SINGLE-PEDICLED FASCIOCUTANEOUS FLAP SURVIVAL IN AGED RAT MODEL OF CHRONIC ALCOHOLISM

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PROGRAM NO: 889.13
ABSTRACT NO: 8562

● INTRODUCTION ●

The use of pedicled skin 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 resection of tumor [1]. Single-pedicled rotational flap remains attached to the donor site via an intact vascular pedicle, which serves as a conduit for supplying nutrients and removing waste from the flap during healing process.

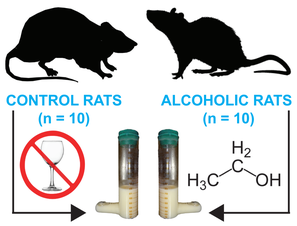
Wound healing occurs through several overlapping but distinct stages: 1) hemostasis; 2) inflammation; 3) proliferation and 4) maturation. The 3rd stage occurs between 3-14 days after an injury, when the wound is 'rebuilt' with new granulation tissue. Different cell types migrate, proliferate and differentiate over an injured dermis. Many of them release angiogenic growth factors, such as VEGF, that trigger generation of new capillary blood vessels from the pre-existing vasculature to provide nutrients and oxygen to active cells with greatly increased metabolic demands [2, 3].

Various systemic factors, such as patient's old age, obesity, chronic diseases, smoking, stress etc., can prolong inflammatory phase of wound healing and impair angiogenesis [4]. Inadequate blood supply is the primary intrinsic factor affecting ischemic flap survival. In addition, under chronic hypoxic and inflammatory conditions, excessive ROS production, persistent presence of TGFβ and pro-inflammatory cytokines, such as IL-6, IL-1β and TNFα, result in enhanced synthesis and proteolytic activity of matrix metalloproteinases (MMPs) [5, 6]. Highly degradative environment, which decreases formation of granulation tissue, fibroblast proliferation and collagen production, compromises cutaneous tissue repair and predisposes a flap to ischemic necrosis. Skin flap failure remains a significant clinical problem in surgery, which should be overcome by understanding the molecular mechanisms underlying successful flap integration [7].

◆ GOAL OF STUDY ◆

Both acute and chronic ehtanol intake increases ROS generation [8], but it may differently modulate immune cell activation and cytokine production that in turn may improve or delay wound healing [9-11]. Here we aimed to determine if there is a statistically significant correlation between chronic alcohol-induced changes at the cellular and molecular level in aged skin and a fate of single-pedicled composite (fasciocutaneous) flap.

❖ EXPERIMENTAL MODEL OF ISCHEMIC FLAP ❖



Impact of chronic alcohol intake on composite flap survival and underlying signaling events was examined in isocaloric pair-fed >1 year old Sprague-Dawley rats that consumed Lieber-DeCarli '82 liquid 1000 kcal/L diet consisting of 15.1% protein, 35.9% fat and either 49% carbohydrates (control group) or 13.5% carbohydrates and 35.5% ethanol (alcoholic group). Ten pairs of rats that were fed such diet for at least one year underwent surgery, where a 3:8 cm width to length ratio pedicled fasciocutaneous flap based on the inferior epigastric artery was raised in each anaesthetized rat and then rotated 60° degrees into a "defect" site that was created in the ventral surface (Fig. 1A-C). Whole piece of skin excised from a defect site on day 0 was used as control for protein basal levels (W). In addition, four sterile polyvinyl alcohol (PVA) sponges were placed in different locations under the laid flap. On post-operative day (POD) 9 (end-point) the remaining sutures were removed, an entire flap was harvested, the PVA sponges were excised and placed in the lysis buffer containing protease and phosphatase inhibitors. The animal was subsequently euthanized in CO_2 chamber. Each flap was photopgraphed under standardized conditions, separated into proximal (P), middle (M) and distal (D) thirds (segments) (Fig. 1D), and snap frozen. For protein analysis, the flap segments were individually crushed into fine powder under liquid nitrogen, and then homogenized using Potter-Elvehjem Grinding Chambers in a modified RIPA tissue lysis buffer (see Box). Protein expression was analyzed by comparative Multi-strip Western blotting (MSWB) in LDS-PAGE system [12].

❖ 50 mM HEPES (pH. 7.4)	❖ 0.5 % Sodium deoxycholate
❖ 150 mM NaCl v	❖ 0.1 % SDS
❖ 1 % Triton X-100	❖ 70 mM n-Octyl-beta-D-Glucoside
❖ 1 mM EGTA	❖ Protease inhibitor cocktail III (A.G. Scientific)
❖ 10 % Glycerol	❖ Phosphatase inhibitor cocktail (Roche)

Protein transfer conditions:
❖ 4-12% gradient gel
❖ XCell II™ Blot Module
❖ 30 V constant
❖ 1 hour 30 min

