

A photograph of laboratory glassware, including a large Erlenmeyer flask containing a dark liquid, a graduated cylinder with a reddish-brown liquid, and a beaker with a dark liquid, all set against a white background.

Streptolysin O Enhances Keratinocyte Migration and Proliferation and Promotes Skin Organ Culture Wound Healing

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12053**

Comment on Therapeutic Hormesis

“Unfortunately, benefits from hormesis may be practically impossible to harness. Hormesis usually occurs at doses about five times lower than the toxic threshold, Calabrese has found... But sensitivities can vary from individual to individual by a factor of 10 to 100. A dose that stimulates hormesis in one person may well be toxic to another. That variability is a major reason why hormesis won't add new medicines to our cabinets anytime soon.” (Discover Magazine, Dec. 2002)

WE ULTIMATELY HOPE TO PROVE THESE STATEMENTS TO BE
INCORRECT

What is Streptolysin O (SLO)?

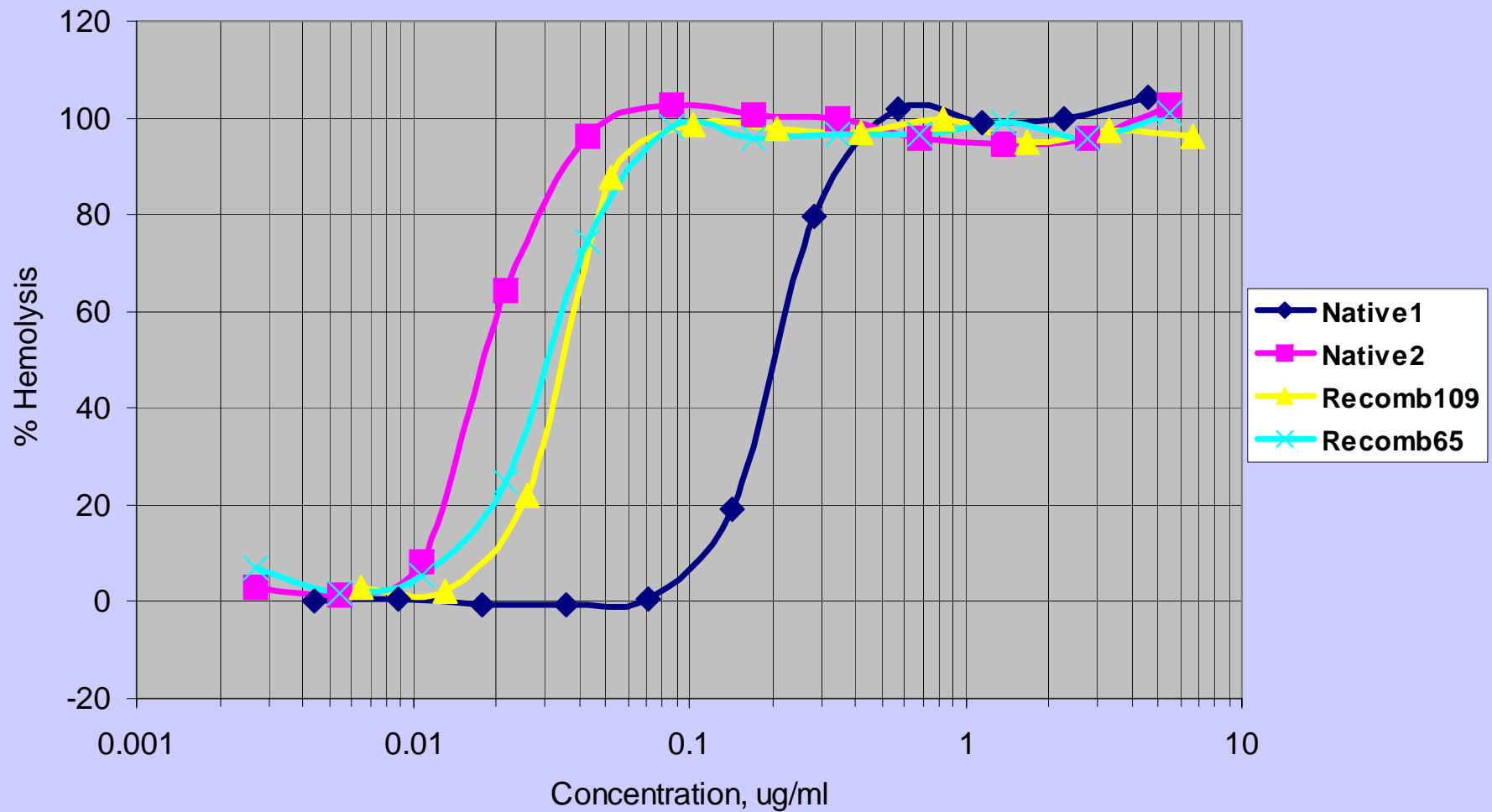
SLO is a hemolytic exotoxin produced by Streptococcus species of groups A, C & G. It is a single chain protein (571 amino acids, 60-70 kDa; a recombinant form is over 100 kDa). Similar toxins are produced by other species of Gram-positive bacteria.

SLO possesses several structural and functional domains including a cholesterol-binding domain.

SLO can, as a thiol-activated, cholesterol-binding agent, form pores in cell membranes in its reduced state (though not post-oxidation).

As red blood cells are particularly susceptible to cytolysis, they are used to determine the cytolytic properties of SLO.

Hemolysis of Sheep Red Blood Cells By Various SLO Preparations (37 C, t = 0 min)



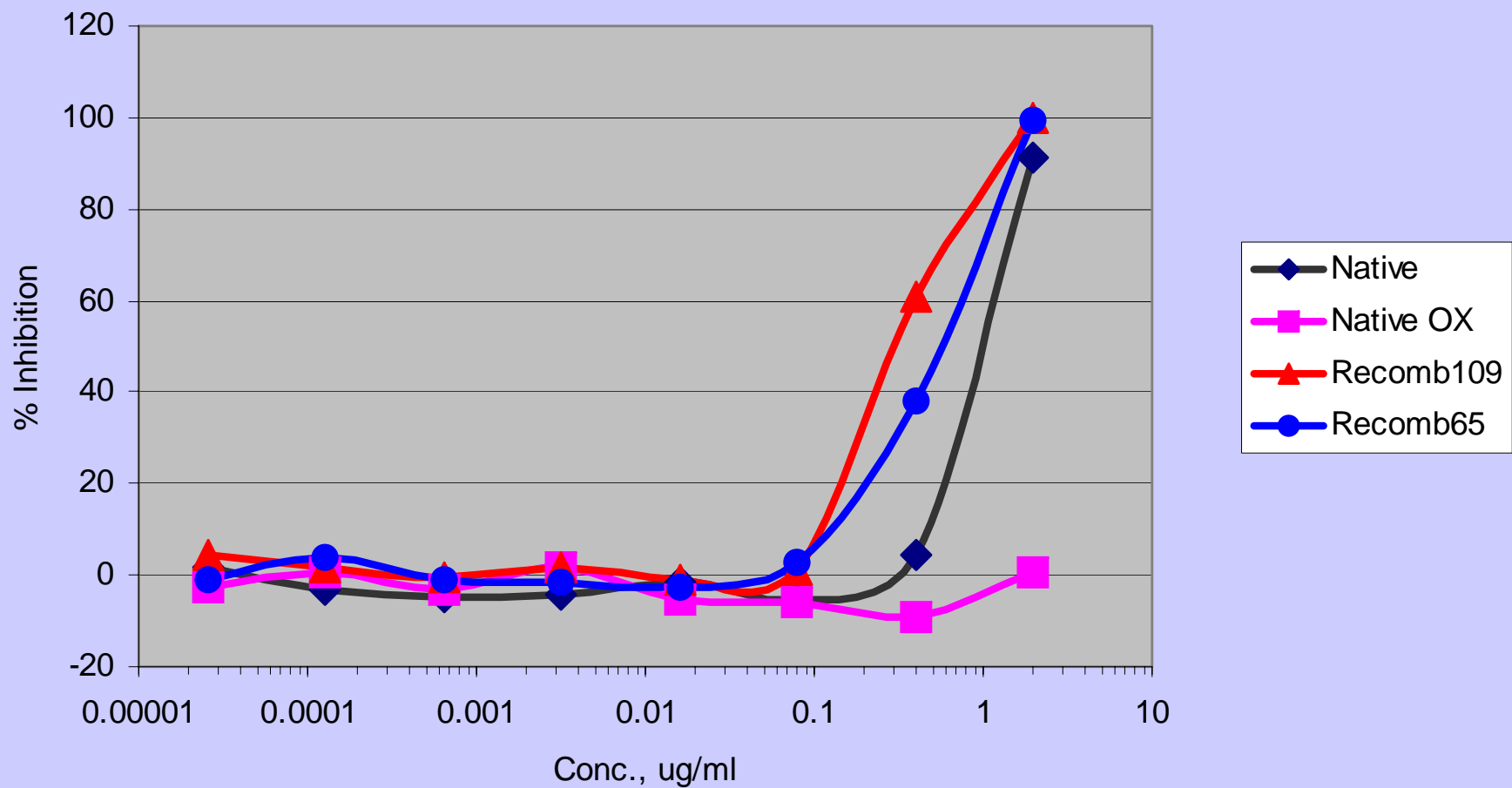
ML-05 (Detoxified Streptolysin O)

ML-05 is a non-hemolytic form of SLO. It is inactivated by controlled oxidation.

