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14. ABSTRACT This final report is essentially the same as the third year report. We first measured dose-time responses in 2-D and 3-D cultures (TASK 1) and discovered that an SM dose of 150uM induced significant apoptotic cell death. We next compared the SM response of 3-D cultures grown in the absence or presence (AlloDerm) of structured basement membrane (BM) (TASK 8) and found that the presence of BM led to resistance to SM-induced damage, suggesting that BM could protect basal keratinocytes from SM-induced apoptosis. To further explore the role of BM in decreased SM susceptibility, primary keratinocytes harvested from Junctional Epidermolysis Bullosa (JEB) patients (#552), that lack a functional $\gamma 2$ chain of laminin 5 and do not adhere to BM, were transduced with retroviral vectors (TASK 4) to restore or abrogate laminin 5-mediated adhesion. We constructed 3-D tissues with these "reverted" JEB cells (TASK 5,6 and 7) and their phenotypic analysis showed that only JEB cells with restored laminin 5 function (F-GAL) were resistant to apoptosis when exposed to SM (150uM), thereby implicating laminin 5-mediated attachment as being important in limiting SM damage. These studies provide important evidence that bioengineered, <i>in vitro</i> tissues mimic many skin alterations previously found <i>in vivo</i> and that adhesion to BM enables epithelial resistance to SM damage.					
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INTRODUCTION

A major reason for our limited understanding of what triggers SM injury is that detailed mechanistic studies using human skin have not been possible for ethical reasons. Therefore, we have used an approach that has allowed us to identify sites and pathways of sulfur mustard (SM)-induced vesication using engineered human skin that mimics the clinical and histologic features of this tissue. Through research conducted this past year, our laboratory has extensively studied the pathophysiology of bioengineered, *in vitro*, 3-D human skin in response to SM by establishing dose/time responses of these human, skin-like tissues that lead to dermal-epidermal separation. We have developed and adapted novel tissue models that have found that structured basement membrane can alter the response to SM injury by making the tissue less susceptible to SM-mediated damage. We used immunohistochemical, morphologic and biochemical analyses to characterize the influence of these ECM and BM substrates on the morphogenesis, survival, differentiation and growth of NHK. We have found that the presence of individual BM components, Type IV and tissues grown on intact complete BM (de-epidermalized dermis) in organotypic cultures supports the survival of these skin-like tissues. Since there is a significant linkage between the pathologic alterations in Junctional Epidermolysis Bullosa (JEB) and SM injury, our understanding of the molecular defects in BM causing JEB should shed light on the pathogenesis of SM-induced blistering. To further explore the role of basement membrane proteins in keratinocyte resistance to SM-induced damage, we directly studied the role of laminin 5 in this process. Primary keratinocytes harvested from patients with the blistering skin disease Junctional Epidermolysis Bullosa (JEB), that lack a functional gamma 2 chain of laminin 5 and are not able to adhere to basement membrane, were transduced with retroviral vectors designed to restore laminin 5-mediated adhesion. We found that only JEB cells in which laminin 5 adhesive function was restored (F-GAL) were resistant to apoptosis when exposed to SM (150 ug/ml), thereby implicating laminin 5-mediated attachment as being important in limiting SM damage. These studies provide important proof of concept that *in vitro* and *in vivo* tissue models mimic many of the tissue alterations previously found in animal models of SM injury. Our findings show that adhesion to basement membrane proteins enables subsets of keratinocytes to resist SM damage. Since we have previously found that only specific subsets of basal keratinocytes underwent cellular damage leading to apoptosis in our *in vivo* engineered tissue models, we have taken an important step towards defining the ECM or BM components that provide survival signals that can protect cells when challenged with SM. These human tissue models will be of great relevance in understanding functional mechanisms of SM injury and in testing new countermeasures to limit its morbidity and mortality.

EXPERIMENTAL RESULTS:

PART I: ESTABLISHMENT OF CONDITIONS UNDER WHICH SULFUR MUSTARD CAN ALTER THE SURVIVAL AND VIABILITY OF NORMAL HUMAN KERATINOCYTES AND JUNCTIONAL EPIDERMOLYSIS BULLOSA CELLS GROWN ON A VARIETY OF BASEMENT MEMBRANE COMPONENTS IN 2-D MONOLAYER CULTURES

TASK 1: To determine the dose/time responses of normal keratinocytes to sulfur mustard exposure

1. Determination of the effects of ethanol on human keratinocytes: Prior to the application of sulfur mustard (SM) on cultured cells, we conducted a series of experiments using various concentrations of pure ethanol to determine the effects of this solvent on keratinocyte growth and survival. This was done

because SM was dissolved in pure ethanol and it was important to determine if this vehicle would induce alterations in cultured cells at the SM concentrations needed. Experiments were carried out in p60 plates and keratinocytes were grown on feeder layers of γ -irradiated 3T3 cells until colonies were 60% confluent. Cultures were then exposed to different doses of ethanol (0.4%, 0.8%, 1.6%) for 30 min and compared to untreated controls. Plates were then rinsed three times with fresh media and were grown for an additional week. Colonies were stained using crystal violet and the diameter of colonies exposed to ethanol doses were measured. ***Based on our experiments we concluded that ethanol did not greatly alter the growth of keratinocytes compared to the non-exposed controls as the size of individual colonies was not altered upon exposure to ethanol (Figure 1).***

2. Staining for cellular apoptosis to determine the dose and time response of cultured keratinocytes to sulfur mustard: To establish the SM doses that could induce alterations in keratinocyte growth, a dose and time study was performed to measure the response of human keratinocytes to SM. Keratinocytes were seeded on sterile coverslips with 3T3 feeder layers (10,000 cells/p60) for six days in p60 plates until the colonies became 70% confluent. Cultures were then exposed to different doses of SM and compared to ethanol-exposed controls. We initially selected four different doses 37.5, 75, 150, 300 μ M of SM and compared these to 0.25, 0.5, 1, 2% ethanol upon exposure for 7 min. Cells on coverslips that were exposed to agents were then processed 24 hours later for immunofluorescent staining for the presence of apoptotic cells using a monoclonal antibody that detects the cleavage product of keratin 18 that is the end result of apoptotic pathways (M30 Cytodeath-stain, Roche, Inc.). Fig. 3 shows the appearance of apoptotic cells that demonstrated M30 staining in the cytoplasm (red stain) for different doses of SM when compared to corresponding ethanol controls. At low SM doses of (37.5 μ M, 75 μ M), fewer apoptotic cells were seen when compared to 150 and 300 μ M SM doses. All doses of ethanol showed fewer apoptotic cells than SM-exposed cells. ***Thus a dose-dependent increase in the number of apoptotic cells was seen after SM treatment. Based on these findings, we selected 150 μ M as a standard SM dose that induced a maximum number of apoptotic cells.***

An additional experiment was performed to study the effect of the length of exposure to SM on cell survival and death. We selected a dose of 150 μ M to study the effects of SM exposure for 1, 3, 7, 14, and 28 min. Staining was performed for apoptotic keratinocytes grown on coverslips using the M30 stain the numbers of apoptotic cells on 3 coverslips were counted for each length of exposure. A sharp increase in the number of apoptotic cells was seen for 7 min exposures when compared to 1 and 3 min exposures. Longer exposures (14 min and 28 min) resulted in an 8–10 fold increase in numbers of apoptotic cells when compared to cultures exposed for 7 min (Fig. 5). In comparison, untreated controls showed a very small number of apoptotic cells while ethanol showed very small changes in the number of apoptotic cells. Fig. 6 demonstrates the appearance M30 positive cell after exposure to SM and to 1% ethanol for 7 min. ***These findings demonstrated that a SM dose of 150 μ M at an exposure time of 7 min was sufficient to induce significant apoptotic cell damage in 2-D cultures of keratinocytes. As a result, it was decided to use 7 min as the standard SM exposure time for all future studies.***

