Lumbar intervertebral disc allograft transplantation: the revascularisation pattern

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Declaration of interest

The authors report no conflicts of interest.

Abstract

Purpose Fresh-frozen intervertebral disc allograft transplantation has been reported to be a viable treatment option for advanced degenerative disc diseases, but rapid degeneration of the postoperative allograft was found. Loss of nutrient supply is believed to be the most likely inducer because the disc allografts have to endure in an ischaemic environment until the nutrient pathway is re-established. The aim of this study was to focus on the revascularisation of the disc allograft after transplantation in goats.

Methods Twenty male goats were used in this study. Intervertebral disc allograft transplantation was performed at L4/L5. Groups of five goats were sacrificed at 1.5, 6 and 12m postoperatively respectively. The transplanted-segments were harvested, fixed, sagittally cut and decalcified for H&E staining and immunochemistry to observe the blood vessel formation at the endplates, anterior outer annulus, posterior outer annulus, inner annulus and the nucleus. The blood vessel density and the sectional vessel area were measured.

Results Blood vessels were first found in the marrow space of the bony endplate and the outer annulus at 1.5 month postoperatively. Then, they were able to penetrate to reach the cartilaginous endplate and the inner annulus after 6 months. Interestingly, the endplate area possessed the most abundant blood vessels, with the highest level of vessel density and area at the final follow-up. None of these newly-formed vessels invaded the nucleus during the observation period.

Conclusions Revascularisation of the postoperative disc allograft has been

determined, but its pattern was different from that in adult normal discs, suggesting that the typical nutrient diffusion pattern may be affected after transplantation.

Key words: Intervertebral disc; allograft; transplantation; revascularisation; blood vessels

1. Introduction

Revascularisation is the reestablishment of the blood supply to a body part or organ that has suffered ischaemia; vasculogenesis, angiogenesis, arteriogenesis and collateral growth, distinct but complementary processes, act in concert to establish a functional vascular network and to govern post-ischaemic revascularisation [1]. It is one of the major challenges for the therapeutic success of organ transplantation such as heart, meniscus and bone allografting [2-4]. During bone allografting, revascularisation obviously affects the graft incorporation [5]. Limited revascularisation would result in insufficient remodelling, leading to non-union, weakening or even stress fracture in the bone allograft over time [6]. In contrast, increasing rapid revascularisation of the bone allograft could further enhance bone defect repair and remodelling [7, 8].

Unlike bone, the normal intervertebral disc is avascular and completely fed by the surrounding vasculature. As shown in Figure.1, the capillaries at the disc's margins provide nutrients for the disc cells *via* two distinct pathways; the cells of the outer annulus receive their nutrients from the capillaries in the soft tissues surrounding the

disc, but the only contact with the blood supply for most of the disc cells is via capillaries arising in the vertebral bodies [9, 10]. They penetrate the subchondral bone through marrow spaces and terminate in loops adjacent to the cartilaginous endplate (EP) [11].

Based on the success of large animal studies [12-14], the use of whole intervertebral disc allograft transplantation in humans for severe cervical disc degeneration has produced good clinical results in a pilot clinical trial with more than 10 years of follow-up [15, 16]. Nevertheless, rapid degeneration of the allograft was seen in some cases, which is thought to be related to a lack of timely return of the nutrient supply [10]. Because osteotomies were performed in the adjacent vertebral bodies during surgery, the postoperative intervertebral disc allograft was thus immersed in an ischemic environment until the nutrient supply was re-established [14]. As the prerequisite for nutrition reestablishment, revascularisation is essential for the survival of the postoperative disc allograft. However, little is known as to whether the host vascular network could reach and nourish the disc allograft after transplantation. Hence, the aim of this study was to focus on the revascularisation of the postoperative disc allograft, which in turn enabled the understanding of how the nutrient supply was re-established.

In this work, intervertebral disc allograft transplantation was performed in the lumbar spine of 15 goats using the newly-developed surgical technique [17]. After transplantation for 1.5, 6 and 12 months, groups of five goats were sacrificed; the transplanted segments were then harvested for histological staining and

immunochemistry to observe the blood vessel formation in the different areas of the postoperative disc allograft.