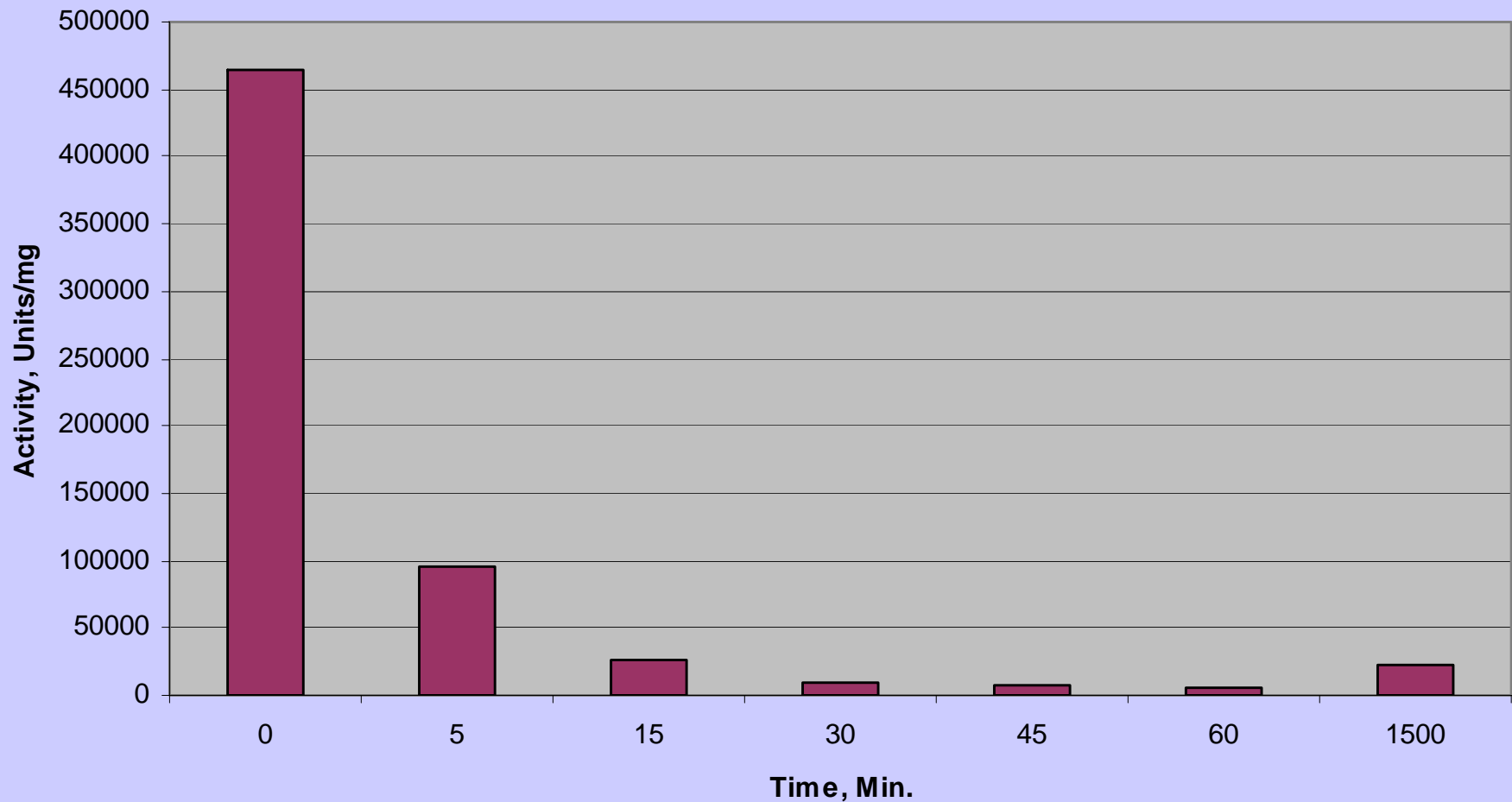
Initial therapeutic interest in SLO was as a treatment for motor deficit disorders. Patients treated with ML-05 observed beneficial changes in scar appearance including size reduction and improved healing.

ML-05 has previously been shown to slow the excessive production of collagen in experimental scleroderma/fibrosis animal models (*Nonlinearity* 2004 Apr-Jun;2(2): 67-87).

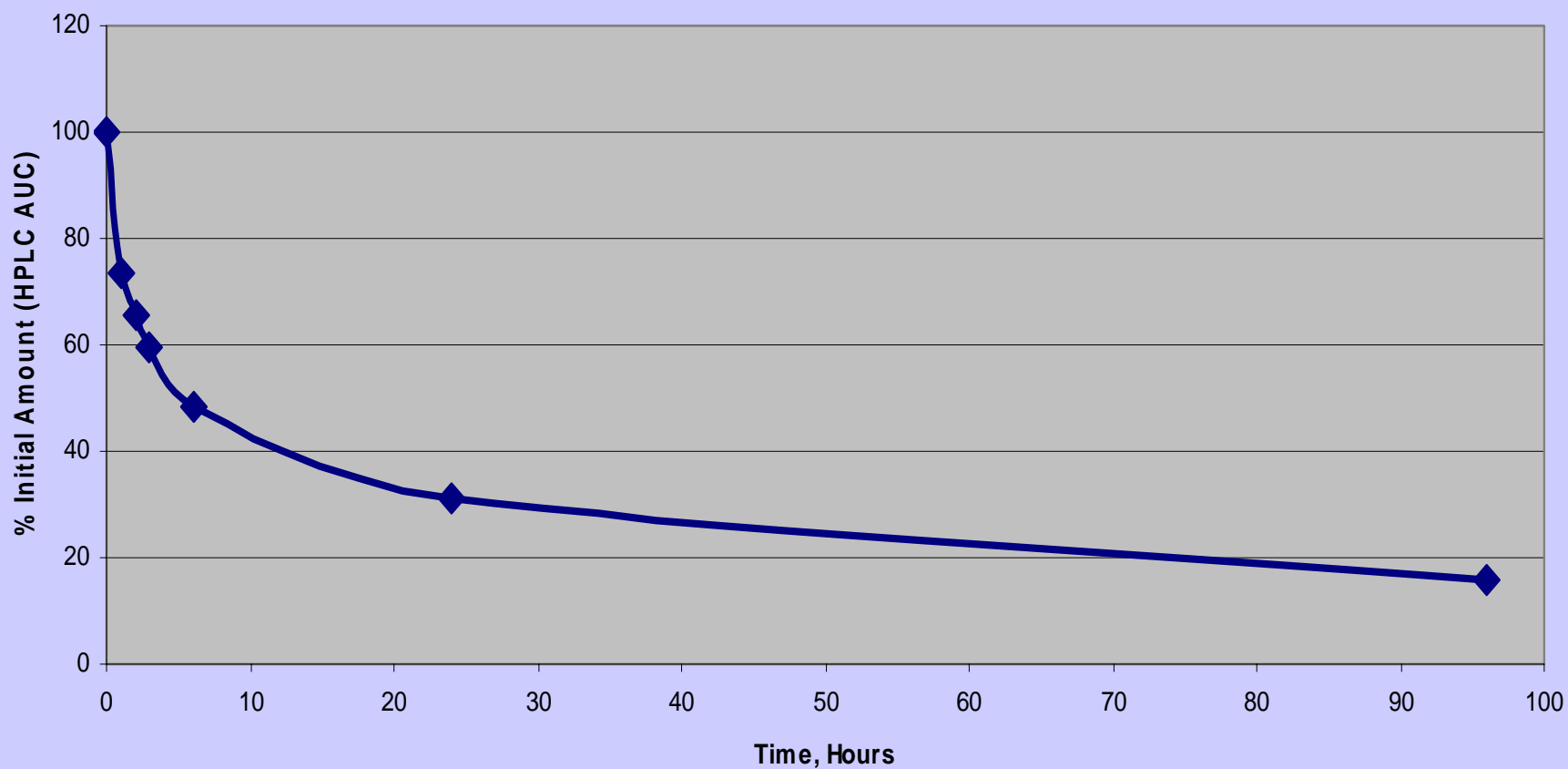
Cytotoxicity of Various SLO Preparations to Normal Diploid Human Fibroblast Cells



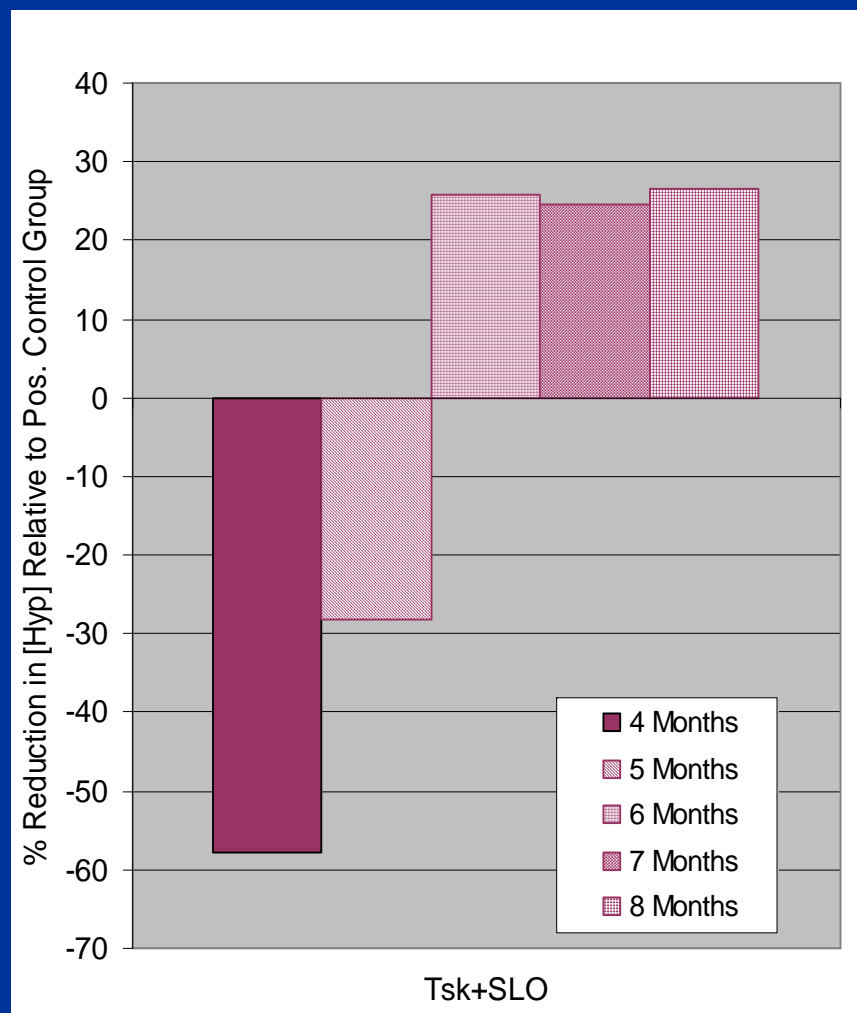
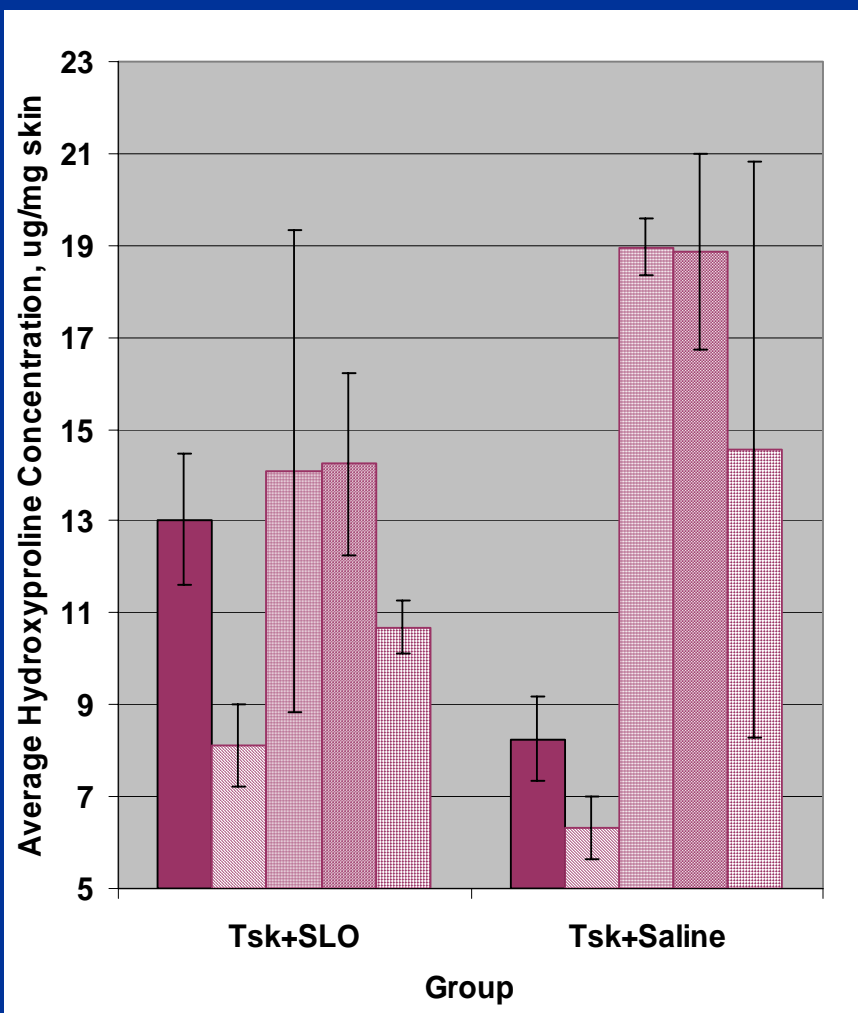
Hemolysis by Native SLO Oxygenated for Up To 60 Minutes (and Reversion After 24 Hr at 4 C)



