The development of neovascularisation in flap prefabrication with vascular implantation: an experimental study

N. Kostakoglou, S. Manek and C. J. Green
Northwick Park Institute for Medical Research, Northwick Park Hospital, Harrow, UK

SUMMARY. Femoral arteriovenous pedicles were implanted beneath the abdominal skin in rats (n = 30) to investigate the development of neovascularisation over time. New vessel formation was assessed by microangiography and quantified under light microscopy. Flap viability was assessed by dye injection studies at different time intervals. Two weeks after implantation of the vascular pedicles, neovascularisation was confined to the vicinity of the arteriovenous pedicle only and none of the flaps were viable. At 4 and 6 weeks, 4 out of 6 flaps were viable, with new vessel formation being widespread throughout the subcutaneous tissue. All flaps were viable at 8 and 12 weeks. Neovascularisation in the dermis up to the epidermis was observed after 8 weeks. Neovascularisation in the panniculus carnosus showed a steady increase after 6 weeks. There was a decrease in the total number of vessels at 12 weeks compared to 8 weeks but at 12 weeks the diameters of the vessels were considerably larger. Flap survival was best predicted by the amount of neovascularisation in the panniculus carnosus. In this experimental model, it is concluded that prefabricated flaps should be raised at 8 weeks instead of 4 or 6 weeks after the implantation of an arteriovenous pedicle, to reduce the risk of flap failure.

Neovascularisation is a process which is frequently exploited in the daily practice of plastic surgeons. Erol described the transformation of an ordinary skin graft into a pedicled flap through neovascularisation. Later, tissue modelling employing this phenomenon was called prefabrication. Different vascular carriers, e.g. omentum, muscle, arteriovenous pedicles and vascularised fascia, have been used to induce neovascularisation in simple or composite flaps. Successful clinical applications of the method have also been reported.

Experimental and clinical implantation of an arteriovenous pedicle into a skin flap was reported by Yao. Several studies have since been done to investigate neovascularisation with different modifications of this particular method of prefabrication. However, there is still controversy about when to raise such prefabricated flaps. Also, there are few studies about the development of neovascularisation over time. We therefore investigated the time course of neovascularisation with implantation of arteriovenous pedicles. Special attention was given to the neovascularisation process in different tissue layers and its relevance to flap viability.

Materials and methods

Thirty male Lewis rats weighing 300-400 g were used in this study. The rats were randomly allocated to 5 groups of 6 animals each. Anaesthesia was achieved by fentanyl and fluanisone (1.2 ml/kg IM) and diazepam (5 mg/kg intraperitoneal).

In all animals, a 3 x 2 cm rectangular skin flap was designed obliquely on the abdominal wall so that the short side would lie parallel to the inguinal ligament (Fig. 1A). The right femoral artery, vein and nerve were dissected as a bundle starting from the inguinal ligament down to the ankle and ligated distally. The reason for including the femoral nerve in the pedicle was to avoid extensive dissection in the pedicle which might lead to vasospasm and thrombosis. The epigastric vessels were divided close to the femoral vessels to increase the reach of the pedicle further distally to the tip of the skin flap. This arteriovenous bundle was then turned over and passed through a tunnel created by a haemostat in the subcutaneous tissue at the site of the planned skin flap (Fig. 1B). The subcutaneous tunnel was 2-3 mm wide to allow the passage of the pedicle only. A special effort was made not to raise the flap completely to avoid the effect of the delay phenomenon. The tip of the arteriovenous bundle was secured to the distal end of the flap site by a 6/0 polypropylene suture. A 3 mm wide silicone sheet was placed beneath the pedicle so it could be located easily at the second stage. The skin incision was sutured and the animals were returned to their cages.

