objective pointed towards the eyepiece.

Results

The experiment did not end well in the case of the first two rats. After 360 minutes of observation while the vein was still clamped, no change was observed. Hypothesizing a technical error, the methodology of preparation of the flap was therefore reviewed. The error was identified in the failed ligation of a side of the pedicle which ensured adequate venous discharge, even with complete occlusion of the lower surface of the epigastric vein. The first two rats were therefore excluded from the study. The technique was modified as described in the Materials and Methods section. In the remaining six rats, it was observed that the changes in the color of the skin and blood occur almost at the same time. Changes in the color of the blood occur between 15 and 30 minutes after occlusion, while changes in skin color occur between 30 to 45 minutes after occlusion. The two events are therefore separated by a variable timescale between 15 and 40 minutes. Considering that monitoring of the flap is hourly, it is unlikely that a difference of so few minutes could be clinically significant. It is also inconceivable to monitor the flap every 15 minutes, as the benefits of this would be doubtful, and would not exceed the resource commitment required to carry this out. The progression of skin color from the second degree to the next one on the scale occurred on average after 30 minutes. The flaps did not become completely blue in all cases before release of the clamp.

In light of the initial feedback showing an insignificant difference between the changes in skin color and in those of the color of the blood, it was thought better to complete the model by extending the observation period to include the period in which the blood outflow is restored. By doing this, the recanalization following exploration and subsequent monitoring to avert a second thrombotic event, is simulated. In this context, it was observed that once the blood flow was restored (release of the clamps = anastomotic recanalization), compared with an immediate return to red blood flow from the pin prick, there was a slow and frequently incomplete - limited to the period of observation – return to the rosy skin color.

In this specific clinical situation, in which the mottled coloration may remain at length, even after the thrombosis has been dealt with, between the two parameters considered in this study the appearance of blue blood after the pin prick is the only one which enables prompt identification of venous thrombosis.

Discussion

Although it has been thirty years since the first skin flap surgery, the phenomenon of clinical manifestations of venous thrombosis in revascularized free flaps has never been the subject of investigation, except in sporadic studies, which have added little to the scarce understanding of the subject matter. This is despite the fact that venous thrombosis is the most frequent, insidious (*and therefore difficult to identify*), and the most damaging to skin flaps. Moreover, signs of venous impairment are commonly experienced, even in cases of partial deficiency of the venous outflow. The mottling of pedicle flaps is a common finding in clinical practice which is cleared up without any outcomes [1].

Experience of such events leads most to adopt a “wait-and-see” approach when it comes to mottled free flaps, in the hope that they will clear up by themselves. This aspect of venous occlusion has in fact been investigated by Russell et al.[2]. In an article on *Plastic and Reconstructive Surgery* from June of 2006, Russell et al. used a pig as an experimental model of partial and total venous occlusion of the flap area. The intention was to provide a basis for future studies of the phenomenon of venous thrombosis and for attempts to identify a methodology which enables certain and prompt diagnosis. Their results showed that total occlusion of the vein resulted in a worsening in the mottling of the skin, while partial occlusion showed the mottling to cease and maintain a plateau once a certain degree had been reached. As it is a likely assumption that, in the case of a vascular anastomosis, the partial obstruction is only a stage in the progression to complete occlusion, the authors conclude that mottling is always an indication that immediate exploration is required [3].

A speedy diagnosis of venous thrombosis is fundamental in saving the flap. A venous thrombosis is frequently cleared up and venous outflow restored unless sufficient time has passed for the occurrence of phenomena such as an untreatable arterio-venous thrombosis or structural changes to the microcirculation compartment which render the restoration of blood flow pointless.

The purpose of this study is to identify the first clear sign of venous thrombosis in a murine model. This involves complete venous occlusion of the pedicle of an inguinal flap, postoperatively and after the restoration of blood flow following a period of stasis, so as to simulate the monitoring of re-thrombosis after a thrombosed anastomotic recanalization. The initial hypothesis that dark blood from the pin prick would precede a change in skin color was confirmed. However, the difference of 15-30 minutes in the onset of such phenomena is not of any great practical use given that, in clinical practice, monitoring of flaps is conducted hourly. In light of the findings by Russell et al. [2], it appears more appropriate to compare the appearance of blue blood after the pin prick to the progression of mottling rather than to the mottling in itself. Mottling is a common occurrence which often clears up by itself and is not an incontrovertible sign of venous thrombosis, as has been demonstrated by Russell et al. [2]. In our study, the progression of the mottling, defined here by an initial bluish coloration (B, Table 2) to the next stage of a bluish coloration of less than 50% of the flap (B+, Table 2), could be observed in a variable time period from 15 to 90 minutes. Considering that the first changes in skin color occurred after 30-45 minutes and the blood turned blue after 15-30 minutes there is an advantage of 45-135 minutes in the findings of blue blood after the pin prick when compared with the progression of the mottling. This advantage is clinically significant because of the time saved with hourly observation by diagnosing thrombosis through the appearance of blue blood and not through the progression of the mottled skin color.