2. Materials and methods

2.1 Animals

The research proposal has been approved by Committee on the Use of Live Animals in Teaching & Research, the University of Hong Kong (CULATR1872-09). Twenty male goats (Hainan East Goats) between 6 and 9 months weighing between 17.5 and 25 kg were used in this study. Five goats served as disc allograft donors and 15 goats were used as allograft recipients.

2.2 Intervertebral disc allograft transplantation

Fresh-frozen intervertebral disc allograft transplantation was performed at lumbar spine (L4/L5) of 15 goats using the surgical technique previously described [17]. During surgery, the "retro-psoas" approach was used to expose the lumbar discs. After the recipient slot was prepared, the allograft with the most compatible size was selected, thawed and implanted without internal fixation. Two temporary screws were inserted into the upper and lower vertebral bodies for placement of a distraction device and removed after the transplantation was done (Figure. 2A). Before surgery, the L4 and L5 transverse processes could be clearly located by palpation; after surgery, the disc-transplanted level was further confirmed by radiography. The goats were allowed free mobilization immediately after recovering from anaesthesia.

2.3 Specimen harvest, histology and immunochemistry

Groups of five goats were sacrificed at 1.5, 6 and 12 months postoperatively. The disc-transplanted segments were harvested immediately and fixed in 4% paraformaldehyde in phosphate-buffered saline for 7 days. The transplanted allograft was separated and cut mid-sagittally using a band saw (EXAKT 300CP Band System, Germany); one half of the transplanted allograft was decalcified, dehydrated, embedded in paraffin wax and finally cut into 5-μm-thick sections for hematoxylin and eosin (HE) staining to observe the newly-formed blood vessels. To detect VEGF expression, slides were exposed to mouse anti-sheep vascular endothelial growth factor (VEGF) antibody (1:200) (NB100-648, Novus Biologicals, USA) at 4°C overnight; horseradish peroxidase-labeled secondary antibody (Gene Tech, Shanghai, China) was then added and incubated for 30 min. Brown color was developed using diaminobenzidine (DAB) as substrate; finally the sections were counterstained with hematoxylin and mounted with a cover slip for microscopic observation. For the control group, five intervertebral disc allografts without implantation were fixed, decalcified, dehydrated, embedded and cut for histological and immunochemistrial analysis to observe the normal vascular pattern of discs.

2.4 Blood vessel density and area measurement

Blood vessels were detected by the evaluation of HE-stained sections, and identified by the lumenal structures with the presence of erythrocytes following the literatures [4, 18-20]. The disc-transplanted segments after 1.5, 6 and 12 months were divided into four areas for blood vessel analysis, as follows: the EP, anterior outer annulus fibrosus (AF), posterior outer AF and inner AF (Figure. 2B). As the bony EP had disappeared

after disc allograft transplantation after 12 months [21], the capillary buds from the vertebral bodies in direct contact with the disc via soft matrix tissue were selected for blood vessels analysis. Using the Image J software (1.47V), the density and the sectional area of the blood vessels were measured. The blood vessel density was calculated by dividing the total number of blood vessels by the area of each observation area according to the literatures [18, 22, 23]. At each allograft specimen, values reported for the four areas of the allograft correspond to the average values obtained from three continuous HE-stained sections.

2.5 Statistics

All data were given as mean ± SD. One-way ANOVA with a LSD *post-hoc* analysis was carried out to compare the value of new blood vessel density and sectional area among different time slots after transplantation, respectively. Statistical analysis was performed using SPSS 16.0 software and significance was accepted when $P<0.05$.

3. Results

All the disc allografts were well seated without subluxation or dislocation throughout the follow-up period [17]; no postoperative complication was seen in the goats after disc allografting [24]. In the disc allograft without implantation, aggregation of the bone marrow stroma and even destruction of the bone marrow architecture were evident; the bone marrow stroma was positive for VEGF, but no obvious lumenal structures with the presence of red blood cells was seen (Figure.3A). After 1.5 months,

sparse newly-formed blood vessels positive for VEGF were embedded inside the granulation tissue at the bone marrow space of bony EP with a density of 12±5 vessels/mm^2 and a mean sectional area of 1027.47±530.87μm^2; no blood vessels formation at the junction between the bony and cartilaginous EP was noted at this time point. A consecutive increase in the vessel density was noted afterwards, ranging from 19±11 vessels/mm^2 at 6 months to 30±12 vessels/mm^2 at 12 months (Figure.3B) (P<0.01). The value of the vessel density, and that of the mean sectional area (3187.04 μm^2) at the last follow-up were highest compared to those at the other two time points (Figure.3C) (P<0.01).