3. MTT viability/survival assays to determine keratinocyte survival after SM exposure: We next performed the MTT assay to evaluate the viability of normal keratinocytes after exposure to SM. In this assay, the yellow tetrazolium salt MTT is reduced in metabolically active cells to form insoluble purple formazan crystals that are solubilized by the addition of a detergent. The purple color can then be quantified by spectrophotometric means and provides a direct measure of cell viability upon exposure to SM. Conversely, a reduction in spectrophotometric measurement reflects the loss of cell viability. We

performed MTT assays using normal human keratinocytes (NHK) at different cell densities to establish if varying cell density could alter cell viability in response to SM. NHK's were plated at densities of 5×10^4 , 2.5×10^4 , 1×10^4 and 1×10^3 and exposed to either 150 μ M of SM or to 1% ethanol control. Fig. 7 demonstrates that cells seeded at low densities (1,000 and 10,000 cells) showed no difference in cell viability when SM exposures were compared to ethanol controls (Fig. 7). However, at high cell densities (25,000 and 50,000 cells), SM exposure significantly decreased cell viability when compared to ethanol-exposed controls. *This demonstrated that the sensitivity of detection of MTT assay required a threshold number of cells greater than 25,000 to yield differences in cell survival between SM- and ethanol-exposed cells. These studies laid the groundwork for all experiments by establishing parameters required for length and concentration of SM exposure needed to alter cell viability and to induce apoptosis.*

TASK 8 : Dose-time response to establish the role of basement membrane components to SM in 2-D cultures

4. The role of basement membrane components on the survival and viability of keratinocytes exposed to sulfur mustard - We next performed experiments to establish the importance of (BM) or extracellular matrix components (EMC) on cell viability and cell survival when exposed to different doses of SM compared to ethanol controls. To establish if the basement membrane (BM) protein Type IV collagen could alter the sensitivity of normal keratinocytes to SM, we seeded different numbers of keratinocytes on Type IV Collagen-coated, 24-well plates and performed MTT viability assays (Fig.8). Results were similar to those seen when cells were plated on tissue culture plastic as SM-exposed cells showed lower viability when compared to those ethanol-exposed at higher cell density (50,000 and 25,000). Similar differences were seen when cells were exposed to SM and ethanol after seeding onto Type I Collagen coated plates (Fig. 9). These experiments demonstrated that an SM dose of 150 μ M for 7 min reduced cell survival compared to ethanol exposure. Fig.10 presents MTT viability assays when 50,000 cells were plated on different substrates including plastic, Type I Collagen, Type IV Collagen, Fibronectin, Laminin and Poly D-lysine in monolayer, 2-D cultures. 24 hours after seeding, cells were exposed to 150 μ M SM or 1% ethanol for 7min. All the plates showed a decrease in cell viability when treated with SM compared to ethanol controls. However, the greatest reduction in viability (50%) upon SM exposure was seen for cells grown on Type IV Collagen. Since cells grown on this substrate also demonstrated the highest cell viability when exposed to ethanol, it is possible that the elevated cell growth on Type IV Collagen made cells more vulnerable to SM damage. Other substrates (Type I Collagen, Fibronectin and Laminin) demonstrated decreased viability upon SM exposure, but to a lesser degree than Type IV Collagen. Poly-D-lysine and plastic-coated dishes demonstrated the smallest loss of viability upon SM exposure. This may be due to the poor attachment of keratinocytes to poly D-lysine-coated plates compared to other substrates.

MTT assays were carried out for varied doses of SM and ethanol on different substrates (Fig. 11). Four different doses of SM were tested (37.5, 75, 150, 300 μ M) and were compared to controls (0.25, 0.5, 1, 2% ethanol) when cells were seeded onto tissue culture plastic, Type I Collagen, Type IV Collagen, Fibronectin and Laminin. As described above, the greatest decrease in viability after SM exposure was seen for cells grown on Type IV Collagen. Interestingly, this was the only substrate that demonstrated loss of cell survival even at low SM doses (37.5 μ M and 75 μ M). Other substrates showed no loss of viability for these low SM doses but did show a moderate decrease at higher SM doses. Thus, while ECM components were protective at low SM doses, they did not provide a survival advantage at higher SM doses. *Significantly, cells grown on the BM component Type IV Collagen demonstrated the*

greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures and intact BM may be required to mediate this event.

TASK 5: Dose-time response to establish the response of JEB cells to low dose SM exposure

5. MTT Assay to determine the response of Junctional Epidermolysis Bullosa (JEB) cells to SM:

There is a significant linkage between the pathologic alterations seen in Junctional Epidermolysis Bullosa (JEB) and those seen in vesicant injury induced by SM. To determine if cells lacking the ability to synthesize a functional laminin 5 molecule would demonstrate an altered sensitivity to SM exposure, we utilized primary keratinocytes that were derived from patients with JEB that were deficient in laminin 5 function. JEB cell lines were initially harvested from the skin of a patient with JEB (552) by the laboratory of Dr. Guerrino Meneguzzi (INSERM, Nice, France). Cells were infected with retroviral vectors that were previously shown in our lab (Progress Report 1) to modify laminin 5 function and restore cell-substrate adhesion in cells that were adhesion-deficient due to the absence of Gamma 2 chain (Phoenix producer cells courtesy of Nolan lab). The following vectors were used to modify laminin 5 function in 552 cells:

Delta BC: Cleaved variant of Gamma 2 chain of laminin 5 with a shortened 80 kd Gamma 2 chain. This generates cells that do not adhere well to connective tissue substrates.

FGAL: Non-cleaved variant of Gamma 2 chain. The chain remains intact and is not cleaved at its BMP-1 site to generate a 155kd chain that restores adhesion.

Pfu: A Gamma 2 mutant that encodes cDNA for the constitutively cleaved form of this chain that has been truncated at the proteolytic cleavage site to generate a 105kd chain. These cells do not adhere well to connective tissue substrates.

Wild type (Gamma 2 WT): This has a full-length Gamma 2 chain and cells infected with this variant restore their laminin 5 function.

Delta C115: This is an empty vector that does not correct laminin 5 due to the absence of Gamma 2 chain and so that cells retain the properties of the mutant cells.

We used the MTT cell proliferation assay to study the effects of SM on JEB cells that were seeded on different BM and ECM components such as Type I Collagen, Type IV Collagen, Fibronectin and Laminin, as well as control plastic plates. Cells were seeded into 24-well plates on these substrates and exposed to 150µM of SM or 1% ethanol on the following day for an exposure time of 7min, rinsed three times with fresh media and incubated for an additional 24 h. Fig. 12 represents results of MTT assays for these JEB cells grown on different ECM or BM substrates. In general, there was an increase in cell viability for the Gamma 2 WT, Delta C115 and FGAL cells, demonstrating that these cells survived the exposure on different substrates to a greater degree when compared to Delta BC and Pfu. However, the degree to which cell viability was altered was dependent on the substrate on which the JEB cells were plated. For example, cells that had restored their laminin 5-mediated adhesive function (FGAL and Gamma 2 WT) showed a minimal loss of viability on Type I and Type IV Collagen and tissue culture plates when compared to JEB cells restored with a gamma 2 chain that did not support adhesion (Delta BC). However, SM-induced loss of cell viability was not as great with Delta BC-restored cells on Fibronectin as had been seen on other substrates. *These findings demonstrated that restoration of adhesive function mediated by laminin 5 was able to provide decreased cell vulnerability and increased cell survival upon SM exposure.*