Despite the small numbers involved in this study, the appearance of dark blood proved to be the earliest, and therefore most effective indicator of venous thrombosis compared to progression of the mottling. However, more numbers are needed in order to quantify the actual amount of benefit and to say with certainty that rather than the progression of the mottling of the skin, the color of the blood following a pin prick must be considered. The context in which the superiority of the pin prick findings over observation of the skin color has been shown without any doubt is in the monitoring of the flap once the vein has been recanalized (on removal of the clamp, in the case of this model). In fact, in this case, once venous flow is restored there is an immediate return to the bright red color of the blood, whereas the skin color undergoes insignificant changes and does not go back to being rosy (limited to the length of the period of observation). In this specific case, assessment of the color of the blood following a pin prick could be a valid indication of re-thrombosis as opposed to changes in skin color which would be difficult to assess as they would be masked by the persistent mottling from the first thrombotic episode. However, this conjecture remains to be verified because it was observed in this study that the mottling does not clear up while the blood returns red straight after recanalization, but it was not demonstrated, even if there is no reason to believe otherwise, that after a second thrombosis (clamping of the model's vein) the blood would instantly become dark. The considerations arising from this study must yet be deemed to be interesting preliminary findings. Definitive and valid conclusions are subject to the analysis of a larger and more statistically significant sample and the resolution of some potential sources of error in the study.

The fact that both observers were always found to be in agreement on the letter to be assigned to the color of the skin and blood is in support of the simplicity and possible objectivity of the methodology. However, a sample of only two observers is too small for confirming the objectivity of the methodology. The fact of having gathered the photos under standard conditions and at predefined moments will allow for future verification of the results using a larger sample of observers.

One of the possible variables which could have influenced the results is the pricking of the flap. The trauma dealt to the tissue by the prick could have influenced the changes in the color of the flap and made some changes permanent, which would have otherwise disappeared without the mechanical trauma. In a continuation of the study it would be opportune to investigate two inguinal flaps at the same time on the same rat, one for changes in skin color, and the other for changes in the color of the blood flowing from the pin pricks. In this way, the variable in the change of the color of the blood will be kept separate from the pricking of the flap. Another potential inaccuracy is the fact that the model represents thrombosis of an anastomosed vessel of a free flap through the occlusion of a healthy vessel of a pedicle flap. In reality, the model does in fact remain effective as it was able to reproduce the final effect of thrombosis, that is to say, the total occlusion of blood flow, which is the object of this study. The temperature of the flap was not investigated because changes in temperature occur at a later time than in the parameters under study, particularly in venous thrombosis, and this is also not an early sign nor of great importance in venous thrombosis. The Doppler monitoring technique was not used in this study as the starting condition was clearly an occlusion of the vein and thus there was no requirement for diagnosis. Instead, the objective clinical manifestations of the occlusion itself were the focus of this study. No control group with vein patency was used. This was considered unnecessary as it is an established fact that a flap whose pedicle is not occluded, damaged, or kneeling, remains rosy and does not undergo changes of either its color or the color of the blood that flows in its microcirculation.

Conclusions

Local flaps are the best choice in plastic surgery when provided with a well-vascularized and sufficiently thick soft tissue [16, 17]. Even if a method may seem the most sensitive and specific, such as the implantable Doppler - the only one able to provide direct and continuous information on the status of anastomosis - it is invalidated by a high number of false positives, that is, anastomosis explored unnecessarily. Even other methods present defects, all due to an incomplete understanding of the phenomenology of venous stasis and the consequent lack of a solid theoretical basis for monitoring it. The result is that, even today, clinical monitoring remains the most reliable and diffuse method used, albeit dependent upon the operator's experience. Demonstration that dark blood from the pin-pricked skin of the flap is an early and sure sign of venous thrombosis, and therefore absolute indication for re-exploration of the flap regardless of the skin color changes of the flap itself, would be very useful in clinical practice and provide the theoretical basis of support to a clinical evaluation that still has an empirical basis. In follow-up studies, with larger numbers, it will also be possible to define the temporal relationship between thrombosis and the bleeding of blue blood after the pin prick, and between this latter stage and the appearance of mottling. In clinical practice, an understanding of the phenomenology of venous stasis in flaps would allow, for example, the thrombosis to be traced back to its onset in order to assess how long the flap has suffered. The implications could be both clinical, in reducing downtime, and experimental, in studying the resistance of different flaps to venous stasis.