The appropriate portion of the host AF and the host anterior longitudinal ligament were preserved to ensure the stability of the disc allograft during surgery. In the control allograft, very few VEGF$^+$ blood vessels were present at the outer AF. At 1.5 months, blood vessels with the presence of erythrocytes were seen at the interfaces of the host ligament and the outermost AF of the allograft; most of them were travelling between the AF lamellae and they were able to penetrate approximately 500 μm into the outer AF (Figure. 4A). The density was 14±6 vessels/mm^2 and the mean sectional area of the blood vessels was 431.06 μm^2 (Figure. 4B). No significant increase in the vessel density was observed after follow-up for 6 and 12 months. Compared with the value of the sectional area of the blood vessels at 1.5 months, a notable elevation was found after 6 months with a mean area of 832.07 μm^2 (Figure. 4C) (P<0.01). Nevertheless, this enhancement was not observed at the final follow up (P>0.05). All the lumenal structures found in the outer AF of the postoperative allograft were

positive for VEGF.

Similar findings were observed at the posterior outer AF of the disc allograft after transplantation (Figure.5). Very rare $VEGF^+$ lumenal structures were found in the posterior outer AF of the allograft before implantation. During surgery, the posterior longitudinal ligament was preserved. After 1.5 months, it was recorded that the blood vessels with the presence of erythrocytes were able to penetrate into the posterior outer AF for approximately 900 μm (Figure.5A). No remarkable change in blood vessel density was seen over time (Figure.5B); the mean values were 27, 20 and 28 vessels/mm^2 at 1.5, 6 and 12 months postoperatively, respectively. Interestingly, the highest level of vessel area was recorded after 6 months, with a mean value of 2019.33 μm^2 (Figure.5C) (P<0.05).

No blood vessels invading the inner AF were documented before implantation and after 1.5 months based on histological results (Figure. 6A-D), but a very small number of $VEGF^+$ vessels with a diameter less than 50 μm were seen in the inner AF of the allograft after 6 months (Figure. 6E-H). The sparse $VEGF^+$ blood vessels still presented until the final follow-up, but they did not penetrate into the nucleus area during the whole observation period (Figure.6G-H). Therefore, although revascularisation of the disc allograft was demonstrated, the pattern was much different from that of normal discs (Figure.1).

Based on these findings, as depicted in Figure.7, it was proposed that blood vessels were first developed in the marrow space of the bony EP and the outer AF after 1.5 months; they were then able to penetrate to reach cartilaginous EP and inner AF after

6 months. None of these blood vessels invaded the centre of allograft; undoubtedly, the EP area possessed the most abundant blood vessels, with the highest level of vessel density and sectional area at the final follow-up.

4. Discussion

The adult normal intervertebral disc obtains the principal nutrients by diffusion from the vertebral capillaries in the highly vascularised vertebral bodies [10, 25]; the vascular buds are distributed through the entire junction of bony and cartilaginous EPs, with the maximum density in the area adjacent to the nucleus pulposus [26]. Reformation of vasculature was documented in the postoperative allograft, but its pattern was changed. The re-established vasculature, perforating the cartilaginous EP, extending into the inner AF and approaching the disc centre, was similar with that found in the intervertebral discs at the fetal to infantile age [27]. But this pattern was different from that in the adult normal discs, which may alter the re-established nutrient supply in the postoperative disc allograft.

The AF comprised highly organised concentric rings of collagen fibres, so that it may be more difficult for the penetration of blood vessels than that in the EP route. After healing, some part of the cortical bone (bony EP) was replaced by trabecular bone [21], blood vessels were then able to grow in the marrow cavity and penetrate adjacent to the cartilaginous EP. Moreover, the vertebral body was highly vascularised. These anatomical features ensure the highest level of blood vessels formation in the EP area. Nevertheless, we should acknowledge that reestablishment

of vascular network is not equivalent to the return of nutrient diffusion into the disc allograft.