After 2, 4, 6, 8 and 12 weeks of prefabrication (n = 6 each), the pedicles were located on the abdominal wall of the rats by palpation. A skin flap with the same design and dimensions as the preoperative markings was raised on the right femoral arteriovenous pedicle. Ten minutes later, the circulation through the flaps was assessed by injection of 1 ml Evans Blue dye from the contralateral femoral vein and observation of blue colouring in the flap tissue for a period of 30 minutes. The flaps were considered viable only when the skin was stained blue. Flaps which showed no change in the normal pinkish skin colour, with blue colouring restricted to the course of the pedicle in the subcutaneous tissue, were considered nonviable. Transposition or free transfer of the flaps to assess their viability were...
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Fig. 1

Figure 1—(A) Design of the rat abdominal skin flap measuring 3 x 2 cm. (B) Femoral arteriovenous bundle ligated at the ankle and turned over to be tunnelled through the subcutaneous tissue.

not considered, as these would have interfered with the quantification of neovascularisation due to the interaction between the flap and its recipient bed. On harvesting the flaps, microangiography was performed by injection of 0.5 ml Micropaque (1:1 diluted with isotonic saline) into the femoral artery which was followed by fixation of the tissues for histopathology.

The prefabricated flaps were divided into two halves to examine new vessel formation in their proximal and distal parts. This examination was to find out whether the site of entry of the implanted pedicle had any influence on neovascularisation. Quantification of neovascularisation was performed by the pathologist (SM) under x25 magnification using a 1 cm² eyepiece graticule with a light microscope. The pathologist did not know which group each specimen was from.

Counting of the vessels was done separately in different tissue layers (pedicle, subcutaneous tissue, panniculus carnosus, dermis). The vascular structures were counted in five different high power fields for all the layers in each flap and the mean number of vessels per unit area was calculated. The unit area was the area seen under x25 magnification using a 1 cm² eyepiece graticule. Abdominal skin flaps of the same design and dimensions as in the experimental groups were harvested from 6 normal rats to serve as controls. Quantification of the vessels was done as described above in all layers, except the pedicle. The Wilcoxon matched pairs signed rank test was used to analyse the difference between the proximal and distal halves of the flaps. Kruskal Wallis analysis of variance and Dunn’s nonparametric post-hoc test were used to analyse the difference in new vessel formation in the various tissue layers at different time intervals. A logistic regression model was used to examine which layers of the flap tissue were predictive of the chances of flap survival.

Results

Flap viability

None of the flaps survived when elevated after 2 weeks of prefabrication. Four out of six flaps (67%) were viable in both the 4 and 6 week groups. Flaps which showed no change in their overall skin colour after dye injection were considered nonviable, although all showed blue colouring in the subcutaneous tissue around the pedicle. All the flaps studied after 8 and 12 weeks were viable with blue colouring of the flap skin following dye injection.

Microangiography

New vessel formation was confined to the tissues around the pedicle 2 weeks after prefabrication (Fig. 2A). In the 4 and 6 week groups, the viable flaps demonstrated widespread vascularity around the arteriovenous pedicle (Figs 2B, C), whereas the nonviable flaps had similar findings to those of the 2 week group. More widespread neovascularisation was observed after 8 and 12 weeks of prefabrication (Figs 2D, E).

Histopathology

In the 2 week group, there was mild to moderate neovascularisation which was confined to the subcutaneous tissue around the pedicle (Fig. 3A). There was no increase in vascularity in the dermis.

In the 4 and 6 week groups, neovascularisation was similar, mainly being widespread in the subcutaneous tissue (Fig. 3B). In two flaps in each group, barium filling was observed above the panniculus carnosus, in the lower dermal vessels.

In the 8 week group, there was abundant new vessel formation in the subcutaneous tissue, as well as an increase in the number of new vessels in the dermis up to the epidermis (Fig. 4A). Approximately half of these vessels were filled with barium.

