Single-pedicled fasciocutaneous flap survival in ageing rat model of voluntary cardiovascular exercise

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● INTRODUCTION ●

Wound healing occurs through several overlapping but distinct stages: 1) hemostasis; 2) inflammation; 3) proliferation and 4) maturation. The 3rd stage occurs between 3-14 days after injury, and is characterized by mobilization, migration, proliferation and differentiation of different cell types over an injured dermis and by the generation of new capillary blood vessels from pre-existing vasculature (angiogenesis) to provide nutrients and oxygen to active cells with greatly increased metabolic demands. Granulation tissue forms below the epithelium and consists of inflammatory cells, fibroblasts and newly formed vessels [1,2].

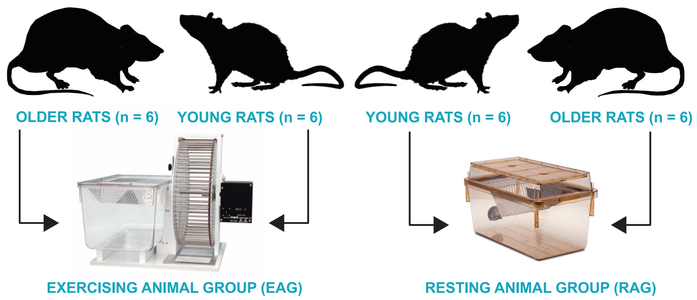
Angiogenesis is potentiated by hypoxia, nitric oxide (NO), certain chemokines and peptide growth factors. Vascular endothelial growth factor (VEGF), released from wound epithelium and from the extracellular matrix by endothelial-derived proteases, activates its cognate protein tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) to stimulate primarily endothelial cell proliferation, survival and increase vascular permeability. Besides VEGF, fibroblast growth factors (FGFs), such as basic FGF (bFGF/FGF-2), FGF-7, FGF-10, are released in early stages of incisional skin repair [2, 3] and transduce signals via 4 FGFR to mediate key events involved in neovascularization.

Pedicled skin flaps remain attached to the donor site via an intact vascular pedicle, which serves as a conduit for supplying nutrients and removing waste from the flap. The use of such flaps has significantly improved the safety and functional outcomes of surgery aimed at restoring function, form and integrity of craniofacial organs after traumatic injuries or cancer [4]. However, many systemic factors, including patient's old age, obesity/malnutrition, chronic diseases, smoking, stress etc., may prolong inflammation phase and proteolytic activity of matrix mettaloproteinases (MMPs), which are known to comromise cutaneous tissue repair and predispose flap to ischemic necrosis, which remains a significant issue in the pathophysiology of flap failure [5].

Cardiovascular exercise reportedly reduces inflammation and hastens wound healing [6-8]. However, its role in pedicled fasciocutaneous flap survival has not been investigated in ageing subjects.

❖ EXPERIMENTAL GOAL & DESIGN ❖

Impact of cardiovascular voluntary cardiovascular exercise on composite flap survival and underlying signaling events was examined in twelve young (2 month old) and twelve older adult (6 month old) Sprague-Dawley rats that were housed individually in cages with or without access to the 13.5" diameter running wheel and a distance counter for 2 weeks prior to surgery, where a 3:8 width to length ratio pedicled fasciocutaneous flap based on the inferior epigastric artery was raised in each rat and then rotated 60° into a defect created in the ventral surface (Fig. 1A-C).



Healthy skin that was excised from a donor site on day 0 was used as control for protein basal levels. 5 mm diameter punch skin biopsies were taken on Post-Operative Days (PODs) 2, 5 and 7, while the entire flaps were harvested on POD 9, and animals were euthanized in CO_2 chamber. Each flap was separated into proximal (P), middle (M) and distal (D) thirds (segments) (Fig. 1D), and snap frozen prior to homogenization in modified RIPA lysis buffer. Protein expression was analyzed by comparative Multi-strip Western blotting [9].

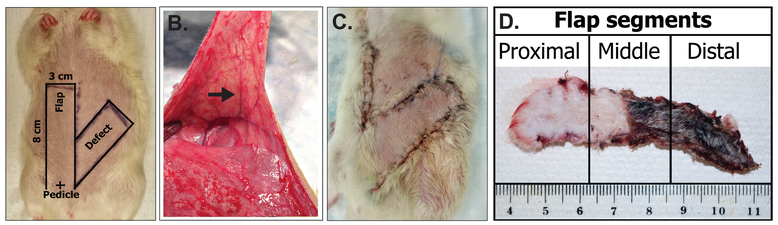


Fig. 1. Pedicled fasciocutaneous flap model. A. Rotational fasciocutaneous flap design. **B.** Raised pedicled flap. Inferior epigastric artery is show by black arrow. **C.** Subject after flap rotation and closure on POD 2. **D.** Excised flap segments.