In light of these findings, we next performed MTT assays for JEB cells on these substrates at an elevated dose (300 μ M) of SM and compared this to control exposures of 2% ethanol (Fig. 13). As a control, we compared the SM response of JEB cells to that of normal keratinocytes, as well. JEB cells whose laminin 5-mediated adhesion was restored (FGAL and Gamma 2 WT) showed a similar susceptibility to this dose of SM, as did cells whose adhesion was not restored (Delta BC). Interestingly, all cells grown on Type IV collagen showed a 2 to 3 fold decrease in cell viability even in the presence of 2% ethanol, suggesting that this substrate rendered cells more susceptible to cell death. ***Thus, it appears that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150 μ M) in 2-D culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300 μ M).***

To further confirm these observations regarding threshold doses of SM, JEB cells were tested on the three substrates that showed the maximum difference in cell viability when treated with SM and ethanol. To accomplish this, JEB cells were exposed to doses of SM (75, 150, 300 μ M) and corresponding doses of ethanol controls (0.5, 1, 2 %) for 7 min. Fig. 14 presents results of the MTT assay for the 5 different JEB cell types and human keratinocytes seeded on three different substrates (Type I Collagen, Type IV Collagen, Fibronectin) that were exposed to 75 μ M of SM or 0.5% ethanol. At lowest SM doses (75 μ M, 150 μ M), FGAL cells showed sensitivity to SM damage on Type I Collagen and Fibronectin, but not on Type IV Collagen (Fig. 14). This supported the observation that restoration of laminin 5-mediated adhesion and resistance to M damage were optimal on proteins present in the BM such as Type IV Collagen at low SM doses. This may occur as Type IV Collagen is known to interact with laminin 5 and may provide apoptosis resistance as due to this adhesive association. This also suggested that restoration of laminin 5 function will not reduce the sensitivity to SM damage when cells are grown on a substrate that is not found in BM, such as Type I Collagen. This apoptosis resistance seen for FGAL cells was lost at higher SM doses (300 μ M, Fig. 16) and was similar to that seen for cells that did not undergo restoration of laminin 5 function (Delta BC) at low SM doses on Type IV Collagen. However, the loss of viability for Delta BC cells was greater than that seen for FGAL cells on Type IV Collagen substrates, even at the elevated SM dose (300 μ M).

A final experiment in monolayer, 2-D culture was performed to compare SM damage between Delta BC and FGAL cells on two substrates (Type I Collagen and Type IV Collagen) that showed the maximum difference in cell viability after SM and ethanol exposure (Fig. 17-20). MTT assays were carried out using 8 different SM doses (75, 150, 300, 450, 600, 750, 900, 1200 μ M) and compared to their corresponding ethanol controls (0.5, 1, 2, 3, 4, 6, 8 %). Both FGAL and Delta BC cells showed a threshold, SM dose below which no decrease in cell viability was seen. Surprisingly this threshold was highest (450 μ M) for Delta BC cells grown on Type IV Collagen plates. In contrast, FGAL cells grown on both Type IV collagen and Type I collagen, as well as Delta BC cells grown on Type I collagen showed a small SM-induced decrease in cell viability below 150 μ M SM. However, as SM dose was increased to 1200 μ M a gradual dose-dependent decrease in cell viability was seen for both cell types on both Type I and Type IV Collagen substrates.

PART II: EXPOSURE OF THREE-DIMENSIONAL, HUMAN ORGANOTYPIC CULTURES HARBORING NORMAL HUMAN KERATINOCYTES AND JEB KERATINOCYTES TO DETERMINE THE ROLE OF BASEMENT MEMBRANE ON THE INDUCTION OF SULFUR MUSTARD INJURY

We next generated 3-D, organotypic cultures to identify pathways of SM induced vesication by using engineered human skin that mimics the clinical and histological features of this tissue. Organotypic cultures grown in the absence of pre-existing BM components ("Raft" cultures) were prepared according to our lab protocol. To accomplish this, early passage human dermal fibroblasts were added to neutralized Type I Collagen to a final concentration of 2.5×10^4 cells per ml. This mixture (3ml) was added to each 35mm well insert of a six-well plate and incubated for 4-6 days in media containing Dulbecco's Modified Eagle's Medium and 10% fetal calf serum, until the collagen matrix showed no further shrinkage. At this time, a total of 5×10^5 normal human epidermal keratinocytes were seeded directly on the contracted collagen gel. Alternatively, to generate cultures in the presence of BM, cells were seeded onto a de-epidermalized human dermis (AlloDerm) that was layered onto the contracted Type I Collagen gel. Organotypic cultures were maintained submerged in low calcium, epidermal growth media for 2 days, submerged for 2 days in normal calcium epidermal growth media and raised, to the air-liquid interface by feeding tissues from below with cornification media for an additional 2 days. At this point, cultures were exposed to different doses of SM or ethanol that were added to fresh media on day 7 of culture. Tissues were exposed for 7 min based on our previous results with 2-D monolayer cultures (see above). In addition to ethanol treated cultures, untreated cultures were used as controls by not adding ethanol to the media. After exposure, tissues were rinsed three times with fresh media and incubated for an additional 24 h. The following day, tissues were pulsed with $10\mu\text{M}$ bromodeoxyuridine (BrdU) 6h prior to terminating experiments to allow assay of proliferation in exposed tissues. Tissues were then bisected and one-half was snap-frozen in liquid nitrogen, while the other half of the tissue was formalin-fixed, paraffin-embedded and Hematoxylin and Eosin sections were prepared.

1. Establishing SM doses that induce tissue damage in 3-D organotypic cultures- morphologic and apoptotic alterations - To gain an understanding of SM doses needed to induce tissue damage in 3-D tissues, cultures were first treated with 75 and $150\mu\text{M}$ SM and compared to untreated cultures and those exposed to 1% ethanol. Figures 21 and 22 demonstrate the appearance of collagen Raft cultures grown in the absence of BM that were either unexposed (A), exposed to $75\mu\text{M}$ (B) or $150\mu\text{M}$ (C) SM or exposed to 1% ethanol (D). Untreated cultures generated a well-stratified epithelium that adhered to the underlying connective tissue. Tissues treated with $75\mu\text{M}$ SM demonstrated an intact epithelium that was well-attached to the underlying connective tissue (B). However, these tissues demonstrated a significant degree of altered tissue organization (B) that was similar to that seen for ethanol-exposed cultures (D). In contrast, cultures exposed to $150\mu\text{M}$ SM demonstrated complete dermal-epidermal separation (C) and an overall thinning of the epithelium. These preliminary findings showed that a $150\mu\text{M}$ dose of SM for 7 min could mimic the vesicating damage induced by SM *in vivo*.