In the 12 week group, there were moderate numbers of blood vessels around the pedicle and in the dermis up to the epidermis. The total number of new vessels in the flaps was less than that seen at 8 weeks, except for a particular increase in the panniculus carnosus (Fig. 4B). Despite the decrease in the total number of vessels in the flap tissue, nearly all vessels had barium in their lumen. The calibres of the vessels were considerably larger when compared to those of 8 weeks (Fig. 4C).
Figure 2—(A) Microangiography performed 2 weeks after prefabrication with implantation of a femoral arteriovenous bundle. Note that the new vessel formation is confined to the vicinity of the pedicle. (B) More widespread neovascularisation around the pedicle at 4 weeks, and (C) at 6 weeks. (D) A mature network of vessels is established after 8 weeks, and (E) 12 weeks of prefabrication. (P: Proximal. D: Distal).

Quantification

Quantification of neovascularisation in the proximal and distal halves of the flaps is shown in Figure 5. The differences between the number of new vessels in the proximal and distal halves of the flaps at all time intervals were not statistically significant.

Quantification of new vessels in the different tissue layers over time is illustrated in Figure 6. The greatest number of new vessels was observed around the pedicle at all time intervals, reaching a maximum at 8 weeks. New vessel formation in the subcutaneous tissue showed a similar course over time to that of the pedicle. The number of blood vessels in the dermis was constant until 6 weeks, then increased to a peak at 8 weeks. The number at 8 weeks was significantly different from the numbers in the dermis at other times and in the controls ($P < 0.05$). The number of vessels
in the panniculus carnosus rose slightly at 4 weeks and then steadily increased from 6 weeks. The number of vessels at 12 weeks was statistically different from the numbers at 2 and 6 weeks and in the controls ($P < 0.01$).

In the backward logistic regression model including all layers of the flaps, flap survival was best predicted by the amount of neovascularisation in the panniculus carnosus ($P = 0.013$).
Discussion

Neovascularisation of simple or composite tissues utilising vessel implantation is a well documented method of flap prefabrication. However, there are contradictory reports in the literature about the exact time when such prefabricated flaps can be raised and transferred. Yao reported that skin flaps, which were tubed and prefabricated by the implantation of a central auricular artery and vein in the frontal region of rabbits, could survive elevation and transfer as early as 9 days after vascular implantation. He elevated prefabricated lower abdominal skin flaps in rabbits 4 weeks after implantation of the femoral artery and vein, while in clinical cases he waited 3–5 weeks before transferring prefabricated flaps. Yao later reported a case where he transferred a free thigh flap 6 weeks after flap prefabrication. Hiras et al. reported that 2 weeks was sufficient to obtain transferrable free skin flaps in rats using vein grafts interposed between the epigastric vessels in rats. Takato et al. raised viable 8 x 10 cm skin flaps from the abdominal wall of rabbits 10 days after prefabrication. Duffy et al. reported near total survival of 1 x 2 cm prefabricated rabbit ear skin flaps 2 weeks after vascular implantation; however, they also observed increased survival in larger flaps as the time between implantation and flap elevation increased. Morrison et al. found a significant improvement in the viability of prefabricated flaps which were raised after 8–12 weeks compared with flaps raised after 4 weeks on rabbit hind limbs.

In the present study, none of the prefabricated flaps survived the elevation 2 weeks after prefabrication, while four out of six flaps were viable at both 4 and 6 weeks after the implantation of an arteriovenous pedicle. Assessment of flap viability was mainly determined by Evans Blue dye injection on an ‘all or none’ basis. Thus, those flaps which turned blue in the skin after 30 minutes were regarded as viable, while all other forms of limited or mottled blue colouring in the subcutaneous tissue together with the pink skin colour of the flap were considered as nonviable. Although dye injection studies sometimes have the disadvantage of underestimating the area of flap survival, our method of assessment is in concordance with the view that capillary blood flow is the principal factor determining flap viability. Therefore, no change in the pink skin colour was accepted as a sign of absence of the dye in the capillary circulation. In other studies, survival of prefabricated flaps was assessed 3–10 days after transposition or free transfer. There is no doubt that survival of flaps after transposition is a more objective way of assessing viability. However, total skin necrosis may not occur in experimental flaps after ligation of the pedicle as a result of neovascularisation from the underlying recipient bed. Therefore, transposition of the flaps to assess survival was not attempted in the present study, as this would have given rise to new vessel formation from the recipient bed, thereby interfering with quantification of neovascularisation in the flap tissues. Microangiographic findings correlated well with the viability of these flaps by demonstrating the vessels to be widespread in those flaps which survived. All flaps were viable after 8–12 weeks of prefabrication.