The VEGFs are all secreted polypeptides and they are essential for early blood vessel formation and angiogenesis [28]; furthermore, it was demonstrated that VEGF was beneficial for the survival of disc cells [29]. In this study, the VEGF expression in the lumenal structure and the surrounding matrix was found after 6 months, which may promote the revascularisation and maintain the cellular survival of the postoperative disc allograft. In the degenerated human disc, the expression of VEGF could be increased by the induction of cytokines [30] which further enhanced angiogenesis by stimulating endogenous endothelial cells. Mild degeneration of the postoperative disc allograft was found in the goat lumbar spine [24], whether the undying mechanism of blood vessel ingrowth is similar with that in the degenerated disc needs further investigation.

In the previous studies, it was found that more than 60% of the disc cells kept the metabolic activity and that the mechanical properties and matrix organization of the allograft could be maintained after cryopreservation [31, 32]. Revascularisation in the postoperative disc allograft suggested that the resident disc cells may obtain nutrients from the penetrating blood vessels directly. But whether the cargos carried by the penetrated blood vessels are good for the cells should be investigated in future research. That is because revascularisation may elevate the regional concentration of pro-inflammatory cytokines, such as interleukins and tumour necrosis factors, which are able to induce the apoptosis or death of EP chondrocytes [33-35]. Furthermore, it

should be emphasised that there was a time lag between when the nutrient pathway was re-established and when the graft cells receive the nutrients. The healing of host–graft interfaces took at least 1.5 months [21]; and the penetration of host blood vessels to the junction between the bony and cartilaginous EPs needed much more time. In patients with severe disc degeneration, the required time should be much longer than that in goats as its larger disc size [36]. Disorders disturbing the microcirculation [37, 38] may further add to the delay in reestablishment of nutrient pathway after disc allografting.

To be honest, there are several limitations in this study. First, the origin of the vasculature in the postoperative allograft was still unclear. Did they originate in the vertebrae or the allograft periphery? Or the vasculature from these two origins worked together to achieve the revascularisation of the postoperative allograft. Second, the correlation between the blood vessel distribution and cell viability/density in the postoperative allograft was not investigated; these data may be helpful to understand the function of the re-established vasculature. Furthermore, the dynamic nutrient perfusion from the penetrating blood vessels to the disc centre was not investigated. This data can be obtained in future experiments using fluorescent probes or enhanced MRI with contrast that could be transported into the disc. Finally, it was not easy to distinguish between the outer and inner AF in the allograft after 6 months, so that quantification of the blood vessels in the inner AF was not performed.

In conclusion, this study documented the revascularisation of the disc allograft after transplantation. Blood vessel formation in the EP area and the AF area was found;

revascularisation in the EP area was the leading route, with the highest level of vessel density and sectional area after 12 months. Nevertheless, the newly-formed vascular network was quite different from that of normal adult discs, suggesting that the typical nutrient diffusion pattern will be affected after transplantation.