To assess the degree of apoptotic cell death in exposed tissues, M30 staining for Rafts exposed to different doses of SM and ethanol was performed from frozen sections. A four-fold increase in apoptotic cells was observed at a dose of $150\mu\text{M}$ of SM when compared to controls and twice as many apoptotic cells were seen with cultures treated with $75\mu\text{M}$ of SM (Fig. 23). We next determined if higher SM doses could increase SM-mediated tissue damage in Raft cultures by performing experiments using $150\mu\text{M}$ and $300\mu\text{M}$ of SM doses in comparison to 1 and 2% ethanol controls (Fig 24, 25). Low power magnification of H&E stained sections after treatment with $300\mu\text{M}$ SM showed a significant degree of tissue damage including complete separation of tissue from the BM zone as well as necrosis of keratinocytes and fibroblasts. Less damage was seen in tissues exposed to $150\mu\text{M}$ SM and there was no separation at the BM interface (Fig.25) To confirm these findings, frozen sections were stained using

the M30 antibody. Numbers of apoptotic cells were greatest in tissues exposed to 300 μ M SM while ethanol-treated tissues showed a very minimal number of apoptotic cells. *These findings established that doses similar to those found to induce SM damage in 2-D cultures of keratinocytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our in vivo studies (REPORT Year 2).*

TASK 8 : Dose-time response to establish the role of basement membrane components to SM in 3-D, organotypic cultures

2. Establishing the role of basement membrane in human skin response to sulfur mustard - In light of these findings, we next studied the effects of SM on organotypic cultures grown in the presence and absence of pre-existing BM components. This would allow comparison to MTT assays carried out on different BM components in 2-D cultures described above. Keratinocytes were seeded on AlloDerm, the de-epidermalized, acellular cadaver dermis derived from human skin that forms intact BM at its dermal-epidermal interface. In this way, it would be possible to determine if BM can protect skin-like tissues from SM-induced damage when compared to tissues grown without BM components. To accomplish this, tissues were grown on AlloDerm, collagen Rafts or on polycarbonate membranes coated with either Type I Collagen, Type IV Collage, Fibronectin or control plastic. After 7 days in culture tissues were exposed to SM (150 μ M) and compared to tissues exposed to 1% ethanol. Fig. 27 represents the low power view of H&E stained tissue sections after SM exposure and Fig. 28 represents the higher magnification view. SM at a dose of 150 μ M induced separation at the BM zone when both collagen Raft (Fig. 27 B, Fig. 28 C) and plastic, non-coated inserts (Fig. 27 D, Fig. 28 B) were compared to the ethanol control (Fig. 27 D, E, Fig. 28 B, D). In contrast, tissues grown on AlloDerm showed that the BM interface was intact (Fig. 27 C, Fig. 28 E) as seen in ethanol-exposed controls (Fig. 27 F, Fig. 28F). Similarly, SM induced separation at the BM zone for tissues grown on different substrates (Type I Collagen, Type IV Collagen, Fibronectin) when compared to ethanol controls (Fig. 29 and Fig. 30).

To confirm these findings a dose of 150 μ M SM was used and compared to 1% ethanol and untreated controls to compare the effect of SM on organotypic cultures on which keratinocytes were grown on Rafts and AlloDerm (Fig. 31 and 32). Tissues treated with 150 μ M SM demonstrated intact tissues (Fig. 31 B) with minimal numbers of damaged, eosinophilic cells in the supra-basal layer (Fig. 32 B arrows) while untreated tissues showed a well-stratified epithelium that was similar to ethanol controls. In contrast, SM-treated Rafts showed a significantly higher number of damaged keratinocytes that displayed nuclear condensation and eosinophilic cytoplasm proving that SM could induce more severe damage to cells grown on Rafts than those grown on AlloDerm. To confirm these findings, we performed immunofluorescent staining using the M30 antibody to assess numbers of apoptotic cells in exposed tissues (Fig. 33 & 34). Rafts treated with SM showed a 10-fold increase in number of apoptotic cells when compared to AlloDerm (Fig. 34), which displayed number of apoptotic cells that were similar to non-treated and ethanol controls. To determine if the induction of apoptosis demonstrated a dose-dependency, we performed experiments on AlloDerm using two different doses of SM along and compared them to controls. AlloDerms exposed to doses of 75 μ M and 150 μ M of SM were similar to untreated and ethanol controls in both tissue morphology (Fig. 35, 36) and upon M30 staining and numbers of apoptotic cells after M30 staining when compared to controls (Fig. 37).

In light of these findings, we next determined the minimal SM dose required for the induction of tissue damage in AlloDerm cultures. We selected 5 different SM doses (75, 150, 300, 600, 1200 μ M) and compared these to ethanol controls (0.5, 1, 2, 4, 8 %) (Fig. 38 and 39). We found that the epithelium remained intact at SM doses of 75 and 150 μ M as induction of SM-mediated damage started at a dose of 300 μ M. At elevated doses of 600 and 1200 μ M SM tissue alterations were more prominent and were characterized by separation of the BM zone. Ethanol treated cultures showed little tissue damage even at elevated levels of ethanol exposure. M30 staining of these cultures showed a gradual increase in the number of apoptotic cells with increasing doses of SM from 75 to 300 μ M SM (Fig. 41). An SM dose of 75 μ M showed 3.8% of apoptotic cells compared to 57.2% observed for 1200 μ M while the maximum percentage of apoptotic cells observed for the highest dose of ethanol (8%) was only 3.6%. This gradual increase in numbers of apoptotic cells showed that AlloDerm tissues were susceptible to SM, but only above a threshold level of SM (600 μ M). ***These findings showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 μ M.***

TASK 5: Dose-time response to establish the response of JEB tissue constructs which were reverted to a normal phenotype by retroviral gene transfer to SM

TASK 6: Assay the response of JEB and normal organotypic cultures to high, vesicating doses of SM

TASK 7: Assay the response of JEB keratinocytes expressing mutated forms of the gamma- chain of laminin 5

3. Establishing the role of laminin 5-mediated adhesion in the response of 3-D tissues to sulfur mustard – the response of JEB mutants to sulfur mustard in 3-D tissues - Since it appeared that BM could protect tissues exposed to SM, we performed a final experiment to determine if JEB cells grown on AlloDerm demonstrated a differential susceptibility to 150 μ M SM (Fig. 42 and 43). All JEB cells showed a degree of epithelial separation at the BM zone when compared to ethanol-exposed control cultures. Normal cells grown on AlloDerm showed protection from SM damage but AlloDerm did not protect JEB cells, (FGAL, Delta BC, Pfu, Delta C115 and Gamma2wt) against the SM induced damage. This was confirmed by M30 staining of these tissues where we observed the induction of apoptosis due to SM compared to ethanol controls (Fig. 45). Paradoxically, FGAL and Gamma 2 WT cells showed the greater number of apoptotic cells among all different JEB cell types. ***This suggests that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to 150 μ M SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM.***

KEY RESEARCH ACCOMPLISHMENTS:

- 1 - We established dose/time responses of normal keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150 μ M) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
- 2 - We established dose/time responses of JEB keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150 μ M) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
- 3 - We have that established cells grown on the BM component Type IV Collagen demonstrated the greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that

individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures.

4 – We have found that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150 μ M in 2-D) culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300 μ M).

5 – We determined that doses similar to those found to induce SM damage in 2-D cultures of keratinocytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our *in vivo* studies (REPORT Year 2).

6 – We have found that showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 μ M.

7 - We found that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to 150 μ M SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM.

REPORTABLE OUTCOMES

PAPERS

1. Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J.A. Analysis of Microenvironmental Factors Contributing to Basement Membrane Assembly and Normalized Epidermal Phenotype, J. INVEST. DERMATOL. 120:923-931, 2003.
2. Greenberg, S., Prabhu, P., Garfield, J., Hamilton, T., Petralli, J., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004).

ABSTRACTS PRESENTED

1. Prabhu, P., Greenberg, S., Lin, N., Garfield, J., Hamilton, T., Petralli, J., and Garlick, J.A. Characterization of the Initial Response of Engineered Human Skin to Sulfur Mustard (Society for Investigative Dermatology, 2004)
2. Kamath, P., Greenberg, S., Petralli, J., Hamilton, T., Garfield, J., Pommeret, O., Meneguzzi, G., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004)

CONCLUSIONS

A major goal of our research studies was to determine the initiating site of SM induced damage that leads to vesicating injury in human skin. YEAR 1 of our research allowed us to generate optimized *in vitro* and *in vivo* human tissue models harboring basement membrane that have made many of the discoveries in this report possible. YEAR 2 of this research focused on characterization of the *in vivo* response of bioengineered human skin to prevesicating and vesicating doses of SM.