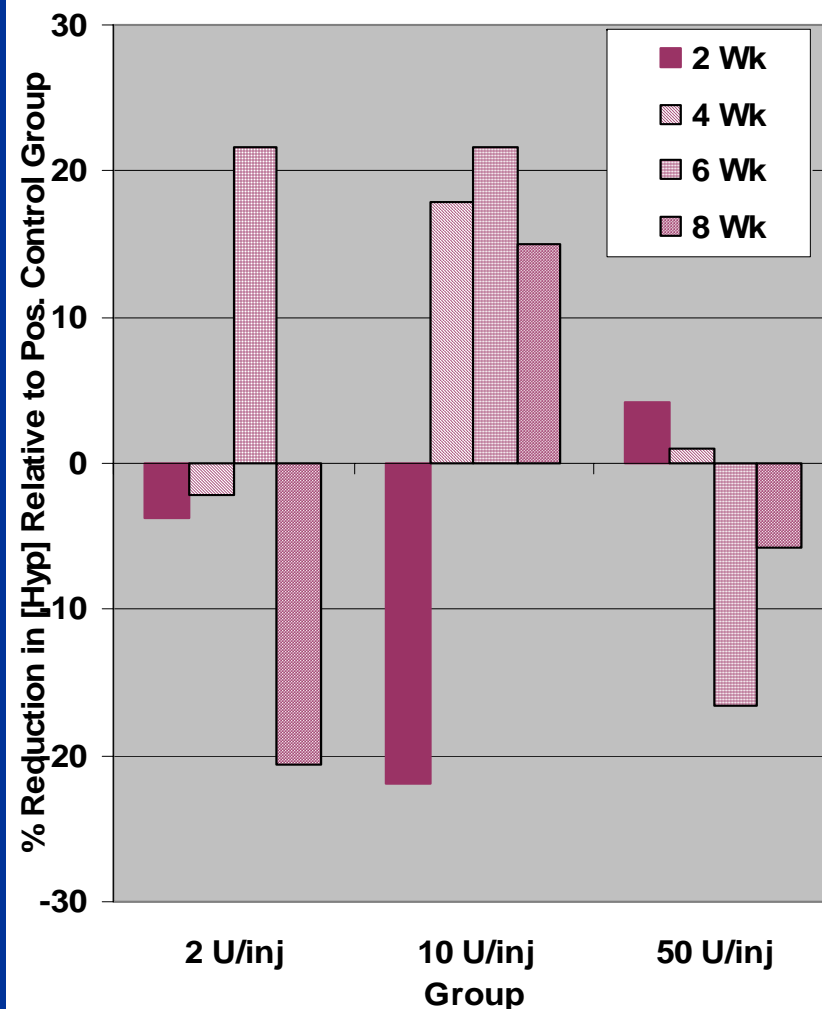
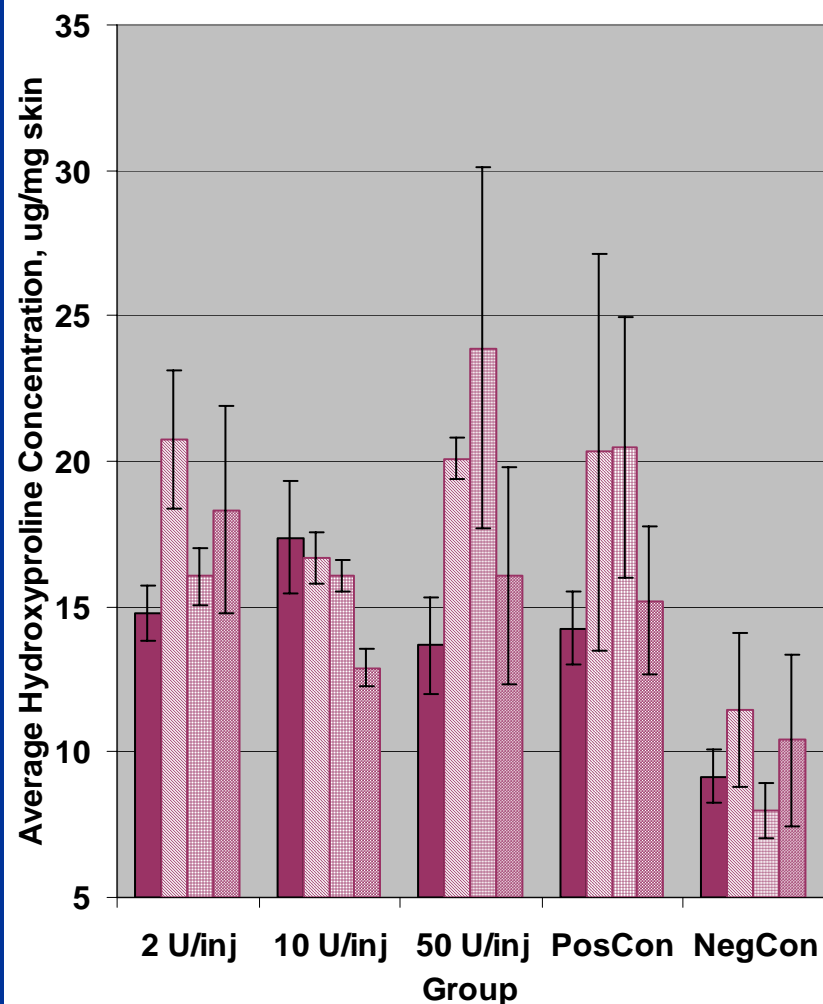
Degradation of SLO Incubated at 37 C Over a 24-Hour Time Period (Based on HPLC Analysis)



Effects of ML-05 Treatment on Mouse Skin Hydroxyproline Levels in the Tsk Scleroderma Model (Cumulative Results)



Effects of ML-05 Treatment on Mouse Skin Hydroxyproline Levels in the Bleomycin- Induced Scleroderma Model (Cumulative Results)



Improved Wound Healing by ML-05

Fibrogenesis (the formation of hypertrophic scars and keloids) during wound healing is related to abnormal collagen deposition. Accordingly, it was hypothesized that ML-05 may be useful in modulating or reducing collagen deposition during the wound healing process. ML-05 may indirectly or directly alter collagen production to facilitate the restoration of normal extracellular matrices within tissues during wound healing via several possible mechanisms.

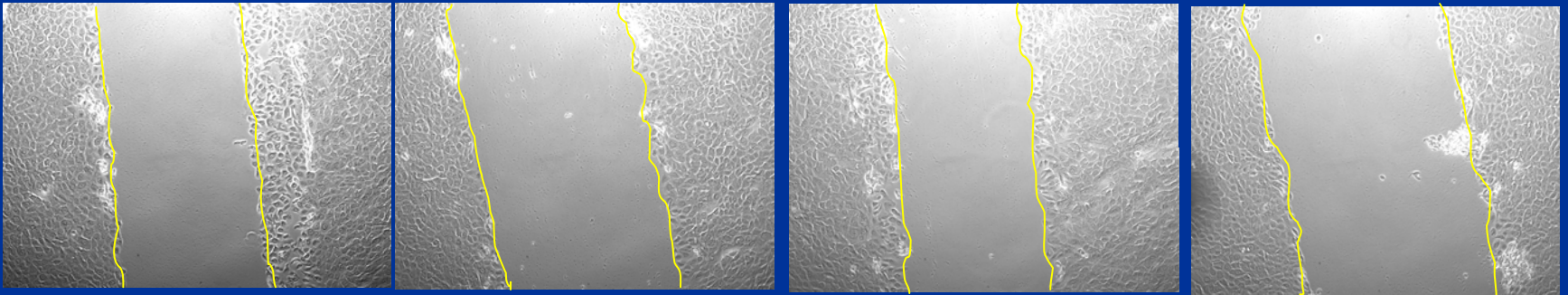
Keratinocyte Scratch Assays

Primary human keratinocytes were grown to 80% confluency in culture dishes. One set of culture dishes was treated with 10 $\mu\text{g/ml}$ Mitomycin C (MMC) for 2 hours (to inhibit cell proliferation and measure migration only). A second set of culture dishes, maintained in the absence of MMC, was used to evaluate both keratinocyte proliferation and migration. Scratches were made with a Pasteur pipette tip. Cells were incubated with four different concentrations of ML-05; PBS was used as a negative control. The scratch fields were photographed at 0, 24 and 48 hours, and cell migration was quantified using computer-assisted image analysis.