The discrepancy between different studies concerning the optimal time for elevation of prefabricated flaps seems to originate from the fact that a wide range of experimental models has been used. In the studies where the flaps were elevated at about 2 weeks, either the delay phenomenon was exploited to induce neovascularisation or the flaps were already raised and left in situ during prefabrication so that new vessel formation from a healthy recipient bed was also induced in addition to the presence of a vascular bundle. Both Hiras et al. and Takato et al. used vein grafts to establish an arteriovenous shunt and tacked these vessels under the flaps raised at the time of the initial operation. In the latter study, the model used was an arterialised venous flap. Duffy et al. and Pribaz et al. implanted vascular pedicles into completely raised...
subdermal pockets in rabbit ears. Only the small flaps (1 x 2 cm) survived almost totally 2 weeks after prefabrication, while larger flaps (1 x 4 cm or 1 x 6 cm) required 6 weeks for near total flap survival. Their results suggest a direct correlation between the surface area of viable flaps and the period of prefabrication.

In a previous study, we also observed a neovascularization time of 2 weeks to be sufficient for small size skin grafts (1 x 2 cm) placed over the inferior epigastric tunnelled through the subcutaneous tissue, carefully avoiding elevation of the flap. By doing so, we aimed to create a model where we could obtain a prefabricated flap with the least possible fibrosis in its substance. The contact surface between the vascular carrier and the flap was restricted to a minimum. Unlike the studies mentioned above, any interaction between an elevated flap and a recipient bed was carefully avoided. Therefore, the additional source of new vessel formation was cancelled in our experimental model. Perhaps this factor played an important role in the relative delay of neovascularisation we have observed. Ono et al. reported similar findings to our study using a similar experimental model. They implanted the femoral vascular bundle beneath the cutaneous pocket, and concluded that the minimum implantation time for a successful prefabricated flap transfer should be between 6 and 8 weeks. Tark et al. reported faster and better neovascularisation with a wide strip of vascularised fascia than a narrow one, thus stressing the importance of broader surface contact of the vascular carrier with the target tissue. When the results of other studies are taken into consideration, it is proposed that the 3 mm wide contact surface between the flap and the vascular pedicle was the main factor leading to later neovascularisation.

The potential role of insertion of a silicone sheet in accelerating neovascularisation has been studied. Itoh observed rapid neovascularisation occurring immediately after insertion of a silicone sheet with the blood supplied mainly from the vessels surrounding the flap. In the same study, he also reported a decrease in the survival rate of the flaps when the silicone sheets were left in place more than 3 weeks. However, his study was limited by the absence of any control groups without silicone sheets. Matoal et al. reported that, despite increased neovascularisation, isolation of the recipient bed from the flap or the vascular carrier did not influence the survival of prefabricated skin flaps. In our study, the reason for using 2-3 mm wide silicone sheets underneath the implanted pedicle was to locate the pedicle more easily at the second stage while elevating the flap. Unlike the previous studies, we observed no complications associated with the silicone sheets. Since we have no cases without a silicone sheet, it is not possible to comment on the effect of the silicone sheet on neovascularisation in our study.