During this year of our research, we received approval for the use of an in-house facility for SM exposures. This laboratory was constructed during YEARS 1 and 2 of our research and final approval for its use was obtained one year ago. This facility greatly facilitated the progress of our work as it allowed all studies with SM to be performed in our laboratory. YEAR 3 of our research has allowed us to establish that intact basement membrane significantly reduces the vulnerability of 3D, human skin-like tissues to vesicating injury. The finding of an increased susceptibility of human skin-like tissues without structured basement membrane to SM-induced vesication indicates that this structure is a critical site for the initiation of SM injury in human skin. Studies in 2D cultures demonstrated that the presence of proteins found in the cutaneous basement membrane zone were not able to reduce the sensitivity to SM damage. In fact, quite the opposite was true, as we determined that cell viability (MTT assay) was lowered and apoptosis was increased (M30 assay) when cells were grown in 2D culture on the basement membrane component Type IV collagen and exposed to 150 μ M of SM. Since it is known that human epidermal keratinocytes have elevated growth on Type IV Collagen, it is possible that this increased proliferation was associated with the elevation of cell damage. Similarly, even in 3D cultures, the presence of basement membrane proteins that were not organized into structured basement membrane were not sufficient to protect keratinocytes from SM damage. We concluded that it was critical to have well-structured basement membrane in 3D (AlloDerm) cultures in order to prevent SM damage leading to vesication. To further establish the role of the basement membrane as a mediator of SM-induced vulnerability, we focused on the role of laminin 5. By using JEB cells restored with a variety of γ 2 chain retroviral constructs, we concluded that restoration of adhesive function mediated by laminin 5 could decrease susceptibility to SM injury. These findings directly implicate laminin 5 and its role in structured basement membrane that mediate adhesive interaction that can prevent SM-induced vesication and can directly modulate SM injury in human skin.

Chart 1: Colony forming efficiency test for diff. Doses of Ethanol.

Plate 1				Plate 2				Plate 3				Plate 4				Plate 5				Plate 6			
Obj.#	Area	Diameter (mean)		Obj.#	Area	Diameter (mean)		Obj.#	Area	Diameter (mean)		Obj.#	Area	Diameter (mean)		Obj.#	Area	Diameter (mean)		Obj.#	Area	Diameter (mean)	
1	303	19.01095		1	723	29.23695		1	368	21.00228		1	693	28.66456		2	333	19.86689		1	685	28.4904	
3	431	22.4343		2	981	35.07506		2	1452	42.55793		2	606	26.8391		3	457	23.60689		2	369	20.75996	
4	297	18.57384		3	274	17.98097		3	1369	40.98646		3	633	27.27014		4	572	25.84705		3	1981	49.6653	
5	655	27.72862		4	497	23.99		4	635	27.42119		4	898	32.7391		5	705	28.69458		4	240	16.88626	
6	719	29.17088		5	271	17.83542		5	190	14.83235		5	685	28.54366		6	1663	45.83457		5	252	17.42439	
7	981	34.67185		6	1158	38.17083		6	788	30.52805		6	299	18.61875		7	587	26.44698		6	616	27.25112	
8	966	32.08782		7	201	15.18621		7	620	26.82806		7	922	34.17847		8	1606	44.38457		7	356	20.56161	
9	100	10.25018		8	1484	42.61647		8	561	25.79769		8	666	28.09636		9	1606	44.38457		8	385	20.78962	
10	218	15.93938		9	784	31.45005		9	1358	41.23758		9	1131	38.86384		10	351	20.38016		9	949	35.24616	
11	248	17.03653		10	753	30.25857		10	354	20.27196		10	230	16.5882		11	333	19.78145		10	351	20.38016	
12	421	22.1994		11	1594	44.31307		11	354	20.27196		11	329	19.83361		12	603	26.66379		11	333	19.78145	
13	341	19.97868		12	327	19.86307		12	1117	36.77989		12	651	27.7603		13	1556	43.41113		12	603	26.66379	
14	105	11.67423		13	223	16.18283		13	773	31.61734		13	773	31.61734		14	679	28.46158		13	1556	43.41113	
15	705	28.99963		14	848	31.66589		14	778	30.46585		14	778	30.46585		15	278	18.55956		14	679	28.46158	
16	575	25.96036		15	213	14.60063		15	557	25.61567		15	245	16.73562		16	330	19.70147		15	278	18.55956	
17	257	17.30348		16	960	25.71567		16	397	21.52352		16	848	32.20823		17	385	20.99992		16	330	19.70147	
18	466	23.09092		17	885	32.77757		17	564	25.9877		17	447	23.98909		18	256	17.35729		17	385	20.99992	
19	340	20.34564		18	302	18.4119		18	1003	36.18402		18	1079	36.96948		19	653	27.54719		18	256	17.35729	
20	252	17.48212		19	1359	40.73583		19	524	24.80816		19	917	34.32692		20	1090	36.01027		19	653	27.54719	
21	749	31.67792		20	838	31.67329		20	838	31.67329		20	570	26.36689		21	1852	47.57377		20	1090	36.01027	
22	295	18.8563		21	314	19.1414		21	1027	36.47375		21	245	16.73562		22	472	23.53242		21	1852	47.57377	
23	252	17.48212		22	567	25.63724		22	263	17.29511		22	245	16.73562		23	385	20.99992		22	472	23.53242	
24	749	31.67792		23	1278	39.90689		23	1027	36.47375		23	1845	44.49541		24	330	19.70147		23	385	20.99992	
25	500	24.01897		24	230	16.0985		24	1027	36.47375		24	266	18.10759		25	496	24.02659		24	330	19.70147	
26	908	33.62228		25	847	33.12015		25	1570	43.80937		25	496	24.02659		26	1032	36.2921		25	496	24.02659	
27	131	14.35246		26	256	17.58948		26	1570	43.80937		26	483	23.76301		27	574	26.1926		26	1032	36.2921	
28	379	21.48697		27	1730	45.71595		27	1128	37.05792		27	483	23.76301		27	574	26.1926		27	574	26.1926	
29	131	14.35246		28	982	35.82237		28	1128	37.05792		28	483	23.76301		28	574	26.1926		28	574	26.1926	
30	599	26.66582		29	34	982	35.82237	29	34	982	35.82237	29	34	982	35.82237	29	34	982	35.82237	29	34	982	35.82237
31	495	24.06542		30	813	31.70016		30	1570	43.80937		30	483	23.76301		30	574	26.1926		30	574	26.1926	
32	338	19.79765		31	982	35.82237		31	1570	43.80937		31	483	23.76301		31	574	26.1926		31	574	26.1926	
33	204	15.55703		32	826	33.93631		32	1570	43.80937		32	483	23.76301		32	574	26.1926		32	574	26.1926	
34	153	12.24627		33	246	16.82804		33	1570	43.80937		33	483	23.76301		33	574	26.1926		33	574	26.1926	
35	196	14.63457		34	908	32.95882		34	1570	43.80937		34	483	23.76301		34	574	26.1926		34	574	26.1926	
36	799	31.08588		35	1714	45.46895		35	1570	43.80937		35	483	23.76301		35	574	26.1926		35	574	26.1926	
37	488	23.7671		36	985	34.64849		36	1570	43.80937		36	483	23.76301		36	574	26.1926		36	574	26.1926	
38	756	29.99797		37	915	33.03319		37	1570	43.80937		37	483	23.76301		37	574	26.1926		37	574	26.1926	
39	61	6.1		38	1123	36.98126		38	1570	43.80937		38	483	23.76301		38	574	26.1926		38	574	26.1926	
40	370	20.51874		39	343	20.56274		39	1570	43.80937		39	483	23.76301		39	574	26.1926		39	574	26.1926	
41	1285	40.55771		40	1019	35.03419		40	1570	43.80937		40	483	23.76301		40	574	26.1926		40	574	26.1926	
42	270	17.70592		41	487	23.96436		41	1570	43.80937		41	483	23.76301		41	574	26.1926		41	574	26.1926	
43	147	12.24352		42	736	29.52664		42	1570	43.80937		42	483	23.76301		42	574	26.1926		42	574	26.1926	
44	196	14.66915		43	1019	35.03419		43	1570	43.80937		43	483	23.76301		43	574	26.1926		43	574	26.1926	
45	254	16.86913		44	634	27.07093		44	1570	43.80937		44	483	23.76301		44	574	26.1926		44	574	26.1926	
46	604	26.59736		45	1322	41.54865		45	1570	43.80937		45	483	23.76301		45	574	26.1926		45	574	26.1926	
47	531	25.03112		46	1726	45.88951		46	1570	43.80937		46	483	23.76301		46	574	26.1926		46	574	26.1926	
48	658	27.99984		47	441	22.72913		47	1570	43.80937		47	483	23.76301		47	574	26.1926		47	574	26.1926	
49	281	17.53221		48	196	14.60063		48	1570	43.80937		48	483	23.76301		48	574	26.1926		48	574	26.1926	
50	232	16.66273		49	32	16		49	1570	43.80937		49	483	23.76301		49	574	26.1926		49	574	26.1926	
51	524	24.8019		50	1730	45.71595		50	1570	43.80937		50	483	23.76301		50	574	26.1926		50	574	26.1926	
52	576	25.94113		51	30	30		51	1570	43.80937		51	483	23.76301		51	574	26.1926		51	574	26.1926	
53	298	18.77829		52	1534	31.11533		52	1570	43.80937		52	483	23.76301		52	574	26.1926		52	574	26.1926	
54	229	16.27969		53	782.7273	29.47727		53	1570	43.80937		53	483	23.76301		53	574	26.1926		53	574	26.1926	
55	100	10.25018		54	447.8669	9.309393		54	1570	43.80937		54	483	23.76301		54	574	26.1926		54	574	26.1926	
56	1285	40.55771		55	34440	1297		55	1570	43.80937		55	483	23.76301		55	574	26.1926		55	574	26.1926	
57	1185	30.30754		56	44	44		56	1570	43.80937		56	483	23.76301		56	574	26.1926		56	574	26.1926	
58	434.56	21.70607		57	782.7273	29.47727		57	1570	43.80937		57	483	23.76301		57	574	26.1926		57	574	26.1926	
59	256.4732	6.597242		58	447.8669	9.309393		58	1570	43.80937		58	483	23.76301		58	574	26.1926		58	574	26.1926	
60	21728	1085.304		59	44	44		59	1570	43.80937		59	483	23.76301		59	574	26.1926		59	574	26.1926	
61	100	10.25018		60	447.8669	9.309393		60	1570	43.80937		60	483	23.76301		60	574	26.1926		60	574	26.1926	
62	1285	40.55771		61	44	44		61	1570	43.80937		61	483	23.76301		61	574	26.1926		61	574	26.1926	