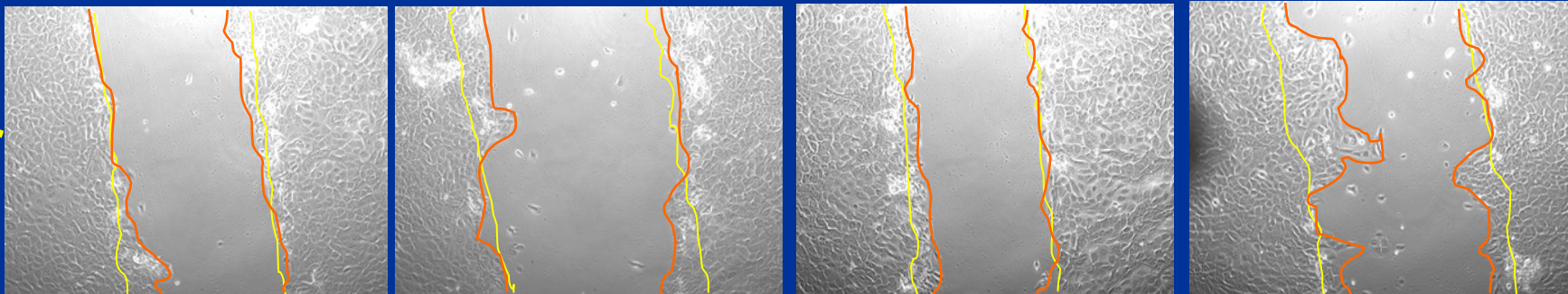
Primary Human Epidermal Keratinocytes

+ mitomycin C **- mitomycin C**

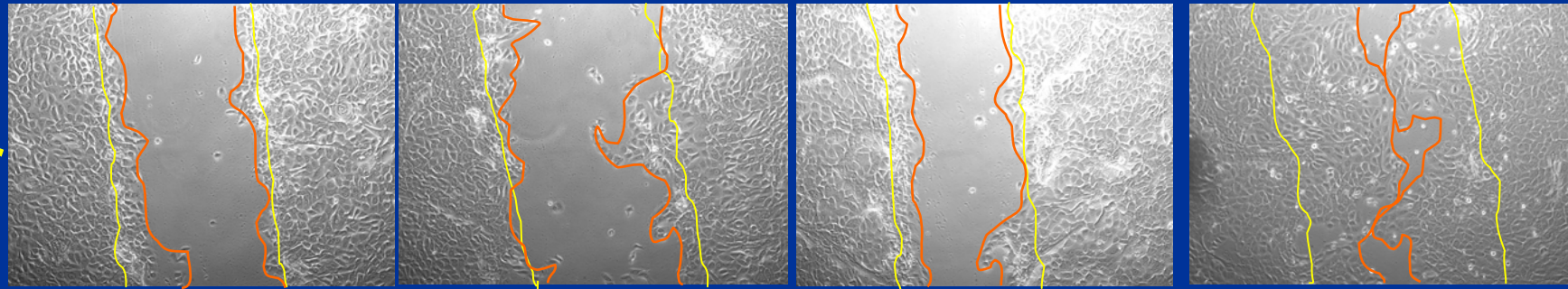
0 hr



24 hr



48 hr



CONTROL

**0.02 U/ml
ML-05**

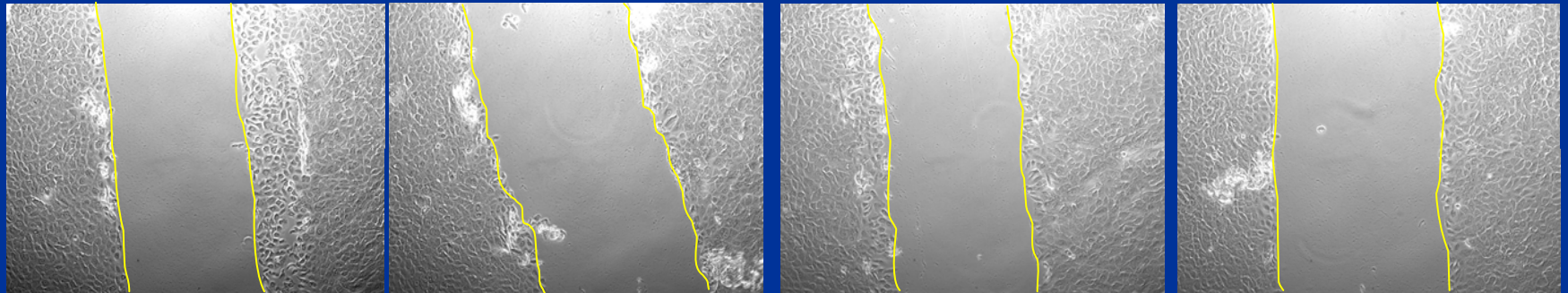
CONTROL

**0.02 U/ml
ML-05**

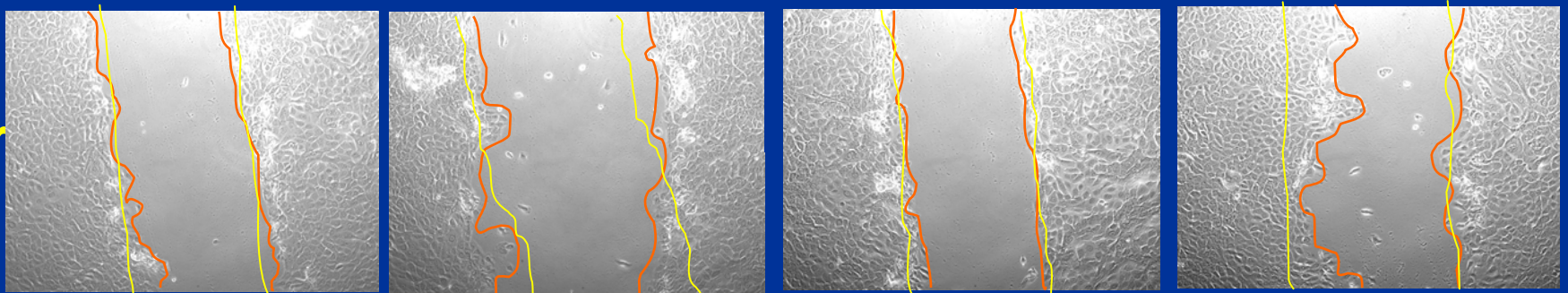
Primary Human Epidermal Keratinocytes

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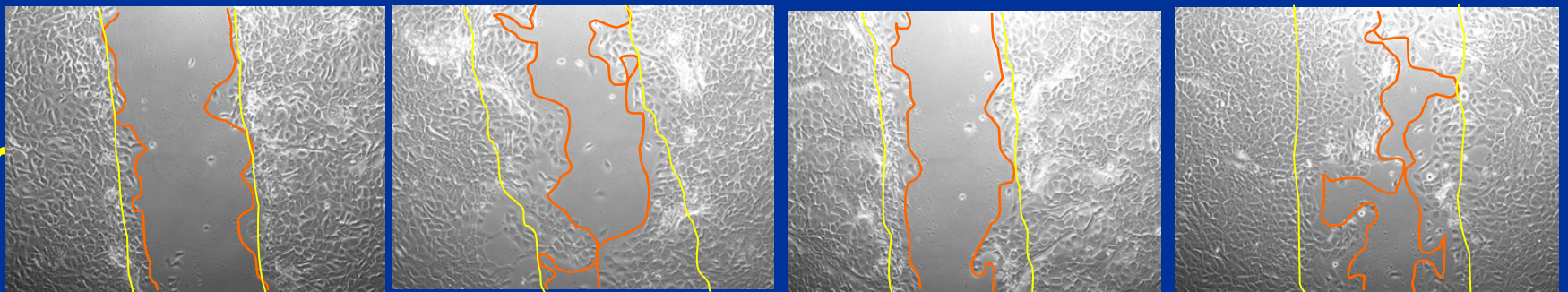
0 hr



24 hr



48 hr



CONTROL

**0.2 U/ml
ML-05**

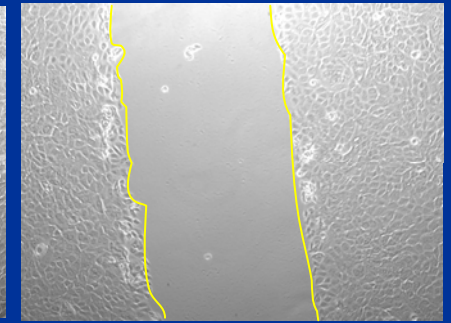
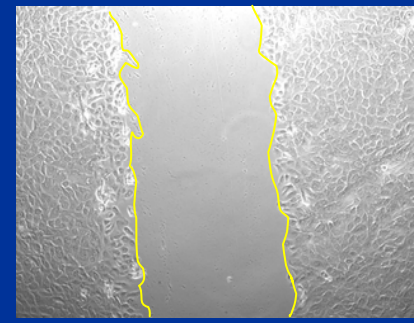
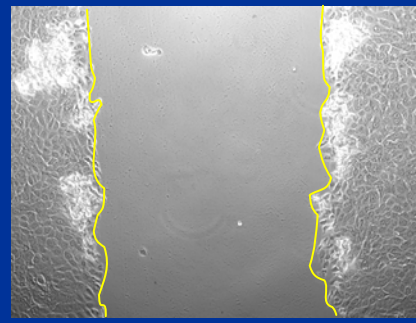
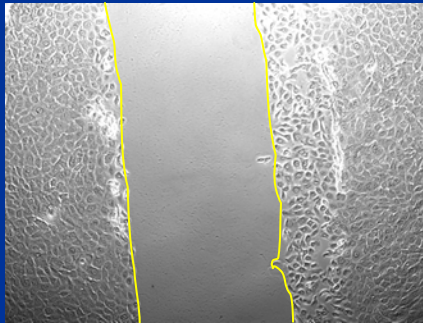
CONTROL

**0.2 U/ml
ML-05**

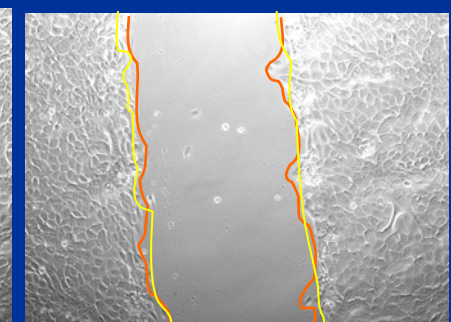
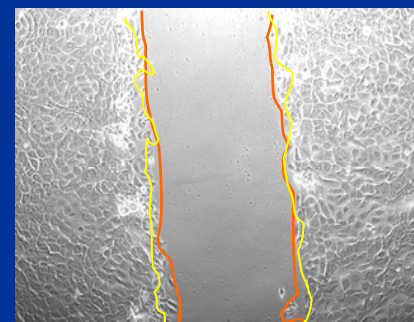
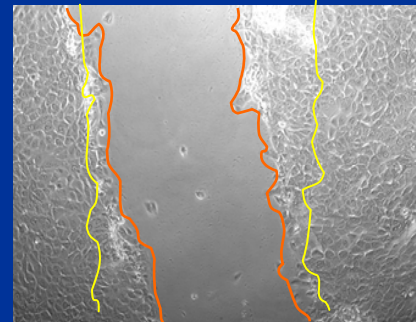
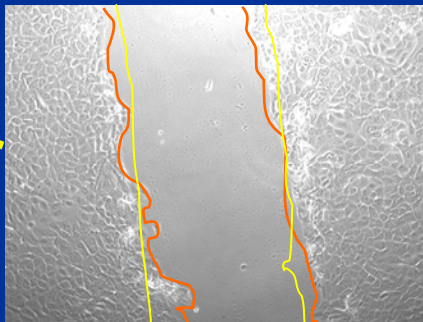
Primary Human Epidermal Keratinocytes

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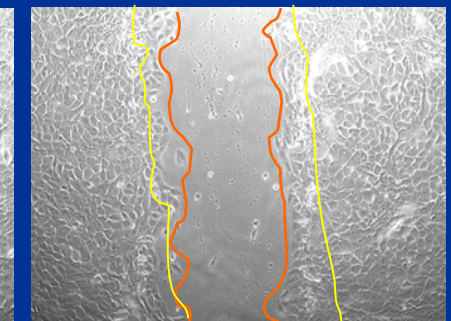
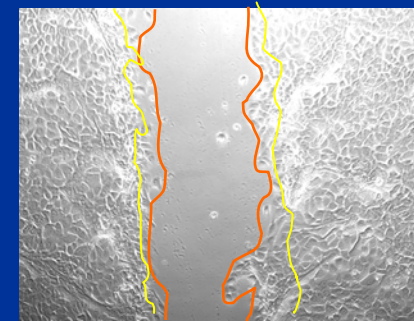
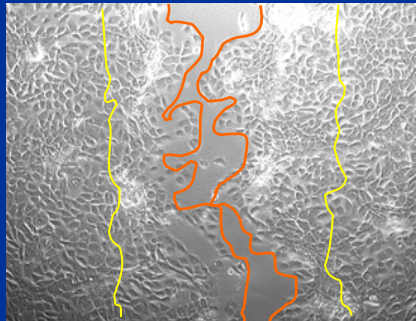
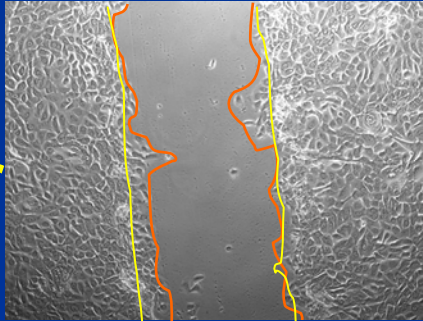
0 hr