⌘ RESULTS: Young vs. Older RAGs ⌘

The survival areas of the flaps were clearly demarcated within 9 days time. The surviving skin was pink-white, tender, and normal in its texture. The necrotic skin was black, rigid, dry, and did not bleed when cut. Specimens from each segment harvested on POD 7 were put into 10% PBS-buffered formalin, fixed for 24 h, embedded in paraffin and stained with Hematoxylin and Eosin (H&E). Acellular areas were pink (negative for hematoxylin-positive nuclei). Flap necrosis was considered to be full-thickness when involved the epidermis, adnexal structures of dermis and subcutaneous adipose tissue. Histologically, obvious inflammation was present in flaps' fascia with neutrophil infiltration along with monocytes and sparse macrophages. Based on planimetric analysis, the mean flap survival was 97.9% vs. 50.83% in young and older resting animal groups (RAGs), respectively (p = 0.0109).

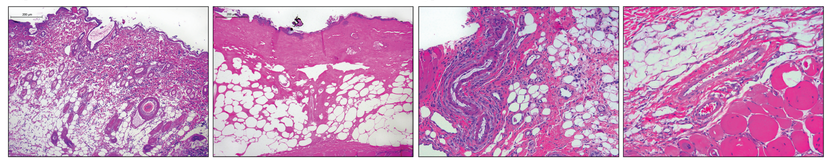
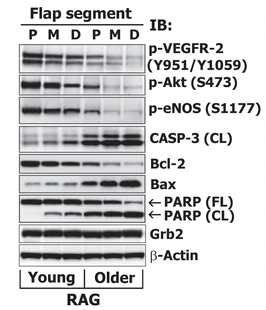


Fig. 2. A. High-power photomicrographs of H&E stained longitudinal sections of representative distal flap segment specimens showing healthy, inflammatory infiltrate with neovascularization in young (*left panel*) resting rats and acellular, full-thickness necrosis in older (*right panel*) rats on POD 7. **B.** Magnified image of H&E stained main arterial pedicle and associated arterioles in young (*left panel*) and older (*right panel*) resting rat proximal segments demonstrating disease-free vascular endothelium and wall.



A plecktrin homology (PH) domain-containing serine/threonine kinase Akt/PKB controls diverse cellular functions, including nutrient metabolism, cell growth, motility, programmed death, eNOS-dependent vasodilation & angiogenesis [10]. In response to stimulation by growth factors, including VEGF & bFGF, Akt is recruited to the plasma membrane by binding to phospholipids that are generated by phosphatidylinositide 3-kinase (PI3K). There Akt is activated by sequential phosphorylation within its activation loop at Thr308 residue and within its C-terminus at Ser473. PI3K/Akt activation increases transcription of main anti-apoptotic Bcl-2 protein and inhibits mitochondrial translocation of pro-apoptotic Bax protein, thereby preventing the disruption of the inner mitochondrial membrane potential, cytochrome c release and the activation of cysteinyl-aspartate proteases (caspases) that cleave various proteins. Intact, 116 kDa PARP-1 is involved in DNA repair helping cells to maintain their viability [11] and plays a positive role in angiogenic process [12]. Proteolytic cleavage of full-length (FL) PARP-1 into two fragments by activated executioner caspases, such as active cleaved (CL) Caspase-3 (CASP-3), facilitates cellular disassembly and serves as a biochemical marker of apoptosis (Fig. 3)

Fig. 3. Correlation between downregulated VEGFR-2/PI3K/Akt signaling axis and increased cellular markers of apoptosis in fasciocutaneous flaps of young and older resting rats. Representative blots of pooled samples (n = 3 per group) are shown.

⌘ RESULTS: EAG vs. RAG ⌘

Young rats in exercising animal group (YE) voluntarily traveled the mean distance of 32.7 km, while the older rats markedly differed in their ability to run the comparable distances, and therefore were divided into 2 subgroups: A) Long distance exercising older rats (OE) (n = 3) who on average traveled 19.21 km and B) Short distance exercising older rats (SOE) (n = 3) who ran ~6.48 km. Interestingly, while younger rats expressed high levels of basal VEGF and low levels of bFGF, older rats had lower basal VEGF and much higher bFGF levels. Cardiovascular exercise accelerated endogenous 42/38 kDa VEGF-A expression in older rats (Fig. 4, left panel), whereas in young animals VEGF readily reached saturation levels. By contrast to old rats, young animals showed inducible bFGF response (Fig. 4, right panel), which correlated with the increased activation of Akt and Bcl-2 expression (Fig. 5).