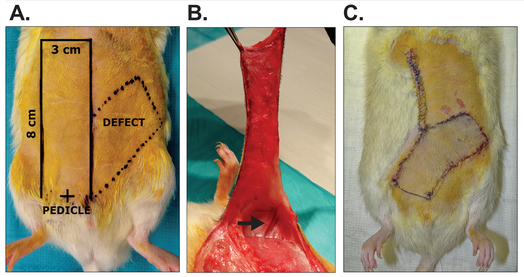
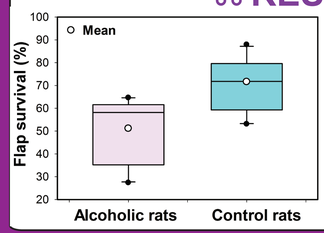


Fig. 1. Pedicled fasciocutaneous flap model. **A.**Rotational fasciocutaneous flap design. **B.** Raised pedicled flap. Inferior epigastric artery is show by black arrow. **C.** Subject after flap rotation and closure on POD 0. **D.** Representative photos of relatively viable and necrotic flaps and their segments after harvest on POD 9.

⌘ RESULTS: Flap survival ⌘



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. Flap survival was determined as percentage of the non-necrotic flap/total flap area in mm² (Fig. 2). Based on planimetric analysis, mean flap survival of rats that were fed alcohol diet was significantly lower than that of control rats - 51.21% ± 4.50% SEM (standard error) versus 70.16% ± 3.57% SEM (P = 0.004, t-test assuming equal variance).

Fig. 2. Percentage of flap survival in experimental animal groups. The results are expressed in box plots as mean ± SD (standard deviation), median (black line), minimal and maximal values (black circles) for each group (n = 9 per group).

⌘ RESULTS: Histology and IHC of flaps ⌘

Specimens from each segment harvested on POD 9 were put into 10% PBS-buffered formalin, fixed for 24 hours, embedded in paraffin and stained with Masson's Trichrome, H&E or antibodies against endothelial cell marker CD31. The acellular areas were pink and negative for hematoxylin-positive nuclei. Segment necrosis was considered to be full-thickness when involved epidermis, adnexal structures of dermis and subcutaneous adipose tissue (Fig. 3). Inflammatory infiltrate in flaps' fascia contained neutrophils, monocytes and sparse macrophages.

GT - granulation tissue; SC - scab; E- epithelium; SF - subcutaneous fat; PC - panniculus carnosus (muscle layer in superficial fascia); D - dermis;

KEY colors in Masson's Trichrome (MT) staining:
❖ BRIGHT RED - cytoplasm, muscle, erythrocytes, keratin
❖ DARK PURPLE - cell nuclei; ❖ BLUE - collagen fibers

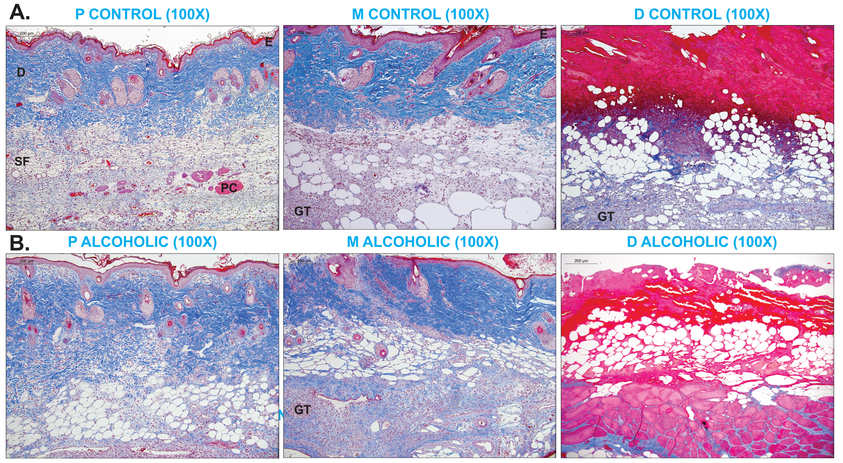


Fig. 3. Low-power photomicrographs of MT stained longitudinal sections of representative proximal (P), middle (M) and distal (D) flap segment specimens from control (A) and alcoholic (B) animal groups. Majority of D segments of control rats showed some degreee of cellularity, whereas most alcohlolic rat specimens demonstrated full-thickness acellular necrosis.

⌘ RESULTS: Protein analysis ⌘

First, certain protein markers (see box below) were detected by immunoblotting (IB) in different flap segments to see how their expression reflects the severity of visible necrosis (Fig. 4A). Then the levels of these proteins were compared in control and alcoholic animal pairs separately (Fig. 4B). Finally, an expression of selected proteins in a given flap segment was simultaneously detected in the lysates of every rat pair by using MSWB (Fig. 4C). Protein signal intensities were calculated, normalized and plotted (Figs. 4, 5).