24 hr



48 hr



CONTROL

**2 U/ml
ML-05**

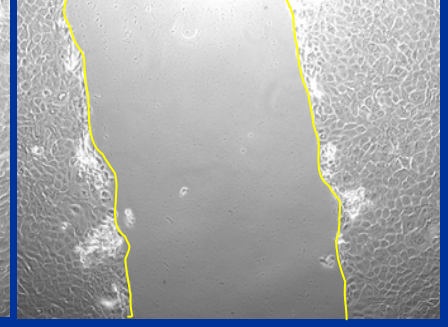
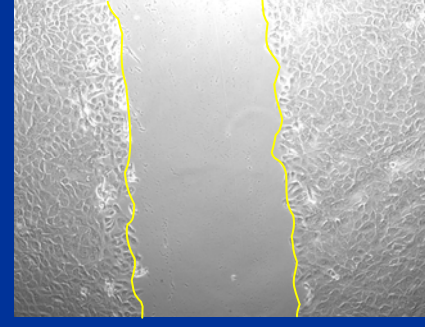
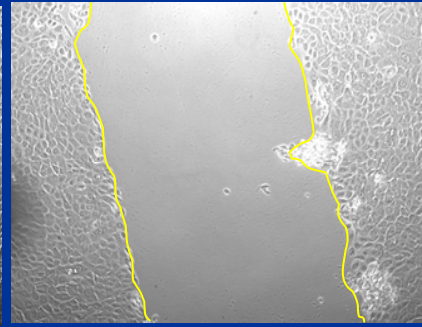
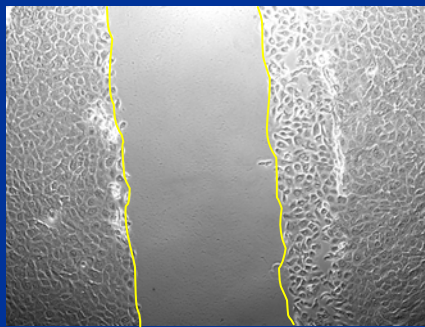
CONTROL

**2 U/ml
ML-05**

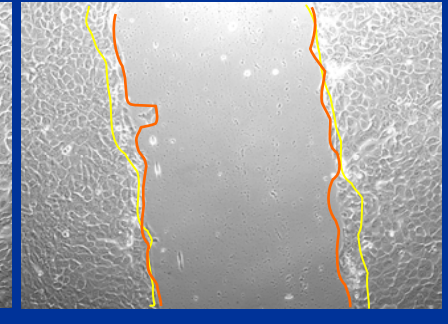
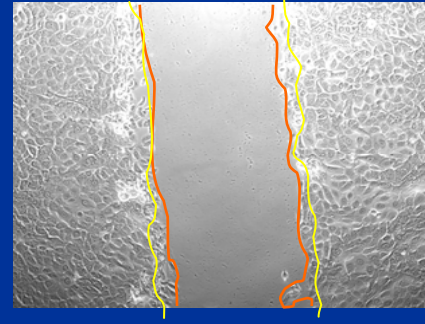
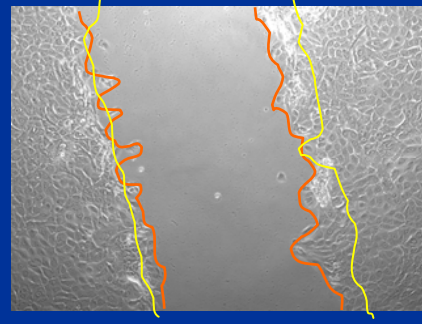
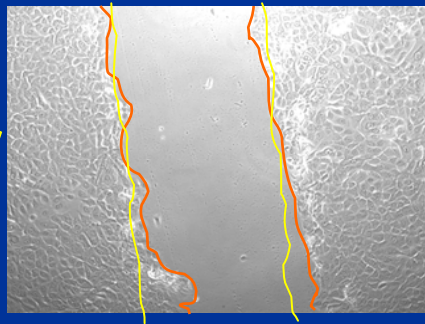
Primary Human Epidermal Keratinocytes

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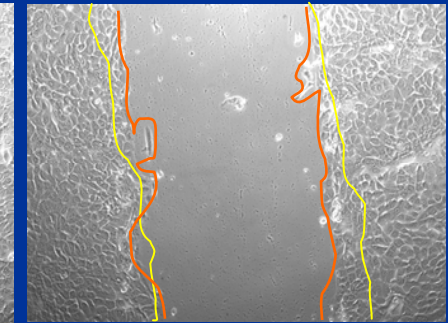
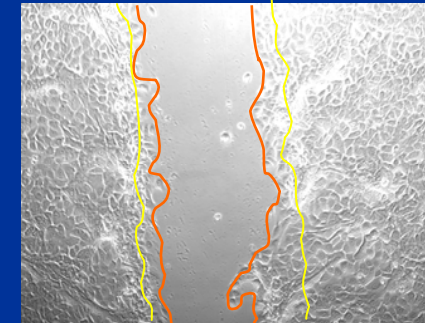
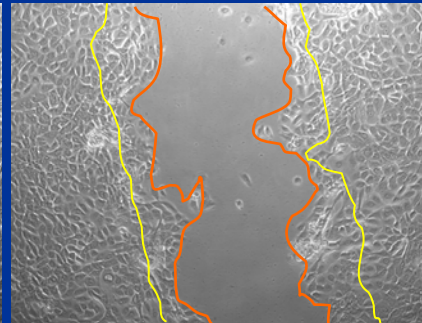
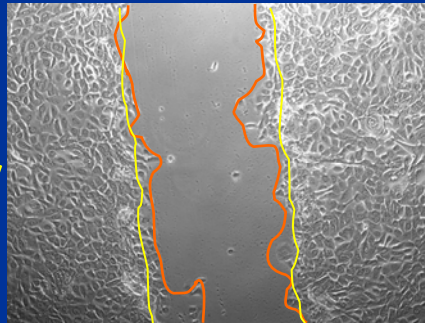
0 hr