References

1.Kroll SS, Schusterman MA, Reece GP, Miller MJ, Evans GR, Robb GL, Baldwin BJ. Timing of Pedicle Thrombosis and Flap Loss after Free-Tissue Transfer. *Plast Reconstr Surg* 1996; 98: 1230-1233.

2.Russell JA, Conforti ML, Connor NP, Hartig GK. Cutaneous tissue flap viability following partial venous obstruction. *Plast Reconstr Surg* 2006; 117: 2259-2266.

3.Disa JJ, Cordeiro PG, Hidalgo DA. Efficacy of Conventional Monitoring Techniques in Free Tissue Transfer: An 11-Year Experience in 750 Consecutive Cases. *Plast Reconstr Surg* 1999; 104: 97 – 101.

4.Eren S, Rübben A, Krein R, Larkin G, Hettich R. Assessment of microcirculation of an axial skin flap using indocyanine green fluorenscence angiography. *Plast Reconstr Surg* 1995; 96: 1636.

5.Rübben A, Eren S, Krein R, Younossi H, Böhler U, Wienert V. Infrared videofluoroangiography of the skin with indocyanine green: Rat random cutaneous flap model and results in man. *Microvasc Res* 1994; 47: 240.

6.Payette JR, Kohlenberg E, Leonardi L, Pabbies A, Kerr P, Liu KZ, Sowa MG. Assessment of Skin Flaps Using Optically Based Methods for Measuring Blood Flow and Oxygenation. *Plast Reconstr Surg* 2005; 115: 539-546.

7.Hölzle F, Loeffelbein DJ, Nolte D, Wolff KD. Free flap monitoring using simultaneuous non-invasive laser Doppler flowmetry and tissue spectrophotometry. J *Craniomaxillofac Surg* 2005; 34: 25-33.

8.Yuen JC, Feng Z . Monitoring Free Flaps Using Laser Doppler Flowmeter: Five-Year Experience. *Plast Reconstr Surg* 2000; 105: 55-61.

9.Irwin MS, Thorniley MS, Doré CJ, Green CJ. Near infra-red spectroscopy: a non-invasive monitor of perfusion and oxygenation within the microcirculation of limb and flaps. *Br J Plast Surg* 1995; 48: 14-22.

10.Machens HG, Mailaender P, Reimer R, Pallua N, Lei Y, Berger A. Postoperative Blood Flow Monitoring after Free-Tissue Transfer by Means of the Hydrogen Clearance Tecnique. *Plast Reconstr Surg* 1997; 99 : 493-505.

11.Setälä LP, Korvenoja EM, Härmä MA, Alhava EM, Uusaro AV, Tenhunen JJ. Glucose, Lactate and Piruvate Response in an Experimental Model of Microvascular Flap Ischemia and Reperfusion: a Microdialysis Study. *Microsurgery* 2004: 223-231.

12.Swartz WM, Izquierdo R, Miller MJ. Implantable venous Doppler microvascular monitoring: Laboratory investigation and clinical results. *Plast Reconstr Surg* 1994; 93: 152.

13. Swartz WM, Jones NF, Cherup L, Klein A. Direct monitorin of microvascular anastomes with the 20-mHz ultrasonic Doppler probe: An experimental and clinical study. *Plast Reconstr Surg* 1988; 81: 149.

14.Kind GM, Buntic RF, Buncke GM, Cooper TM, Siko PP, Buncke HJ Jr. The Effect of an Implantable Doppler Probe on the Salvage of microvascular Tissue Transplants. *Plast Reconstr Surg* 1998; 101: 1268-1273.

15.Niitsuma K, Kuwahara M, Iioka H, Yurugi S Use of Plastic Baskets for Rat Anestesi. *Plast Reconstr Surg* 2006; 117: 1076-1078.

16.Polotto S. Nipple-areola complex reconstructive techniques applied to breast reconstruction: a systematic review. Euromediterranean Biomedical Journal 2012, 7(5):15-25.

17.Polotto S. Scar revision principles applied to excisional, irregularization and v-to-y advancement flap techniques. Capsula Eburnea 2011, 6(19):90-97.