With regard to the time course of neovascularisation in different tissue layers, our study has shown that new vessel formation starts around the arteriovenous pedicle. Early in the course of prefabrication some of these vessels formed communications as demonstrated by microangiography. After 4-6 weeks, neovascularisation became widespread in the subcutaneous tissues with larger vessels and denser vessel configuration. Two out of six flaps at both time intervals had new vessels in the lower dermis. However, there was no statistically significant increase in the number of new vessels in the dermis and panniculus carnosus until the end of 6 weeks. Regarding the relationship between neovascularisation and viability of the flaps, 4-6 weeks could be considered a critical turning point where communications between the vessels of the subcutaneous tissues and the dermal plexus start to form. The increase in the number of new vessels in the dermis and panniculus carnosus at 8 weeks seems to enable a more mature circulation to form, thereby guaranteeing the viability of the prefabricated flap. This was supported by the logistic regression test, which revealed that the panniculus carnosus was the most predictive layer in determining the chance of flap survival. In addition, this study has interestingly shown that maturation of circulation continues until 12 weeks with an increase in the calibre of newly formed vessels.

In this study, allocation of 6 animals to each time interval increased the minimum difference necessary to be detected as statistically significant. Thus, application of nonparametric tests was more suitable, and this has decreased the power of the statistical analyses. From the histopathological observations made in this study, it seems that the panniculus carnosus acts as a barrier to the upgrowth of blood vessels in the early stages. However, it later serves as a pool where newly formed vessels of the subcutaneous tissue and dermis meet to form a network. Regarding the similar number of vessels in the pedicle area and subcutaneous tissue at 2, 4 and 6 weeks, despite the difference in flap viability, it may be concluded that the amount of new vessels in these particular layers is not always an indicator of better vascularity. The peak observed at 8 weeks in these particular layers may be due to either the variability of response in the small groups of animals or the interconnection between the dermis and panniculus carnosus vessels with the vessels of subcutaneous tissue.

In this study, there was no significant difference between the number of new vessels in the proximal and distal halves of the flaps at all time intervals. This suggests that the neovascularisation process is not influenced by the direction of flow in the pedicle, but rather triggered by the interaction between the pedicle wall and subcutaneous tissue throughout the whole length of the vessels. Morrison et al. reported a similar pattern of neovascularisation in prefabrication by the vascular implantation method and suggested that the inflammatory response provoked by perivascular dissection might be the responsible factor for new vessel formation.

From a clinical point of view, the goal of tissue prefabrication is tailoring tissue to the patient's requirements without being restricted to natural vascular territories. The main advantage of prefabrication with vascular implantation is the likelihood of having a near normal, thin skin flap without considerable fibrosis in its substance. To achieve such a flap it seems
reasonable to avoid raising the tissue while performing the vascular implantation as it was done in this study. The main drawback of the method is the long waiting period during the first stage and the possible lack of a suitable arteriovenous pedicle. However, the latter could be overcome by liberal use of vein grafts.

In conclusion, the present study has shown that it may be possible to elevate flaps prefabricated by the vascular implantation method at 4–6 weeks. Nevertheless, one should better wait for 8 weeks for a more mature circulation to develop, to reduce the risk of flap failure.

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References


The Authors

Naci Kostakogiá MD, Associate Professor of Plastic Surgery, Hacettepe University Medical School, Ankara, Turkey. Formerly Microsurgical Research Fellow in the Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex.

Sanjiv Maneck BSc, MBBS, FRCPATH, Senior Registrar in Histopathology, Radcliffe Infirmary, Oxford. Formerly Research Pathologist in the Section of Surgical Research, Northwick Park Hospital, Harrow, Middlesex.

Colin J. Green PhD, DSc, FRCS (Hon), FRCVS, FRCPath. Professor and Head of the Institute, Northwick Park Institute for Medical Research. Harrow, Middlesex.

Correspondence to Dr Naci Kostakogiá, Bilkent-1, Evleri E-5, Blok D-18, Ankara 06533, Turkey.

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