24 hr



48 hr



CONTROL

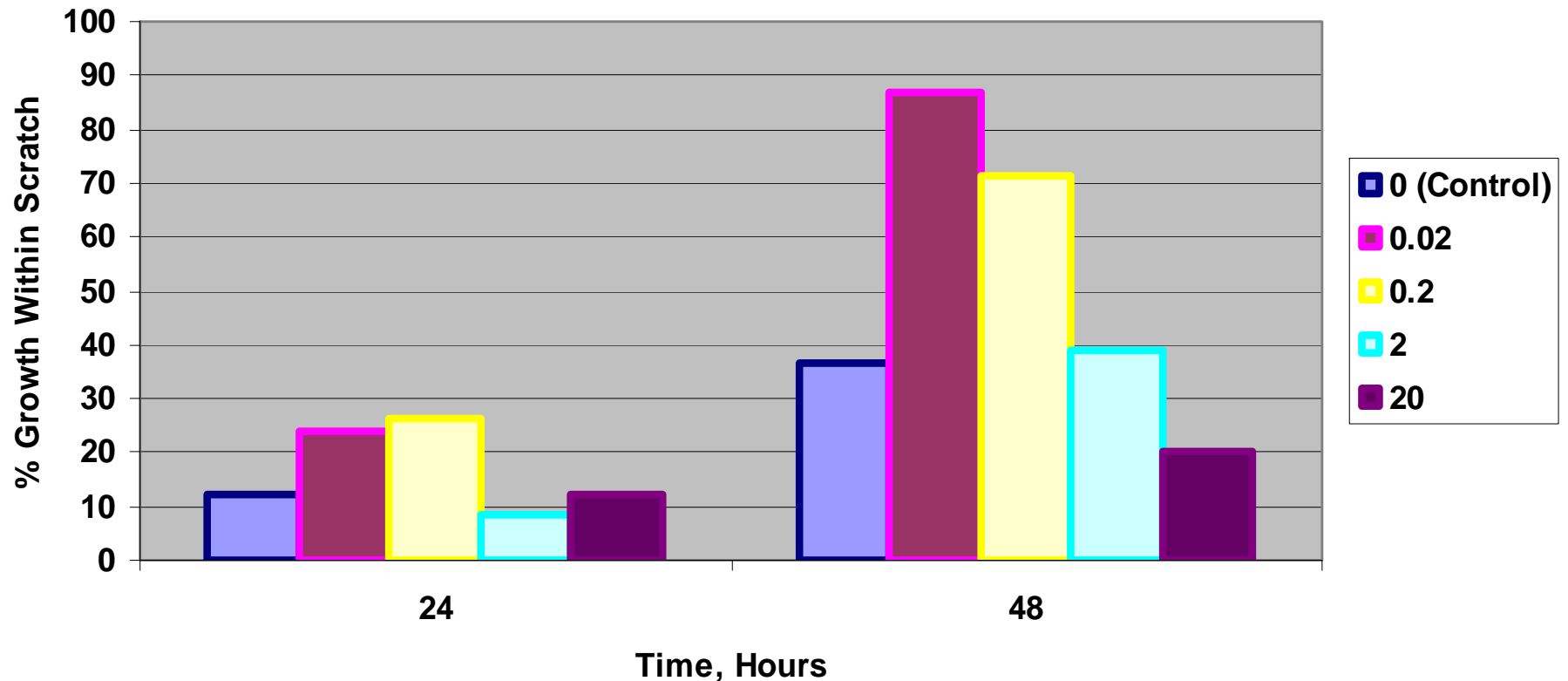
**20 U/ml
ML-05**

CONTROL

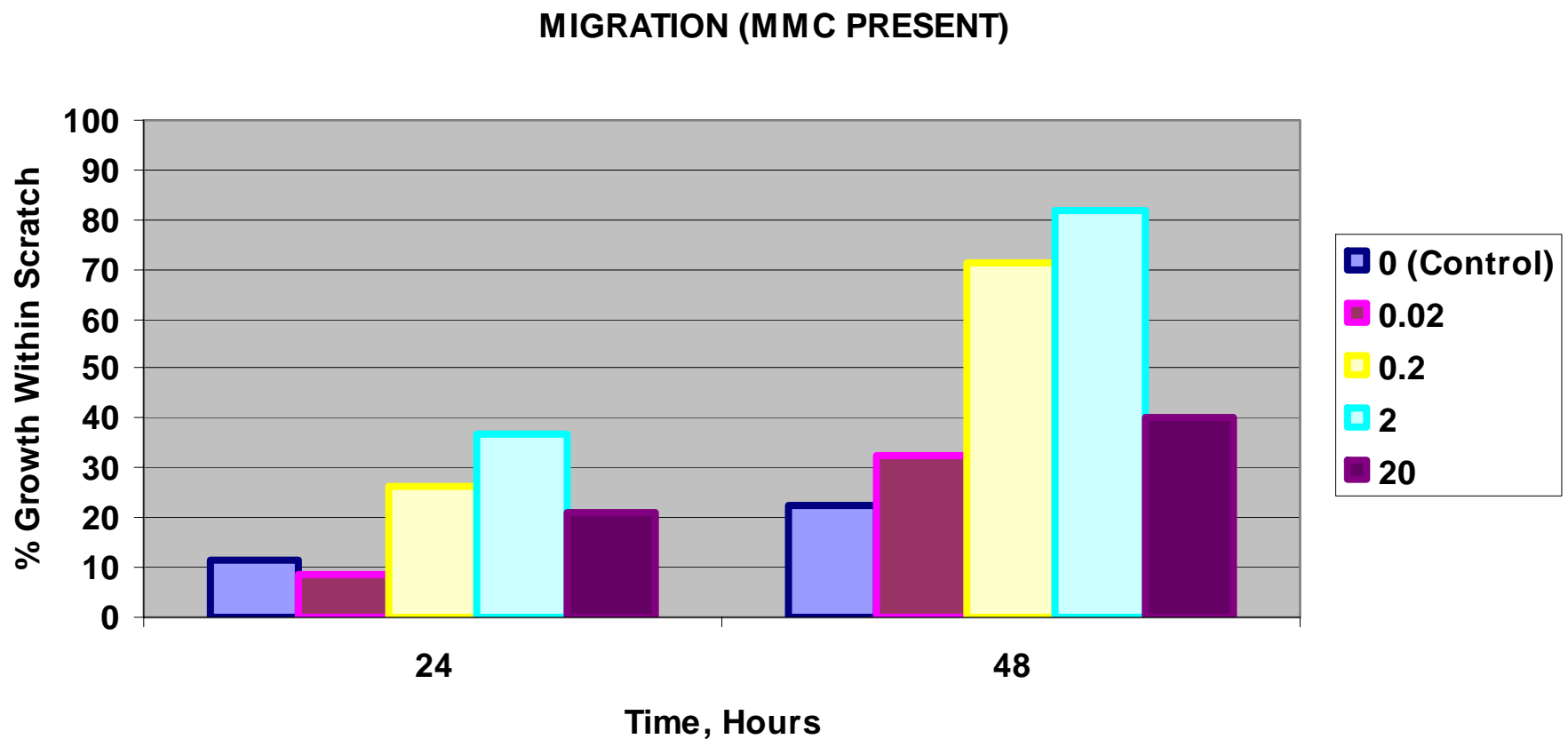
**20 U/ml
ML-05**

Activity of ML-05 in Primary Keratinocyte Scratch Assays

PROLIFERATION & MIGRATION (NO MMC)



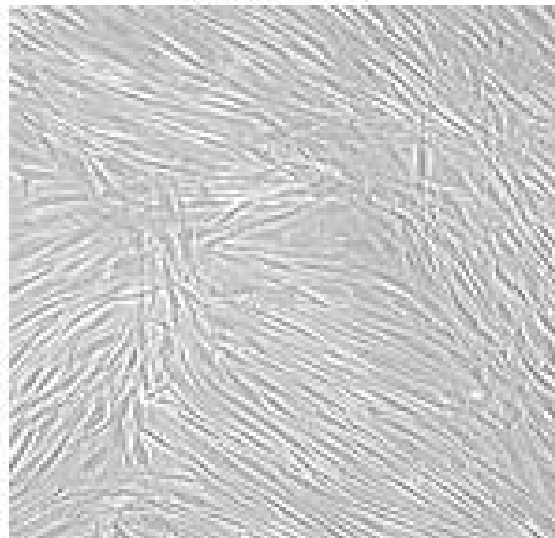
Activity of ML-05 in Primary Keratinocyte Scratch Assays



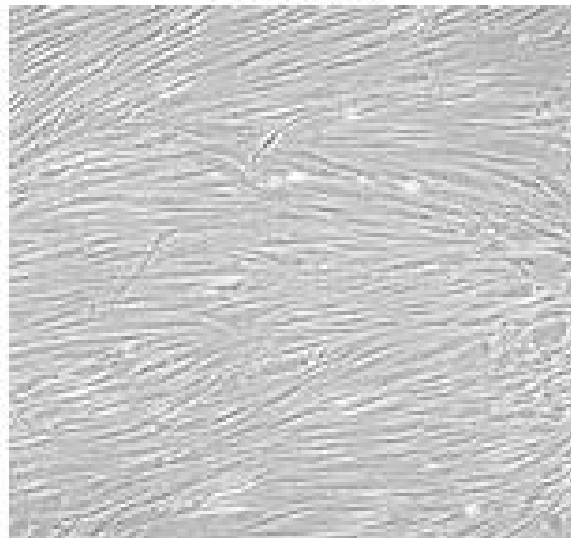
Fibroblast Scratch Assays

Performed using normal human diploid fibroblasts instead of epidermal keratinocytes. ML-05 did not affect either migration or proliferation of dermal fibroblasts, indicating that the effects of ML-05 on cell migration/proliferation may be keratinocyte-specific. However, the spatial orientation of fibroblasts within the scratch zones appeared to be more orderly if ML-05 was present.

Control



ML-05



Human Skin Organ Culture Wound Assay

Specimens of normal human skin obtained from reduction mammoplasty (about 1 cm² each) were wounded by the creation of 4 mm punch biopsies in the center through the reticular dermis. Skin samples were incubated with four different concentrations of ML-05 using two different treatment conditions. Indirect treatment of the epidermis through the dermis (dermal exposure) was achieved by adding ML-05 to the medium; direct treatment of the wound surface (topical exposure) involved the topical application of ML-05 to the wound site. PBS was the negative control.

For each condition, two experiments were conducted, one with explants being evaluated over 4 days post-treatment, and a second with explants evaluated over a 6-day period. Wounds were quantified daily by computer-assisted planimetry.



UNTREATED CONTROLS (DERMAL EXPOSURE)



DAY 0

DAY 1

DAY 2

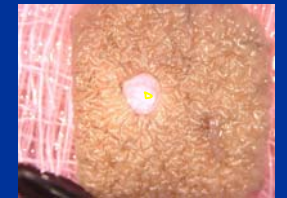
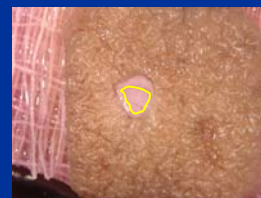
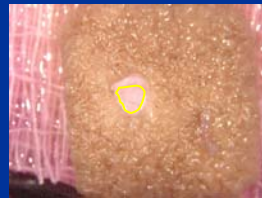
DAY 3

DAY 4

DAY 5

DAY 6

DERMAL EXPOSURE: D = 0.2 U/ml



DAY 0

DAY 1

DAY 2

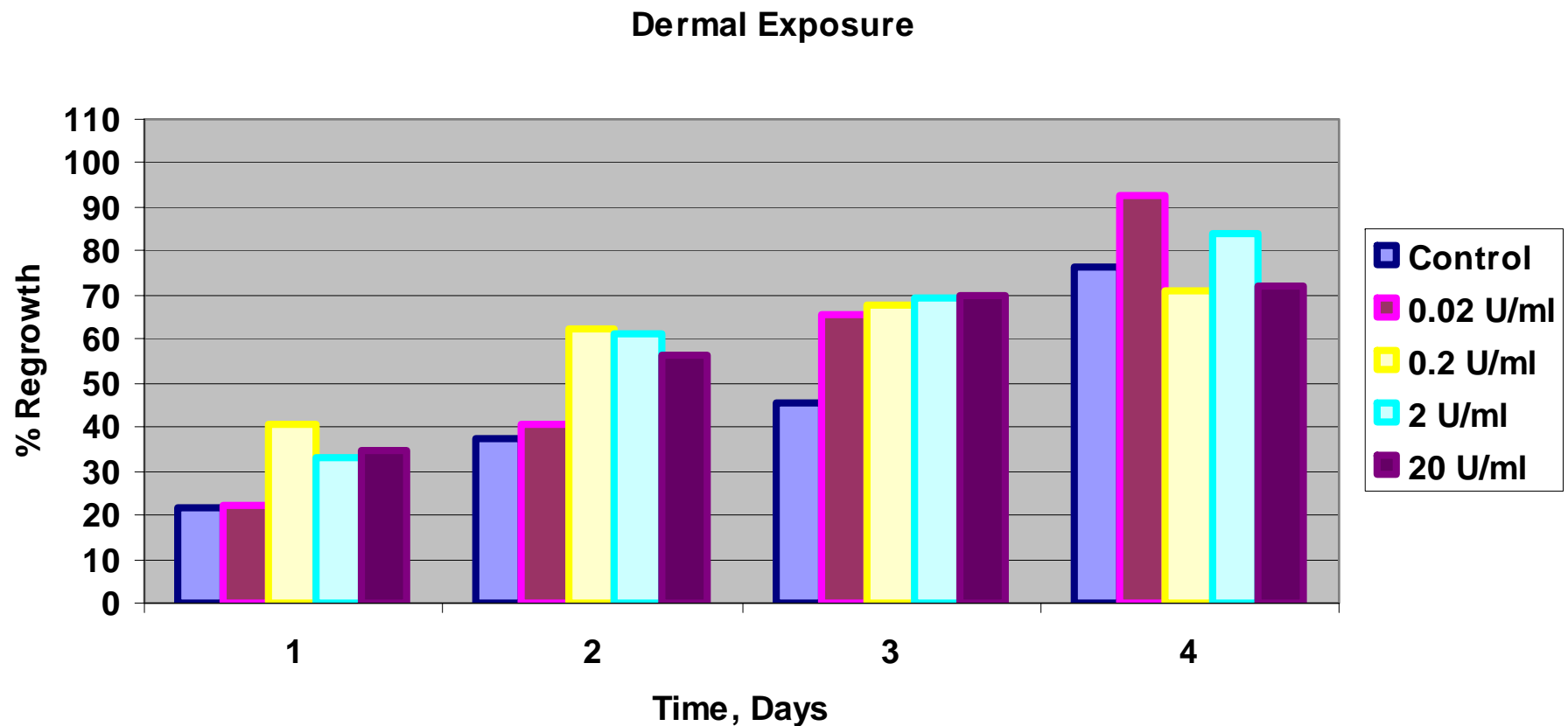
DAY 3

DAY 4

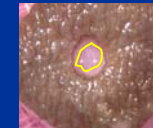
DAY 5

DAY 6

Activity of ML-05 in Skin Explant Wound Healing Assays



UNTREATED CONTROLS (TOPICAL EXPOSURE)