❖ Cleved (CL) Caspase-9 - apoptosis;
❖ p-Akt (S473) & p-STAT3 (Y705) - cell survival;
❖ Src family kinases (SFK) (Y416) & Hsp27 - wound contraction;
❖ TGFβ & PAI - 1 - fibrosis and apoptosis;
❖ p-ERK1/2 (T202/Y204) - cell proliferation;
❖ p-PLC-γ (S1248) & p-p38 MAPK (T180/Y182)- cell motility;
❖ p-JNK1 (p46) (T183/Y185) - TNFα–induced cell death [13-14];
❖ IL-1β & TNFα - tissue inflammation and death;
❖ VEGF & FGF-2 - angiogenesis;
❖ CD144 (Vascular endothelial (VE) Cadherin) - vascularity
❖ MMP-9 (produced by activated neutrophils & macrophages) - ECM, including viable and non viable collagen degradation; marker of chronic wound and inflammation;
❖ α-SMA - marker of vascular smooth muscle cells and of fibroblast differentiation into myofibroblasts;
❖ Collagen type I - deposited by fibroblasts; marker of wound healing.

⌘ RESULTS: Protein analysis ⌘

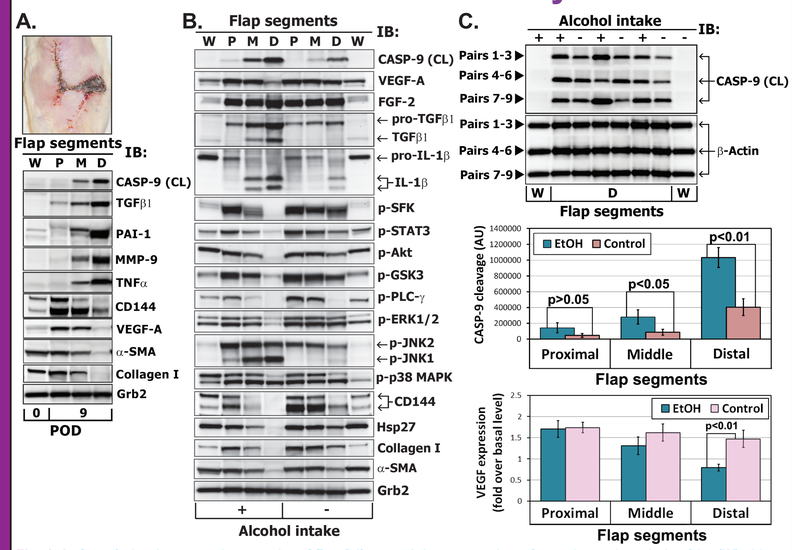


Fig. 4. **A.** Correlation between the severity of flap failure and the expression of protein markers in healthy (W) skin prior to wounding and proximal (P), middle (M) or distal (D) flap segments at 9th day after raising flap. **B.** Comparison of protein expression in flap segments of control and alcoholic paired rats. Representative 1 pair with flap survival rates of 73.44% (alcohol intake -) and 36.61% (alcohol intake +) is shown. W - basal conditions. **C.** Caspase-9 cleavage under basal (W) conditions and in distal flap segments of alcoholic and control rats at POD 9. **D.** VEGF-A levels under basal (W) conditions and in different flap segments of alcoholic and control rats at POD 9.

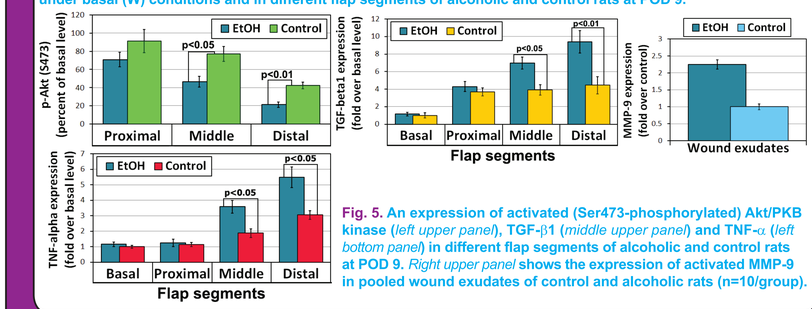


Fig. 5. An expression of activated (Ser473-phosphorylated) Akt/PKB kinase (*left upper panel*), TGF-β1 (*middle upper panel*) and TNF-α (*left bottom panel*) in different flap segments of alcoholic and control rats at POD 9. *Right upper panel* shows the expression of activated MMP-9 in pooled wound exudates of control and alcoholic rats (n=10/group).

■ CONCLUSION ■

Chronic alcohol consumption sustains cellular production of pro-inflammatory cytokines, inhibits the anti-apoptotic PI3-kinase/Akt signaling, augments activated MMP-9 levels in wound exudates by 2.25-fold and significantly decreases single-pedicled fasciocutaneous rotational flap survival in aged rats.

ACKNOWLEDGEMENTS

This research was supported by Thomas Jefferson University Department of Otolaryngology - Head & Neck Surgery.

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THANK YOU for your time!

♦ REFERENCES ♦

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