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Prefabricated flaps and neoangiogenesis initiated via venous grafts in arteriovenous loops

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Abstract

New developments in regenerative medicine are bound to revolutionize the way we approach loss of function and form in human organisms. Especially in the field of reconstructive plastic surgery new biotechnologies find their way from bench to bed. Biofabrication is an evolving field that aims to combine natural biologic processes with bioartificial constructs with the scope of reconstituting tissue without having to rely on autotransplantation. In this brief review we present the concepts of intrinsic *vs.* extrinsic neovascularization and we discuss the use of neovascularization in three dimensional matrices. In a clinical context matrix flaps for application in reconstructive surgery can be fabricated this way.

Keywords: Plastic surgery, flap prefabrication, tissue engineering, arteriovenous loop, venous graft, neoangiogenesis

INTRODUCTION

Due to a steadily increasing life span not only in developed countries tissue wear-out or tissue loss becomes a growing problem to preserve sufficient quality of life. This holds especially true for elderly patients[1]. The interdisciplinary field of Tissue Engineering (TE) and Regenerative Medicine (RM) is one area where the hope for cure of these problems is seen. Within this specialty of life sciences "Biofabrication" recently has been added as an evolving research field that aims to optimize spatial cell and growth factor delivery into laboratory grown constructs to mimic the natural consistency of tissue like structures. According to Groll *et al.*[2], from a research strategy perspective, Biofabrication within TE and RM aims at exploiting automated processes, for
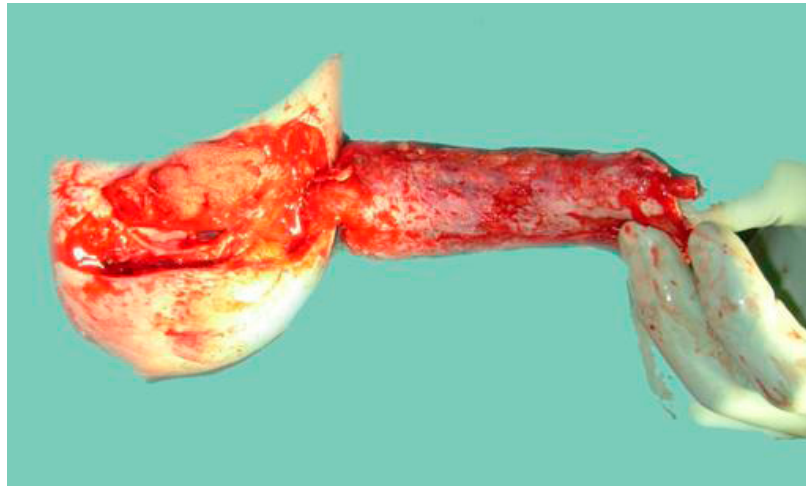
Figure 1. Prefabricated flap with skin grafted undersurface of radial arm forearm free flap to reconstruct the lower lip after extensive cancer resection

the most part Additive Manufacturing techniques, to generate cell-biomaterial constructs that, through their internal and external spatial arrangement may mature into functional tissue equivalents. Accordingly, these strategies typically target the development of scaffolds or composite constructs which exhibit tissue mimetic hierarchical features[3]. When living single cells, bioactive molecules, biomaterials, or cell-aggregates small enough to be printed are used for fabrication, the mentioned constructs can be achieved by bioprinting as defined by Guillemot *et al.*[4] earlier. While these new techniques offer reasonable advantages in TE and RM utilizing a subsequent maturation process after 3D cell printing it may help to yield structural biologically functional constructs, the clinical translation into relevant patient applications is still lacking[5]. TE constructs still suffer from a lack of vascularity at the time of transplantation into the human recipient.

Similar to our clinical routine with the prefabrication of individualized customized flaps [Figure 1] for transfer our group has repeatedly investigated the prevasculrization of TE scaffolds utilizing an arteriovenous loop to further a clinical translation of laboratory grown tissue substitutes. We have therefore studied the effects of prevascularization in TE constructs using an arterio-venous loop (AV-loop) to 3D vascularize given matrices of relevant size before they are transplanted into the recipient in small and large animal models[6-8] and have successfully transferred this technique into clinical application[9].