DAY 0

DAY 1

DAY 2

DAY 3

DAY 4

DAY 5

DAY 6

TOPICAL EXPOSURE: D = 0.2 U/ml



DAY 0

DAY 1

DAY 2

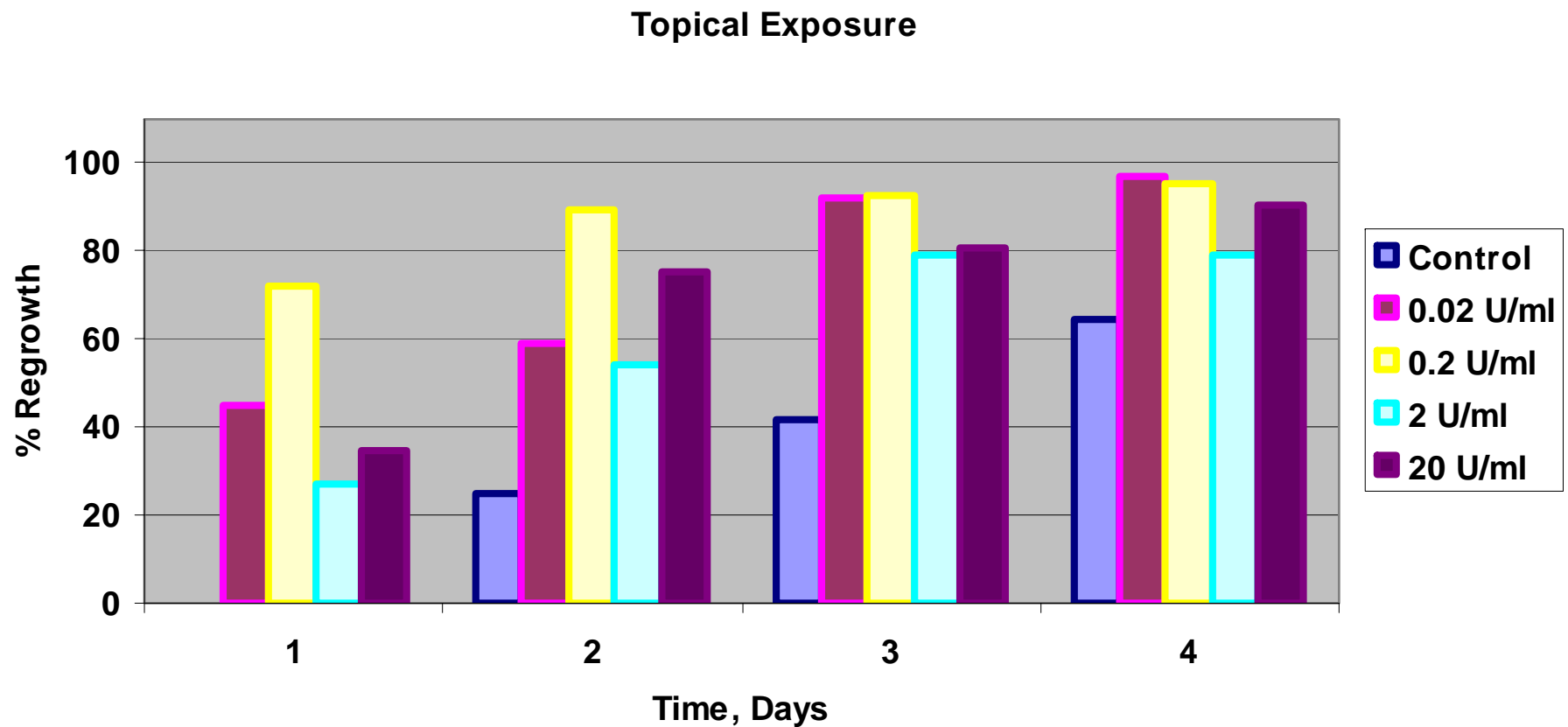
DAY 3

DAY 4

DAY 5

DAY 6

Activity of ML-05 in Skin Explant Wound Healing Assays



Human Skin Organ Culture Histology

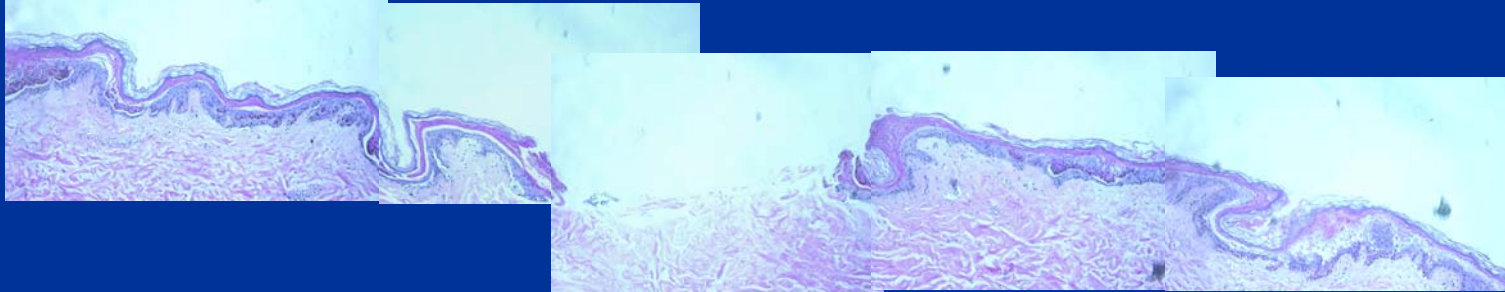
All treatment and control specimens were collected at the end of the experiment (day 4 or day 6) and frozen sections were prepared using OCT compound. 5 mm thick sections were stained with hematoxylin and eosin. The sections were analyzed using a Nikon microscope and digital images were obtained using a Spot RT camera.

Skin Organ Culture Histology Results

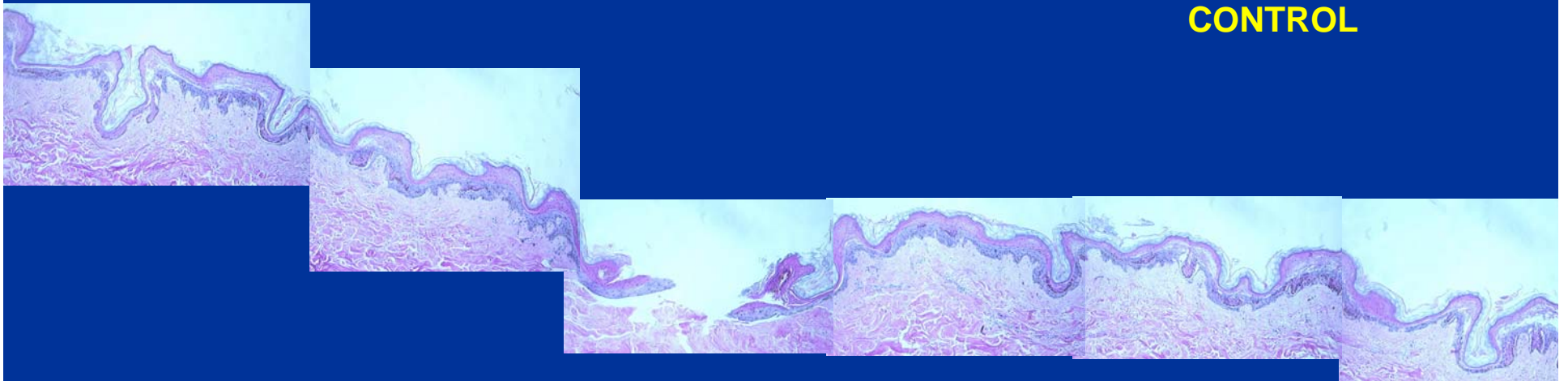
Dermal Exposure: Histological examinations of the day 6 dermal exposure cultures treated with ML-05 showed essentially complete closure and a fully restored epidermis compared to the incompletely healed untreated control.

Topical Exposure: Skin sections showed incomplete wound healing in controls, whereas the two lowest test concentrations of ML-05 (0.02 and 0.2 units/ml) showed a fully restored epidermis. However, at the two higher concentrations (2 and 20 units/ml), incomplete healing was observed, with a fragile epithelium, showing loss of attachment of suprabasal keratinocytes to the basal layer.

DERMAL EXPOSURE, Day 6

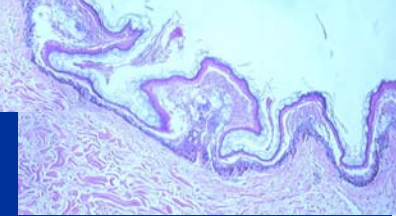
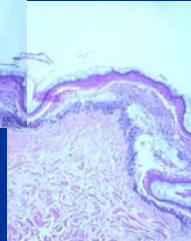
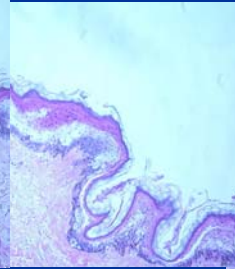
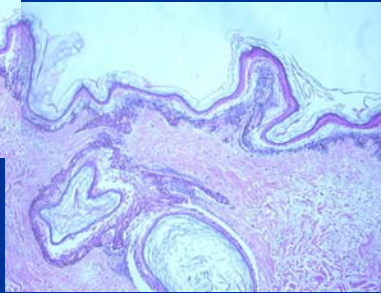
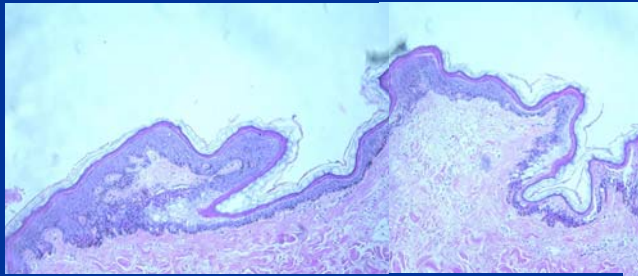


CONTROL

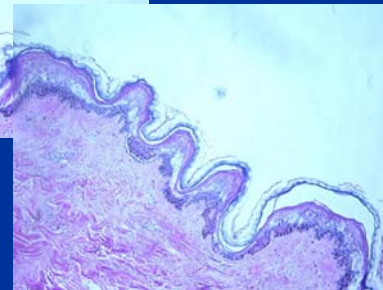
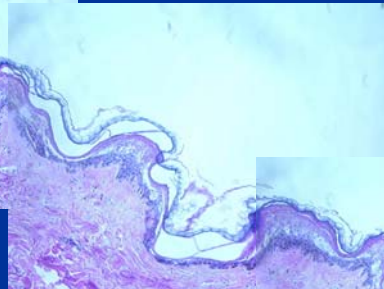
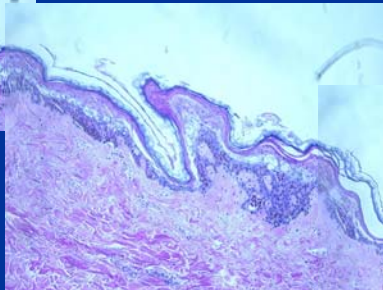
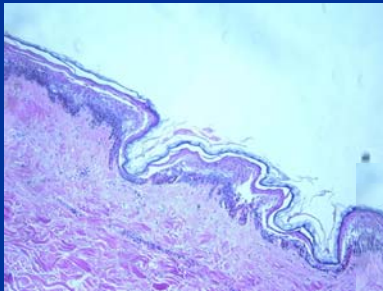