5. Acknowledgments

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Figure legends

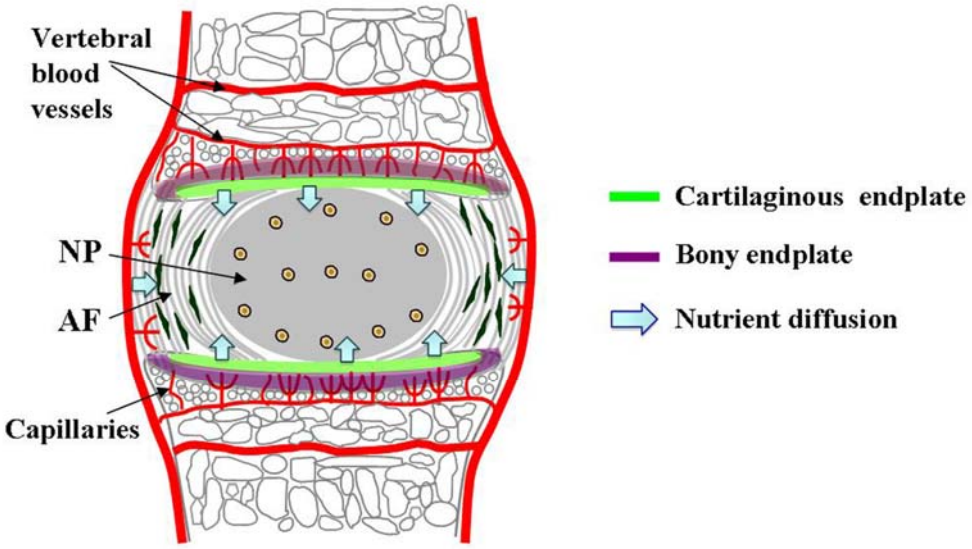

Figure 1. The blood vessels system at the margins of normal disc for nutrition diffusion. This schematic image was modified according to our previous publication [10]. Abbreviations: NP, nucleus pulposus; AF, annulus fibrosus.

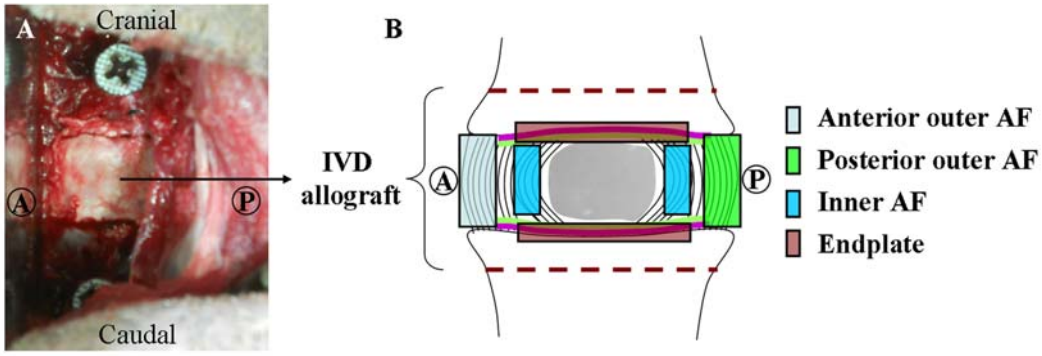

Figure 2. The disc allograft immediately after transplantation (A) and the four areas selected for blood vessel analysis (B). Abbreviations: IVD, intervertebral disc; A, anterior; P, posterior; AF, annulus fibrosus.