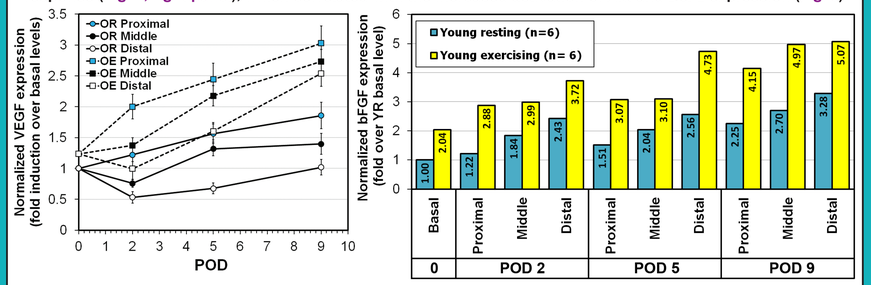


Fig. 4. Left panel. Effects of exercising on time-course of VEGF expression in different flap segments of older rats. Right panel. Effects of exercising on time-course of bFGF expression in different flap segments of young rats.

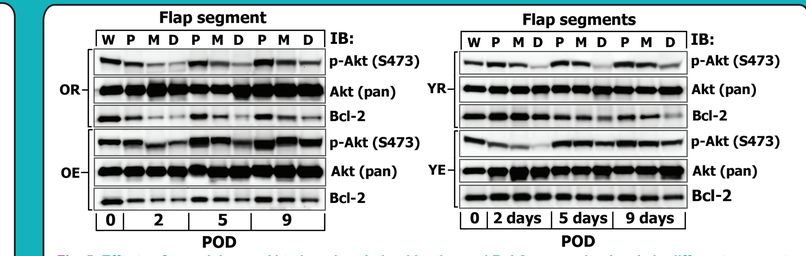


Fig. 5. Effects of exercising on Akt phosphorylation kinetics and Bcl-2 expression levels in different segments of flaps in older (*left panel*) and young (*right panel*) rats. W, whole flap harvested at day 0.

Exercising in old rats increased expression of Heat shock protein 70 (Hsp70) (Fig. 6, upper panel), which plays a key protective role against ischemic tissue injury and positively regulate wound healing [13]. Consistent with augmented VEGF and increased Akt activity, there was a sharp reduction in CASP-9 and PARP-1 cleavage (Fig. 6, lower panel), especially in distal, most necrotic flap segments, where the exercising resulted in higher flap survival rate on average by 1.8 fold (p = 0.0313). Exercising inhibited TGFβ and MMP-9 induction, which may have contributed to restored type I collagen levels. Finally, more intensive exercising augmented phosphorylation of p38 MAPK and STAT3 [14] (Fig. 7).

Fig. 6. Effects of exercising on VEGF-A, Hsp70, TGF-β, MMP-9, 17 kDa large fragment of cleaved active CASP-9, β-Actin (loading control) (*upper panel*) and full-length (FL) and cleaved (CL) PARP-1 form expression in distal segments of the flaps harvested from old resting or exercising rats at different times. Representative blots of pooled samples (n = 6 per group) are shown.

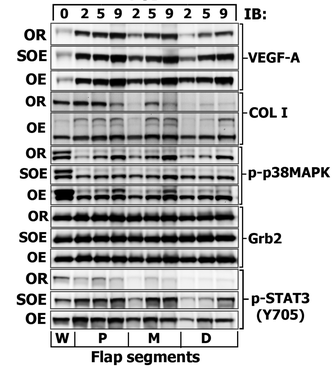


Fig. 7. Effects of exercising intensity on VEGF-A, Type I Collagen expression, p38 MAPK and STAT3 activation, Grb2 (loading control) in different segments of the flaps harvested from old resting (OR), short distance old exercising (SOE) and long distance old exercising (OE) rats at different times. Representative blots of pooled samples (n = 6 per group) are shown.

■ CONCLUSION ■

Voluntary exercising improves single-pedicled fasciocutaneous rotational flap survival in ageing rat model in part through the upregulation of VEGF and bFGF expression followed by the activation of anti-apoptotic PI3K/Akt signaling pathway and through the inhibition of pro-apoptotic TGFβ signaling. Exercising prevents time-dependent activation of Caspases and MMP-9 induction in distal, most prone to necrosis tissue areas, resulting in ameliorated wound healing.

ACKNOWLEDGEMENTS

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THANK YOU for your time!

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