Chart 1: Colony forming efficiency test for diff. Doses of Ethanol.

Plate 7				Plate 8			
Obj. #	Area	Diameter (mean)	Obj. #	Area	Diameter (i)	Obj. #	Diameter (i)
1	311	18.84619	1	981	34.25568	1	981
2	318	19.13212	2	664	27.40764	2	664
3	1861	47.4571	3	627	29.62269	3	627
4	319	19.42469	4	867	32.62684	4	867
6	338	20.03454	5	1857	47.70511	5	1857
7	2393	53.89959	6	724	29.20603	6	724
8	319	19.38202	7	1074	37.28857	7	1074
9	2921	60.42813	8	1434	41.99668	8	1434
10	1095	36.68781	9	526	24.80192	9	526
12	668	28.04055	10	671	28.0817	10	671
13	377	20.92064	11	956	34.29499	11	956
15	2657	56.46736	12	977	34.44915	12	977
18	314	19.39491	13	564	25.91216	13	564
21	1229	38.13656	14	208	15.60507	14	208
22	1235	38.62394	15	1547	44.14896	15	1547
23	470	23.73361	16	452	23.18513	16	452
24	1179	39.52578	17	378	20.97375	17	378
25	564	25.93602	19	1376	41.49363	19	1376
27	637	27.13687	20	612	26.79121	20	612
29	499	24.28621	21	284	18.60869	21	284
30	2715	57.31591	22	2120	50.5307	22	2120
31	521	24.75276	23	874	32.32846	23	874
32	808	30.94194	24	2446	54.7328	24	2446
35	320	19.49257	25	790	30.42181	25	790
36	243	16.84583	26	1243	39.00727	26	1243
38	591	27.60254	27	1324	41.81233	27	1324
39	350	20.67114	29	489	23.73582	29	489
41	404	21.85555	30	805	30.57047	30	805
43	410	21.99871	32	266	18.03897	32	266
44	278	17.76215	34	398	21.72645	34	398
45	620	27.06714	37	904	34.10687	37	904
46	3746	68.06725	38	1326	40.3833	38	1326
47	981	34.38712	39	891	33.26693	39	891
48	757	29.96356	Stats		Diameter (i)		
49	2014	49.61019	Min	208	15.60507		
50	1735	46.23339	(Obj. #)	14	14		
Stats		Area	Max	2446	54.7328		
Min	243	16.84583	(Obj. #)	24	24		
(Obj. #)	36	36	Range	2238	39.12773		
Max	3746	68.06725	Mean	928.9394	32.39751		
(Obj. #)	46	46	Std.Dev	520.7398	9.362057		
Range	3503	51.22141	Sum	30655	1069.118		
Mean	1005.472	32.00173	Samples	33	33		
Std.Dev	893.8641	13.99589					
Sum	36197	1152.062					
Samples	36	36					

Fig. 1: Colony forming efficiency test for diff. Doses of Ethanol.