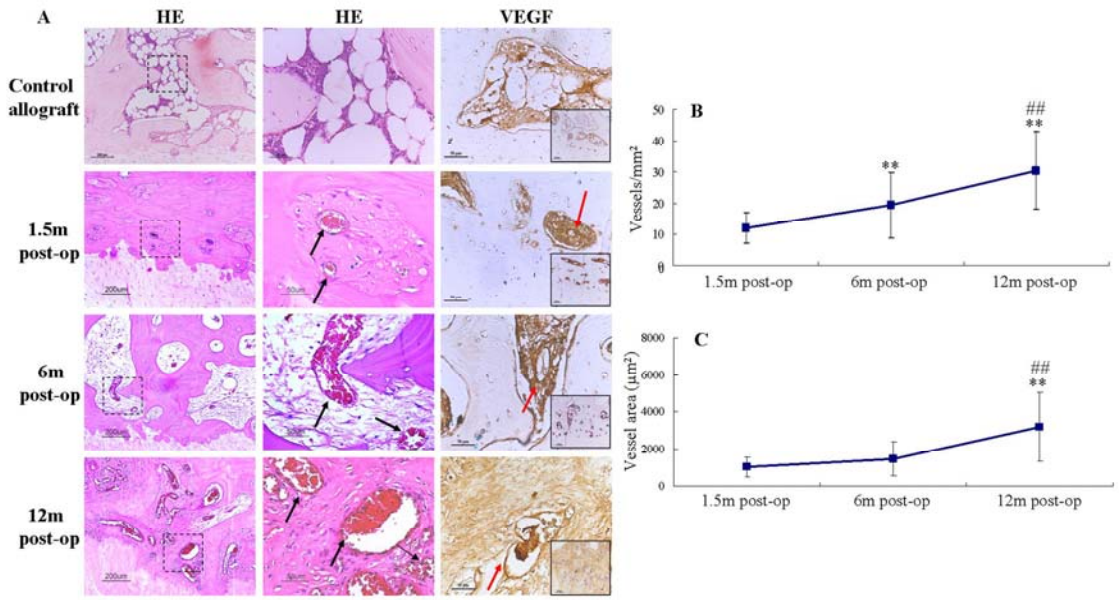

Figure 3. Revascularisation at the EP area of the IVD allograft at 1.5, 6 and 12 months after transplantation. Red blood cell–containing vessels (black arrows) were found at the EP area after 1.5 months and these lumenal structures were positive for VEGF(red arrows) (A); quantification of the blood vessels was carried out from the number of blood vessels per section (B) and the mean sectional area (C). $^{**}P<0.01$ vs. 1.5 months post-transplantation; $^{\#\#}P<0.01$ vs. 6 months post-transplantation.

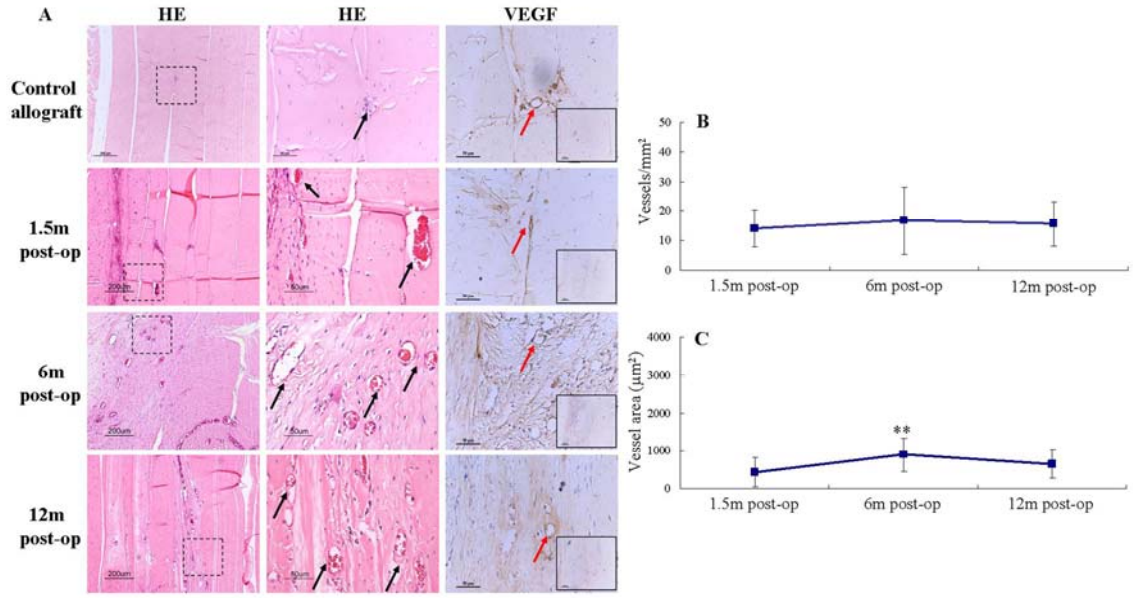

Figure 4. Revascularisation at the anterior outer AF area of the disc allograft at 1.5, 6 and 12 months after transplantation. Black arrows pointed out the blood vessels with the presence of erythrocytes while the red arrows pointed the $VEGF^+$ lumenal structures. $^{**}P<0.01$ vs. 1.5 months post-transplantation.