Nevertheless, efforts have been undertaken to unravel the complex mechanism and receptor network that are involved in neoangiogenesis. To better understand why and how neovascularization effects occur we investigated experimental AV-loop models several times[10-12] [Figure 2]. One insight from these experiments was that the interposition of a vein graft into the loop optimally leads to vascularization of the constructs, while this is not working equally well in the case of flaps with an arteriovenous bundle only[13,14]. To further enhance neovascularization it has also been demonstrated that a combination of intrinsic and extrinsic vascularization even yields faster neovascualrization[15]. Polykandriotis *et al.*[13] have shown that early arterialization and angiogenesis in the AV-loop in a fibrin matrix with an interpositional venous graft (IVG) segment, which was placed into a closed chamber and embedded into a fibrin gel in an animal model, revealed direct luminal neovascular sprouting, evident between day 10 and day 14 from the vein and the IVG but not from the arterial segment.

Investigating the special role of the venous graft itself within the experimental setting of an AV-loop to vascularize TE constructs seems valuable. From clinical observations in prefabricating flaps or transplanting free flaps with arterial or venous extensions or utilizing AV-loops to connect the flaps[16] it seems obvious
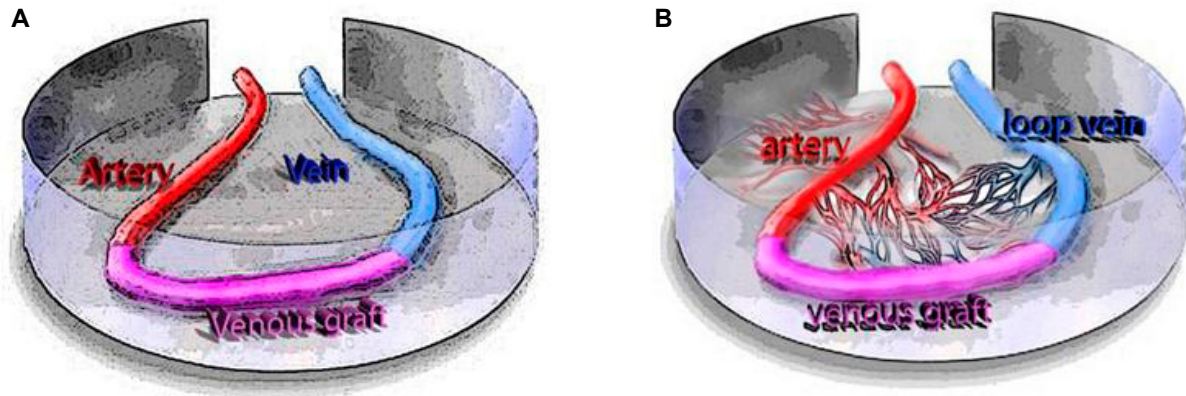
Figure 2. A: The AV-loop in the isolation chamber. V-femoral vein, A-femoral artery, G-venous graft, C-isolation chamber. After exposure of the femoral neurovascular bundle at the medial thigh of the rat, a femoral venous graft is harvested from the contralateral side and interposed between the femoral vessels by anastomoses. The isolation chamber is placed in the medial thigh of the rat and 300 μL of the fibrin matrix are applied at the bottom of the chamber. The arteriovenous fistula is laid onto the clot with the artery and vein exiting through an opening at the proximal pole and are covered with the rest of the fibrin clot. The lid is closed and the chamber with the matrix inside is fixed onto the adductor fascia at the medial thigh; B: after an interval of approximately 6 weeks, neovascular sprouts emerge from the AV-loop and form new fibrovascular tissue

that the interpositional vein graft itself may play a crucial role in neovascularization.

OWN EXPERIENCE OF THE AUTHORS

We have reviewed numerous of our own studies utilizing an AV-loop in small and large animals to 3D vascularize scaffolds or to study principles of neoangiogenesis for tissue generation or in the context of cancer research[3,7,10,11,17-22]. The process of establishing an arteriovenous loop in a rat model is described in detail by Weigand *et al.*[23]. To analyse the phenomenon of neovasculogenesis from such an AV-loop, as previously described in earlier studies[13,14,24-26] AV-loops were created in inbred male Lewis rats weighing approximately 250 g (Charles River, Sulzfeld, Germany) in various study designs. The loops were embedded into a custom-made cylindrical Teflon isolation chamber, which was fixed in the medial thigh of the rat by means of polypropylene 5-0 and in most of the studies 300 μL of the fibrin matrix were placed at the bottom of the chamber. The arteriovenous fistula was then laid onto this clot with the artery and vein exiting through an opening at the proximal pole and was then covered with the rest of the fibrin clot.