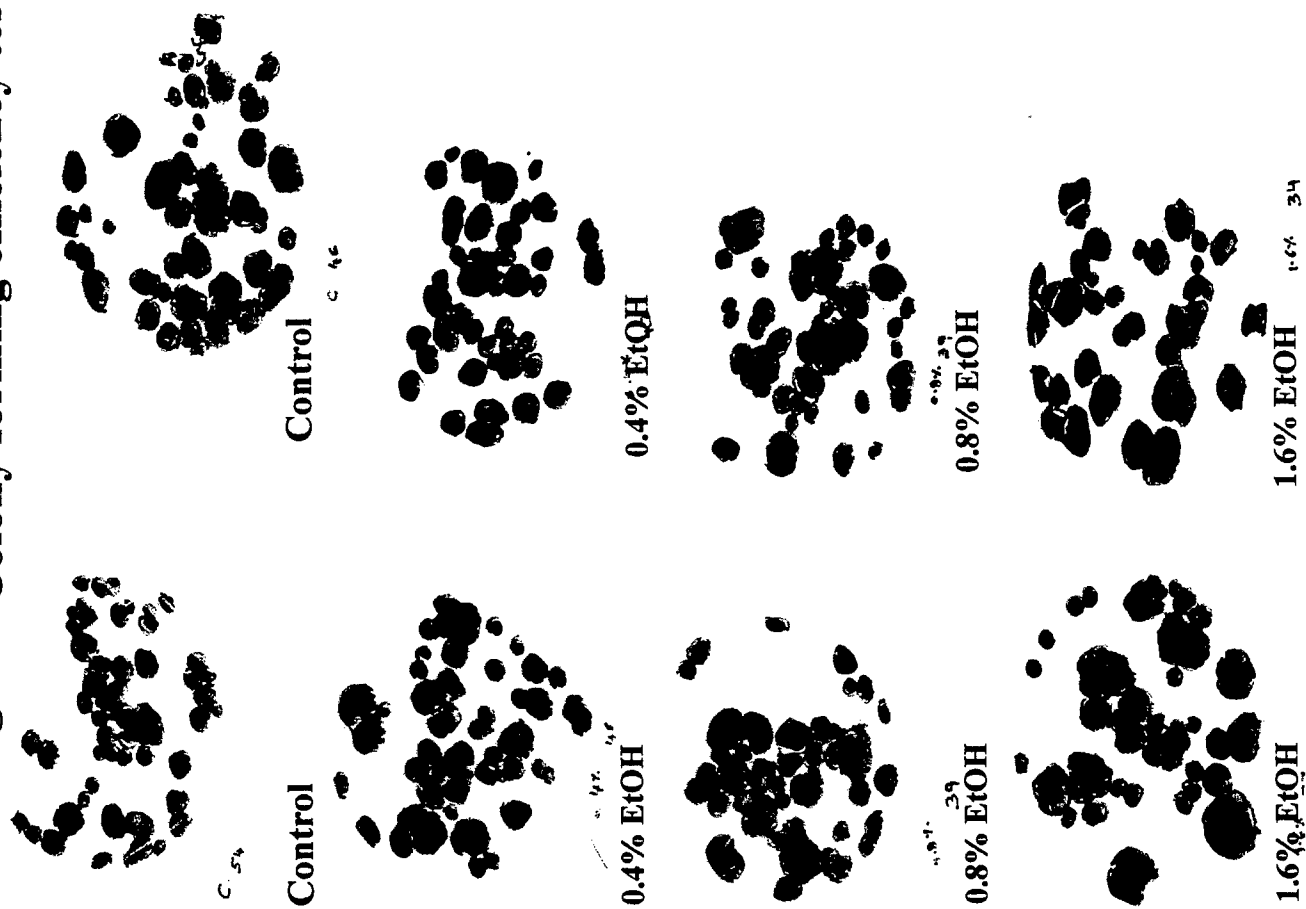


Fig. 1: Colonies shown by the Keratinocytes after the exposure to different doses of ethanol (0.4%, 0.8%, 1.6%) along with untreated control

Fig.2: Graphical representation of colony forming efficiency of keratinocytes exposed to diff.doses of ethanol.

Table:

Chemicals	Ave. no. of colonies.
Control	50
0.4%EtOH	45
0.8%EtOH	39
1.6%EtOH	35

Graph:

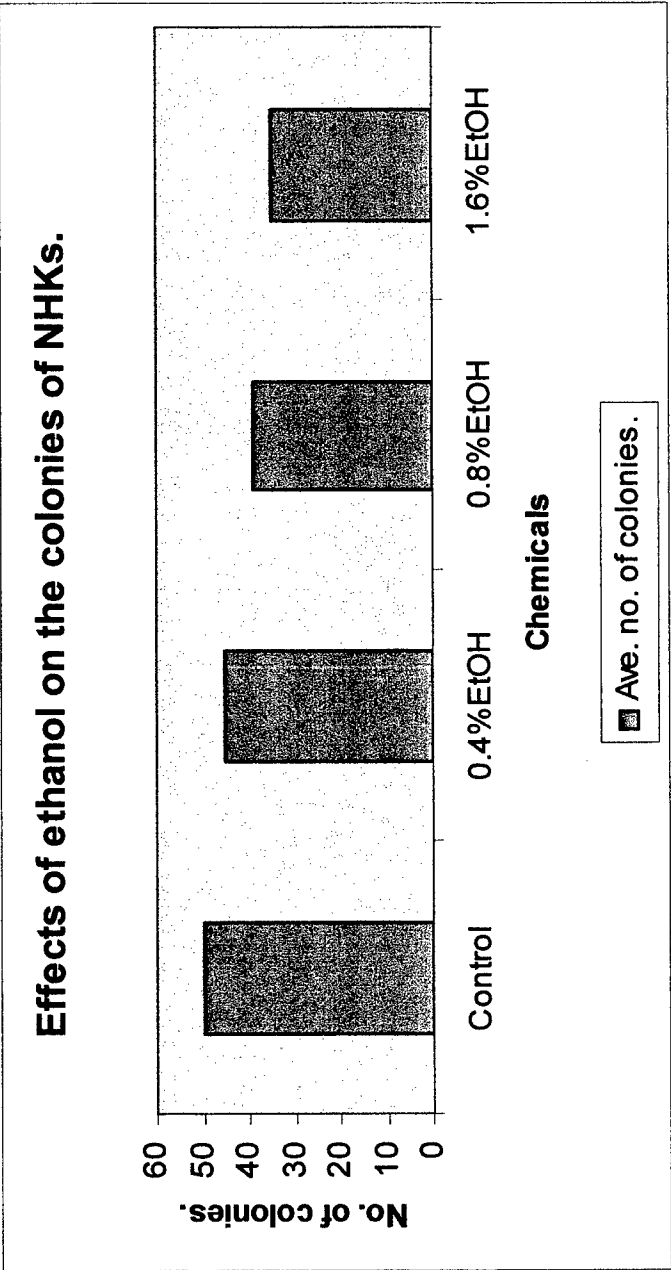
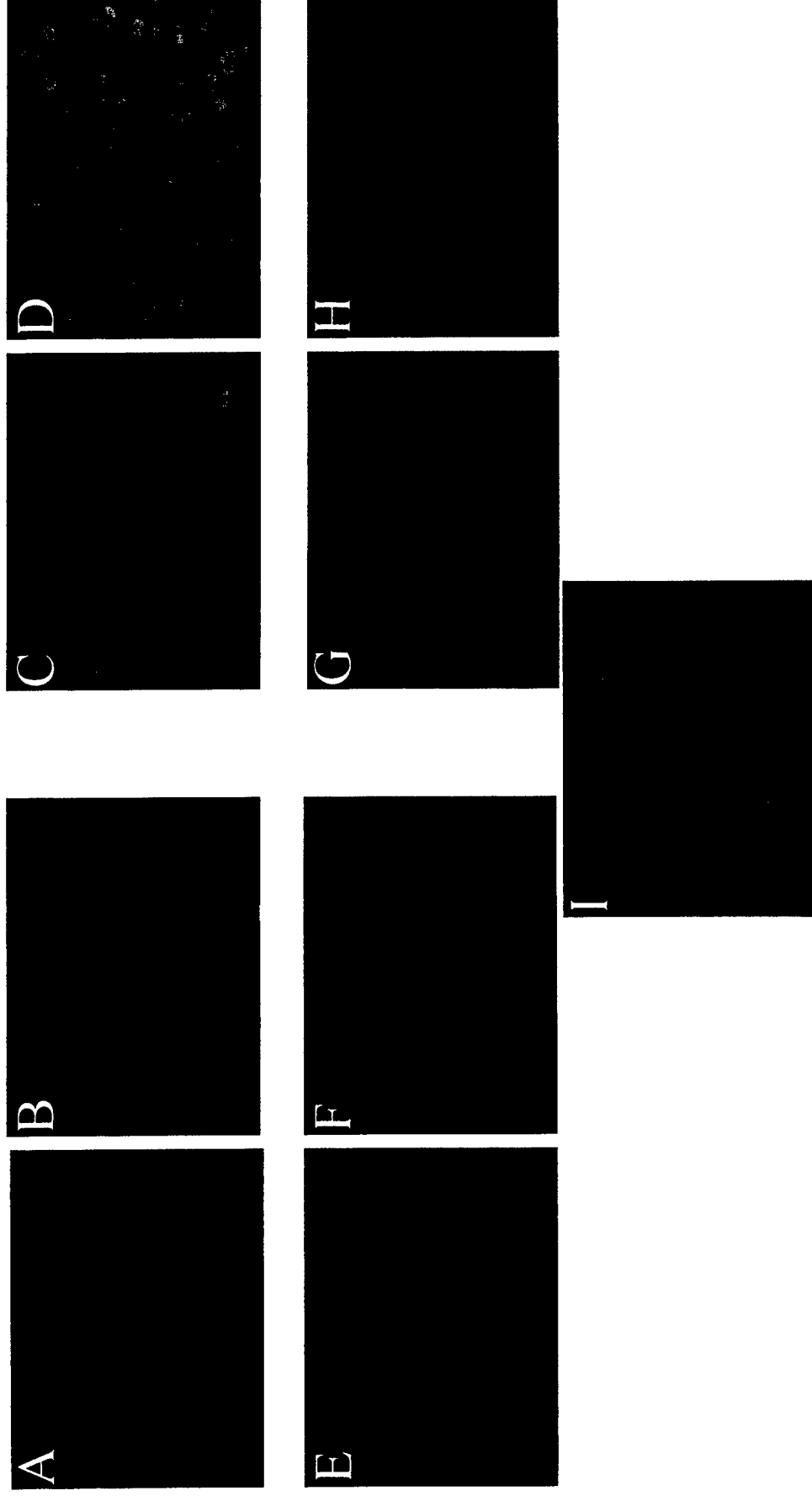