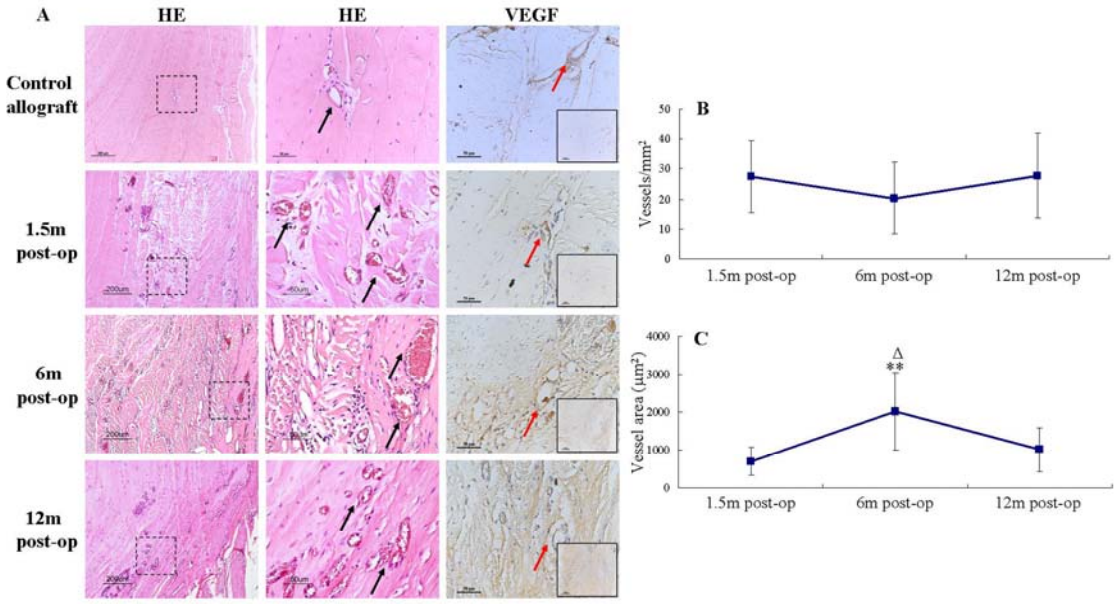

Figure 5. Revascularisation at the posterior outer AF area of the disc allograft at 1.5, 6 and 12 months after transplantation. Black arrows pointed out the blood vessels with the presence of erythrocytes while the red arrows pointed the $VEGF^+$ lumenal structures. $^{**}P<0.01$ vs. 1.5 months post-transplantation; $^{\Delta}P<0.05$ vs. 12 months post-transplantation.