We also performed numerous studies with bone matrices or scaffolds in the small and in the large animal model.

Our primary series utilized the TISSUCOL-Kit 2,0 Immuno (Baxter GmbH, Unterschleißheim, Deutschland) according to the manufacturers recommendations. When using fibrin alone a fibrin clot of 600 μL with end concentrations of 33.7 mg/mL Fibrinogen and 25 IU/mL Thrombin was used as previously described[13,23,27] [Figures 3 and 4].

To investigate the neoangiogenesis and to study the cycle of sprouting and vasculogenesis in this context, 3D computed tomography was performed in order not to sacrifice the experimental animals. Corrosion casts were another method to visualize vasculogenesis. For this end at different time intervals from implantation to explantation, AV-loops were perfused with a low viscosity resin and were processed for scanning electron microscopy of the vessel wall. Controls were performed with immunohistochemistry and by means of computer tomography which allowed linear *in vivo* investigations.

Visualization of angiogenesis phenomena over various time points was attained with the help of the
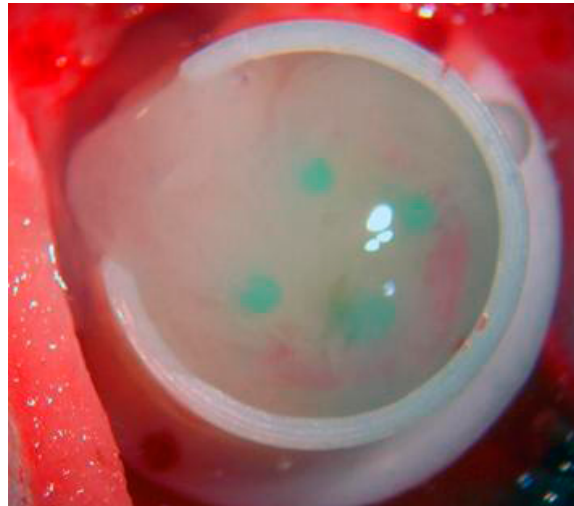
Figure 3. Fibrin matrix within experimental chamber including an arteriovenous loop: at the time of implantation the gel matrix is covering the AV-loop, which is fixed with 4 pins to prevent discloation of the loop
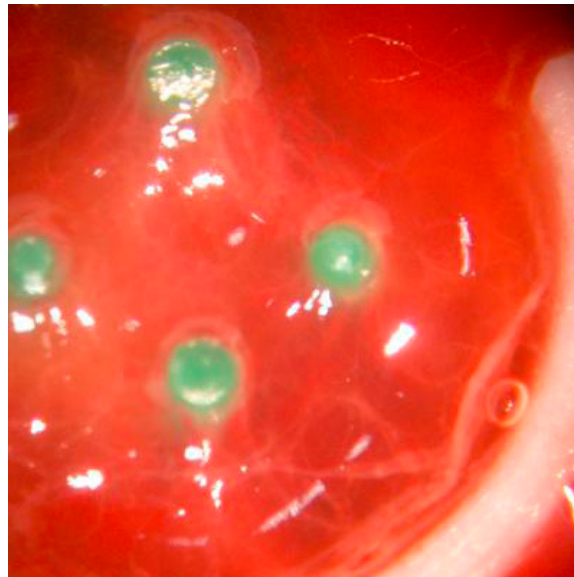

Figure 4. Fibrin matrix within experimental chamber including an arteriovenous loop: at day 12 the matrix is showing red colour as a sign of vascularization

corrosion cast technique [Figure 5]. For this purpose the animals aorta and inferior vena cava were exposed through a median incision from the xiphoid process to the pubic symphysis. The aorta was then cannulated through a 24-gauge catheter and the inf. vena cava was severed. After cannulation of the aorta the vascular system of the rat was rinsed with approximately 200 mL of heparinized Ringer solution (100 IU/mL) under a hydrostatic pressure between 80 and 100 mmHg and until fluid escaping from the severed inf. vena cava was clear. Perfusion of the caudal vascular system with 20 mL of a methylmethacrylate resin in a mixture of 4:1 prepolymerized oligomere to methylmethacrylate monomer (Aldrich Chemie, Germany) and benzoyl peroxide as a catalyst (Mercox, Ladd Research Industries, Burlington, VT) was then performed to gain vascular replicas, which could be investigated with a scanning electron microscopy (SEM). In addition to better detect the phenomena of luminal sprouting from the central vascular axis, microdissection under a microscope was undertaken as a modification of a method described previously[28].