**0.02 U/ml
ML-05**

DERMAL EXPOSURE, Day 6

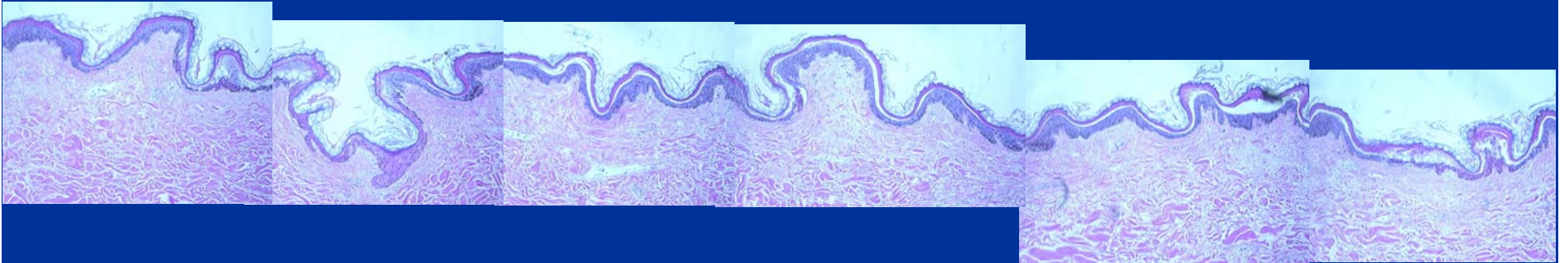


**0.2 U/ml
ML-05**



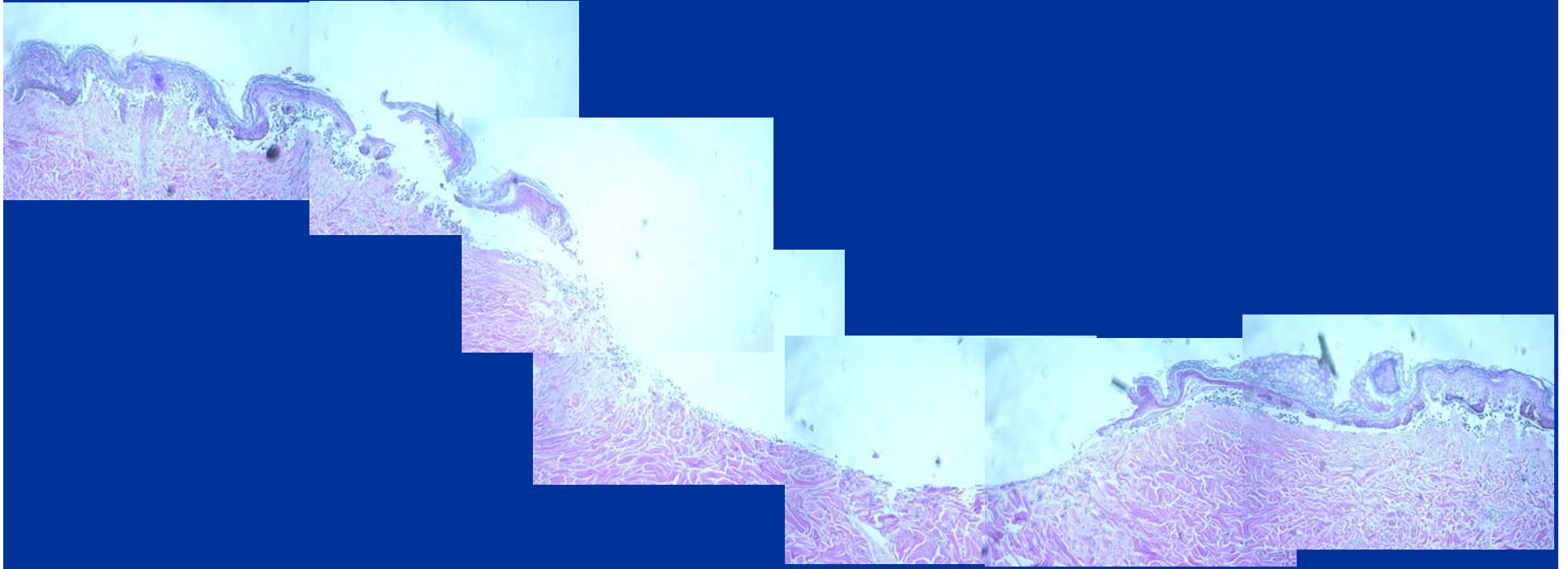
2 U/ml ML-05

DERMAL EXPOSURE, Day 6



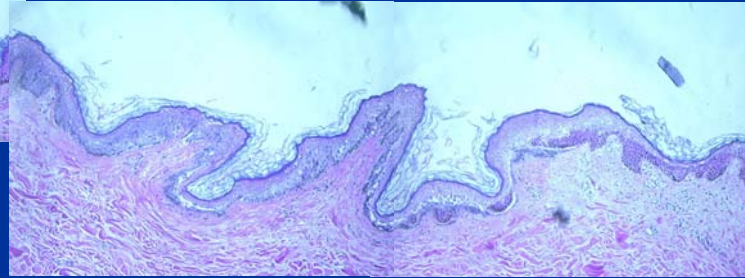
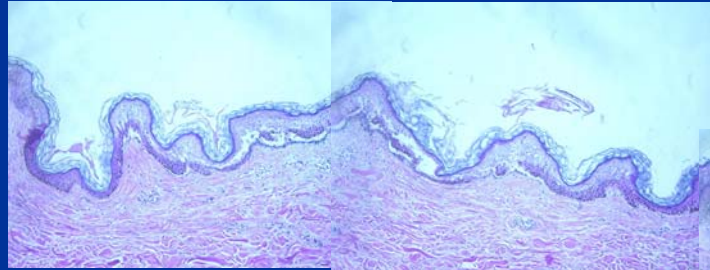
20 U/ml
ML-05

TOPICAL EXPOSURE, Day 6

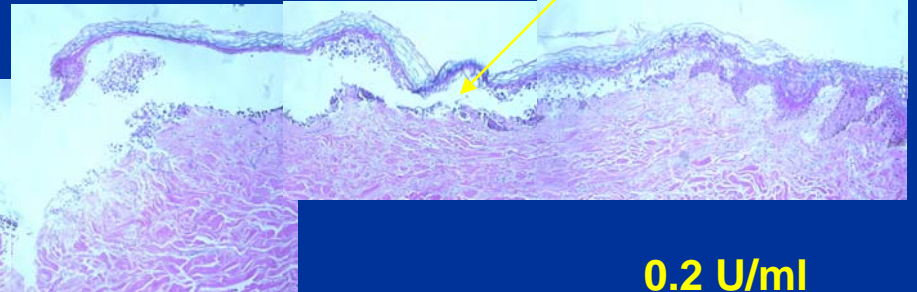
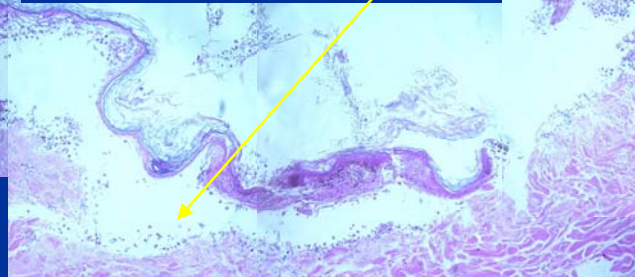
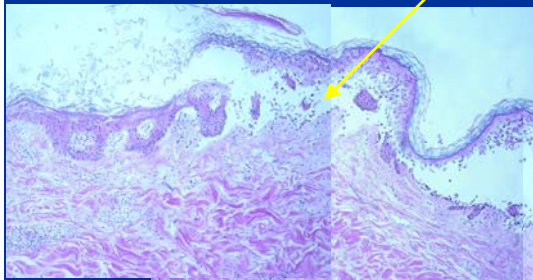


CONTROL

TOPICAL EXPOSURE, Day 6

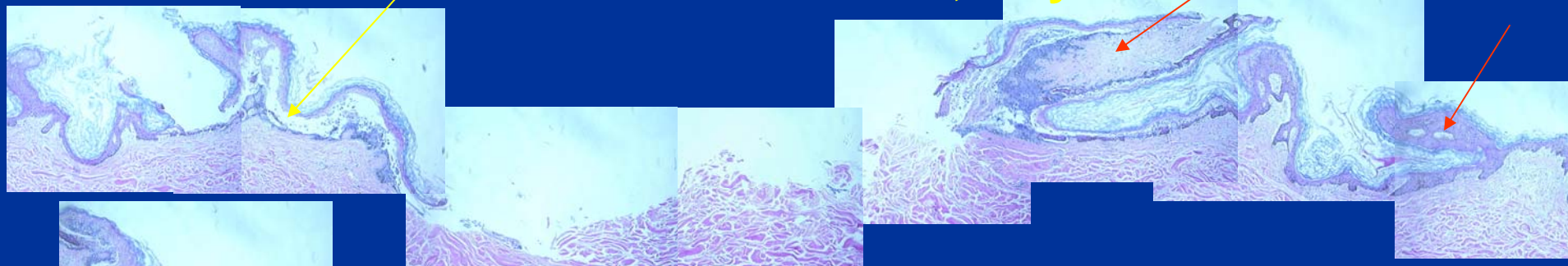


0.02 U/ml
ML-05

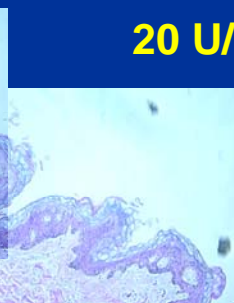
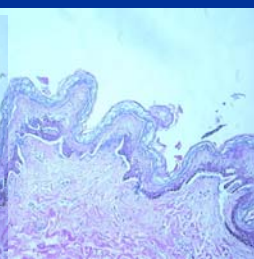
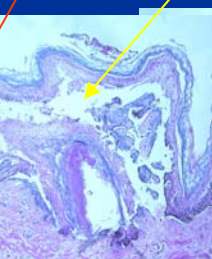
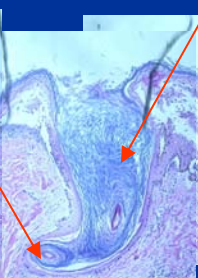
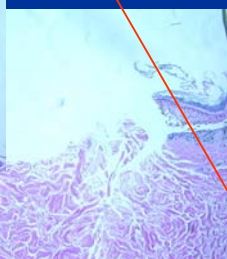
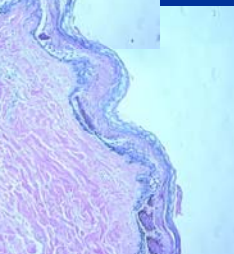
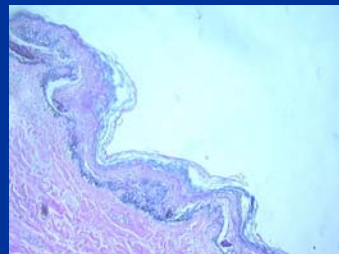


0.2 U/ml
ML-05

TOPICAL EXPOSURE, Day 6



2 U/ml ML-05



20 U/ml ML-05

ML-05: Possible Mechanisms of Action in Promoting Wound Healing

1. As a potential immunomodulator, ML-05 could be affecting cytokine/chemokine expression, neutrophil and epithelial cell migration/proliferation, and other specific responses relevant to wound healing. A variety of cytokines and chemokines are known to have a role in promoting keratinocyte migration, proliferation and reepithelialization during wound healing. The production of several of these cytokines and chemokines can be induced by SLO and related substances.

2. ML-05 may benefit the wound healing process by inducing or maintaining the expression of hyaluronan receptor CD44 in keratinocytes. ML-05 was previously shown to activate CD44 expression in keratinocytes *in vitro*. Upregulation of keratinocyte CD44 could lead to the accumulation of CD44 in the extracellular matrix via proteolytic cleavage, which then can promote the assembly of hyaluronan-rich extracellular matrices through the formation of CD44-hyaluronic acid complexes. These complexes could modulate epithelial cell behavior, including increased mobility, altered adhesion and/or altered proliferation of keratinocytes. Accordingly, such physiological changes occurring in the area of the wound may ultimately result in a restoration of normal extracellular matrix organization in that area.

Summary

Low concentrations of ML-05 promoted healing and reepithelialization in human skin cultures wounded *in vitro* by punch biopsy. ML-05 also promoted proliferation and migration of normal human epidermal keratinocytes but did not improve that of normal human dermal fibroblasts. The results of the present research suggest that in addition to previously shown activity in alleviating diseases involving excess collagen deposition, such as scleroderma, ML-05 may promote wound healing.