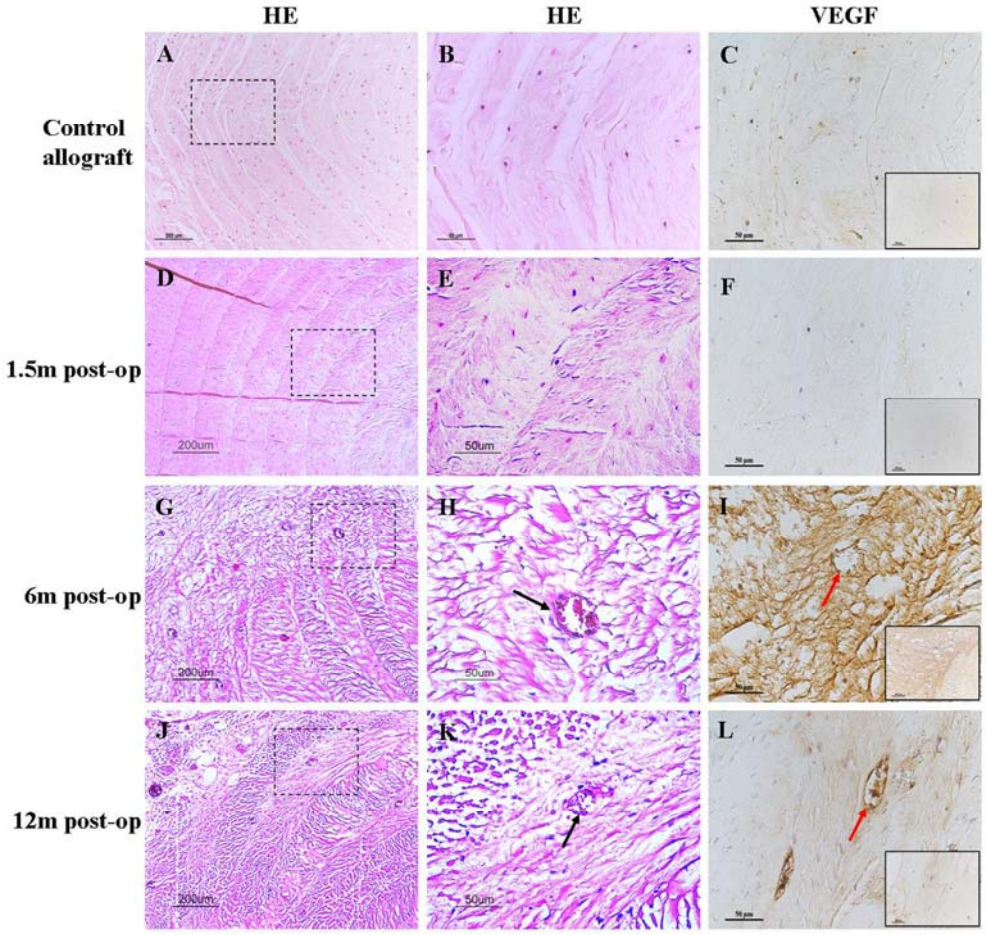

Figure 6. Revascularisation at the inner AF of allografts over time. The black arrows in D and F show the blood vessels. Black arrows pointed out the blood vessels with the presence of erythrocytes while the red arrows pointed the $VEGF^{+}$ lumenal structures. Scale bar in A, D, G and J is 200 µm and in the others is 50µm.

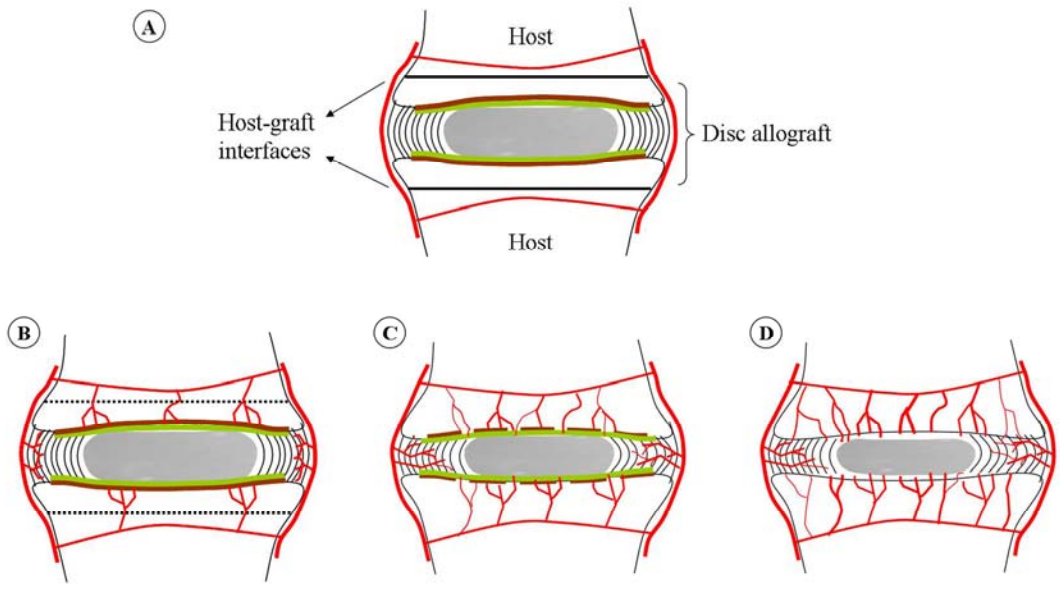

Figure 7. The revascularization pattern of the IVD allograft over time. The IVD allograft was placed in an ischaemic environment immediately after transplantation (A). Based on the above results, the blood vessels were first seen in the marrow space of the bony EP and the outer AF after 1.5 months (B). The vessels then reach the cartilaginous EP and inner AF after 6 months (C). None of these vessels invaded the centre of the allograft at the final follow-up. The EP area demonstrated the most abundant blood vessels with the highest vessel number and area (D). However, the pattern of the newly-formed vasculature in the IVD allograft was different from that in the normal adult IVD in which blood vessels are only present at the outer AF and immediately adjacent to the cartilaginous EP. Abbreviations: AF, annulus fibrosus; EP, endplate; IVD, intervertebral disc.