Figure 5. Example of corrosion cast showing enhanced neovascularization with vessel sprouting and formation of capillary bed rom an experiment with a duration of 6 weeks: vasculogenesis is more pronounced from the venous side than from the arterial side
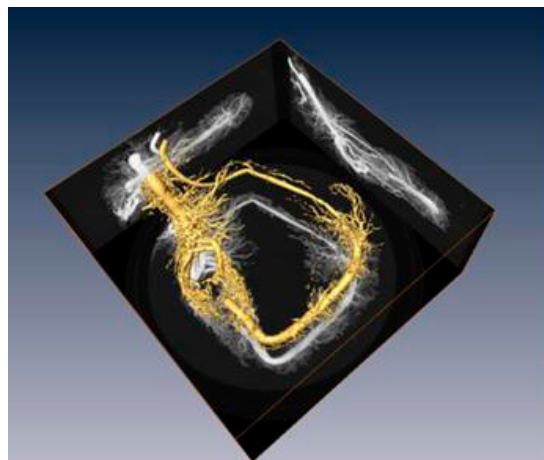
Figure 6. Micro-computed tomography of AV-loop in chamber with visibly enhanced neovascularization including vessel sprouting and formation of a capillary bed from the venous side rather than from the arterial side from an experiment with a duration of 2 weeks

Micro computed tomography (CT) was performed with a FORBILD High Resolution Micro-CT (Erlangen, Germany)[13] [Figure 6]. We have previously shown another possibility to visualize the vessels fully in 3D by calculating an isosurface for all voxels above a given threshold[13].

THE ANGIOGENIC CASCADE IN THE AV LOOP MODEL

In a first line of experiments we verified that vascularization of a confined porous matrix was feasible. For this purpose we combined the AV-loop model, with a porous bone matrix and encased the system in an isolation chamber. Explantation times were set to 2, 4 and 8 weeks[29].

Within 8 weeks post implantation, the entire matrix was usually infiltrated with a fibrovascular tissue. Essentially, the matrix was vascularized. We evaluated the results by means of histomorphometry, immunohistochemistry, corrosion casting, micro-magnetic resonance angiography (MRA) as well as micro-

computed tomography. We noticed an undisturbed perfusion of the extremities during the whole period with a general patency rate of 83.3% of the AV-loops as previously shown[13]. Pushing on, we found out that the AV loop was able to vascularize a fibrin matrix. However, neovascularization seemed to emerge mainly from the venous part and the interpositional graft of the AV-loop and less from the arterial segment[13].

In another line of experiments, after addition of vasoactive growth factors (VEGF and FGF) at the time of implantation we could observe that the angiogenic events were significantly accelerated in the group with the growth factors, in terms of onset of sprouting as well as progression into the phase of remodelling[24].

With micro-CT it was possible to visualize the patency of the vascular axis which could be confirmed both as was macroscopically seen at the time of explantation. When we compared the findings to long term observations after 6 weeks, the results at two weeks demonstrated initial arteriovenous shunting through the graft. This indicates that the neoformation of the capillary network within the constructs in the chamber forms an organoid like structure with an artery and a draining vein.

In the same time, the prevascularized matrices were secondarily loaded with a plethora of different types of stem cells, including osteoblasts and liver stem cells. It was a natural evolution that the AV loop was evaluated in the big animal model. Beier *et al.*[30] performed the AV-loop operations on sheep with great success.

Currently the AV-loop is utilized in selected cases in the clinical setting[9,11,16,31].

IMPORTANCE OF THE AV LOOP MODEL FOR RECONSTRUCTIVE MEDICINE

In general, phenomena of neovascularization and neoangiogenesis play a crucial role in Plastic Surgery and have led to the prefabrication and preformation of customized flaps[32] that are created in situ in the patient[33,34]. Flap prefabrication is a clinically applied method to generate custom made transplants with the help of arterializing tissue either with arteriovenous bundles or arteriovenous loops[35]. It has been described for various tissues, including skin, fasciae, muscle, periosteum and/or bone[36-39]. In the clinical scenario both arterial and venous supercharging have been postulated to potentially improve the survival area of prefabricated flaps and to extend the possibilities of creating custom made tissue flaps[40-42].

The combination of prefabrication and tissue engineering has been investigated intensely[39,43,44]. From this clinically proven method we introduced the technique of prevascularizing scaffolds and constructs in the context of Tissue Engineering with the help of an arteriovenous loop incorporated into a given matrix[3,23]. In small and large animal models we observed that the arteriovenous loop triggers a vivid and rapid angiogenetic response[7,11,17-19]. In our experiments male imbred Lewis rats were used. In personal communications with other groups we were intrigued to hear that on Wistar rats the phenomenon of limp ischaemia was common. Our data shed light on the early phase of angiogenesis between day 2 and day 4, as we could not find vessel sprouting. On the other side between the 10th day up to day 14 a vivid angiogenetic response was seen that led to a rapid induction of a dense neocapillary microvascular network. In our AV-loop model we had expected an early sprouting from capillaries and venules as has been described generally in the context of angiogenesis as soon as 27 h after the angiogenetic trigger[45]. The influence of the fibrin matrix that we used might be causative for this observation.

Tissue engineering (TE) has been a promising research field for more than two decades by now. Nevertheless despite recent advances the translation of TE into daily clinical practice has not yet been achieved. One major obstacle is the step of transplanting a generated tissue construct into a recipient site, where it is dependant from the ingrowth of small vessels into the scaffold. Since this process of revascularization takes several days any cell within a matrix rely on nourishing by diffusion only. Intrinsic prevascularization

will allow cells within a matrix to survive the critical time frame until vascularization has taken place and nourishing substances can be delivered from the recipient into the bioartificial construct[10].

Other studies have used Matrigel as a matrix for the AV-loop and showed enhanced neovascularization[46]. However, for potential clinical applications, matrigel, extracted from the Engelbreth-Holm-Swarm mouse sarcoma, is not a matrix of choice in patients. In contrast, fibrin as a matrix is a commercially available product that has been in clinical use for decades. It possesses several advantages as a potential matrix, some of which have to do with the interplay between controlled fibrinolysis and onward angiogenesis[47]. We have shown that the kinetics of neoangiogenesis and neovascularization can be enhanced by addition of soluble growth factors which boost neovascular growth[48,49]. Fibrin is one possible carrier and has been repeatedly demonstrated to be a suitable carrier for cultured cells, such as in cultured human keratinocyte transplantation[50,51] and muscle TE.

The formation of new blood vessels is a fundamental process in tissue regeneration and in organ development. Angiogenesis is most frequently associated with inflammation, along with hypoxic conditions and cell infiltration, although it remains elusive how molecular and cellular mechanisms underlying inflammatory reactions in detail regulate angiogenetic processes. Hypoxia-inducible factors (HIFs), HIF1 alpha and HIF2 have been investigated as potential indicators of neoangiogenesis in various contexts[52-55].

The influence of mesenchymal stem cells might also play a critical role in neovascularization[56,57]. It has been hypothesized that angiogenesis and vasculogenesis can be studied in the context of TE with regard to similar processes that occur in tumorigenesis and that TE models could help to further clarify these biological events[58]. Phenomena of intimal hyperplasia and thickening of the vessel wall during neoangiogenesis have been well described[59-61]. It has also been noted that myointimal thickening leads to a continuous decrease in the luminal diameter[60], which is an explanation for the decrease in the luminal vessel diameters that we observed following day 7 in our experiments. It is not clear why the intepositional vein graft shows the highest increase in angiogenesis and if there is a critical length of such an AV axis that would limit the clinical applicability. From clinical data in using long bypass grafts for free flap transplantation we suggest that the length of an interpositional vein in such circumstances graft is limited[16]. If bioartificial vessels, which would be desirable in terms of minimizing the donor site morbidity, will become clinically available and will offer the same capacity of sprouting and neoangiogenesis remains yet open.

CONCLUSION

Although the AV-loop model is rather complex and time consuming, it offers the unique advantage of studying neoangiogenesis and vasculogenesis in a controlled environment, to observe the mechanisms of vessel arborisation and remodelling, and to investigate changes in the perfusion pattern in correlation to the specific segments of the AV-loop.

DECLARATIONS

Acknowledgments

Authors' contributions
Prepared this manuscript: Polykandriotis E
One of the first to establish the AV Loop Model: Polykandriotis E, Arkudas A, Horch RE
Edited the manuscript and provided valuable illustrations material, developed and established the AV Model: Arkudas A

Currently working with the AV Loop Model, contributed with her own insight for the future of the AV Loop: Weigand A

Edited parts of the manuscript: Weigand A, Cai A

Rewrote a substantial part of the manuscript: Horch RE

Availability of data and materials

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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