Fig. 3: Immunofluorescent staining for Apoptotic cells induced by diff. Doses of SM, Ethanol and control.

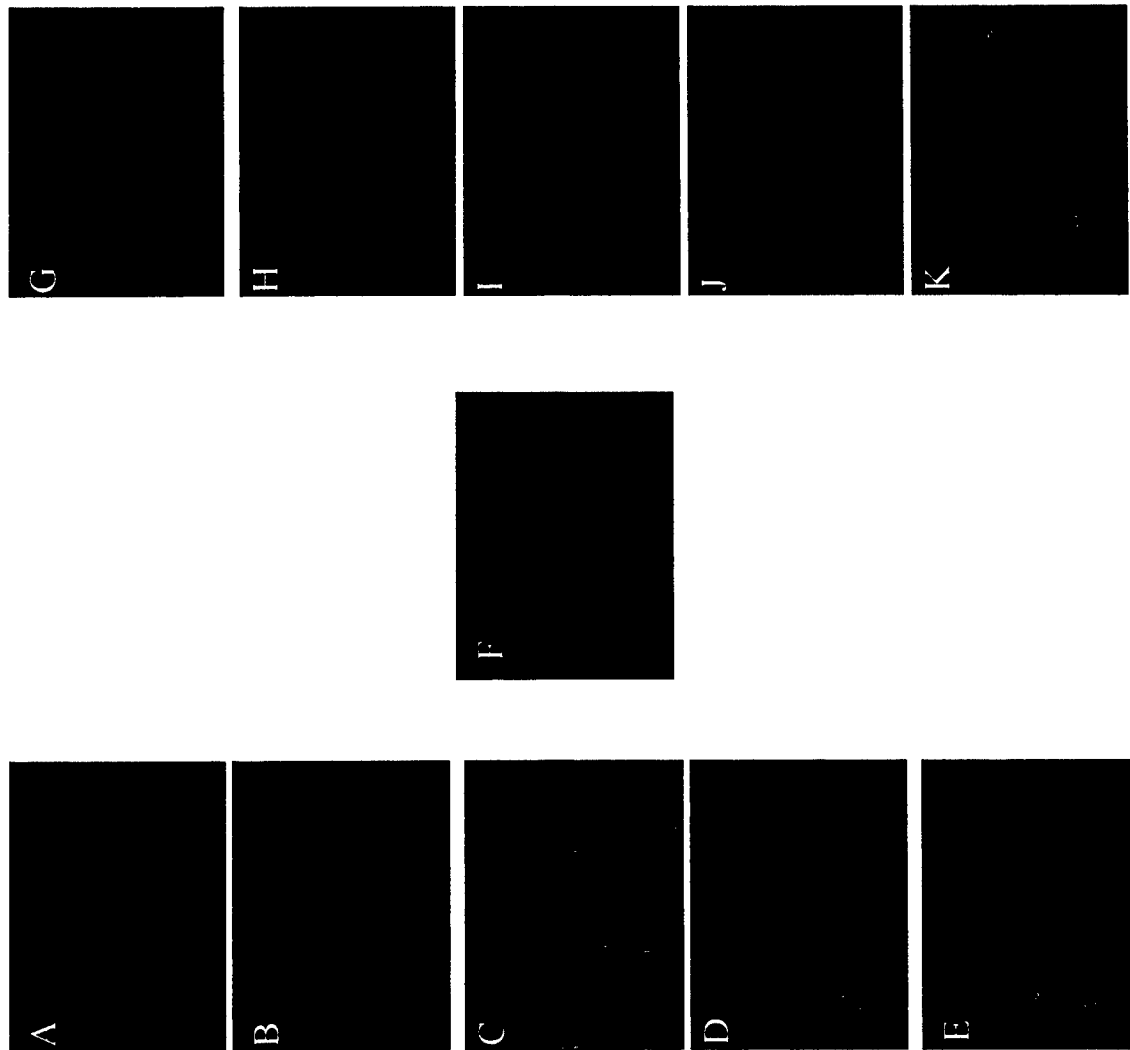


A-D -Varying doses of SM (37.5, 75, 150, 300μm)

E-H – Varying doses of concurrent Ethanol controls (0.25, 0.5, 1, 2%)

I – Untreated control.

Fig. 4: M30 Cytodeath staining for Keratinocytes exposed SM and Ethanol at diff. Sample intervals.



A-E- 1, 3, 7, 14, 28 min. of 150um of SM; G-K - 1, 3, 7, 14, 28 min of 1% Ethanol;
F- Untreated control.

Fig. 5: Apoptotic cell counts (NHK)
Table:

Chemicals	1min.	3.5min.	7min.	14min.	28min.
Control	4	4	4	4	4
SM (150 μ g)	18	69	795	4015	6783
1%EtOH	6	13	15	18	221

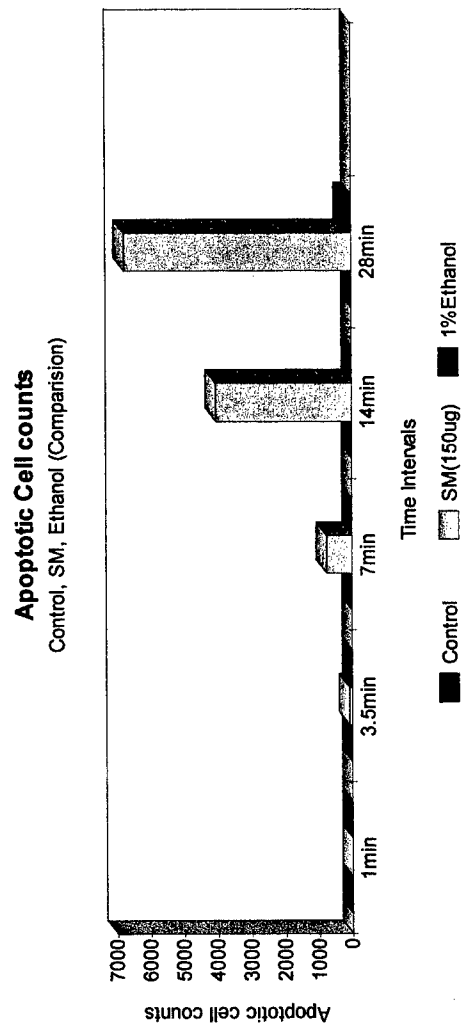
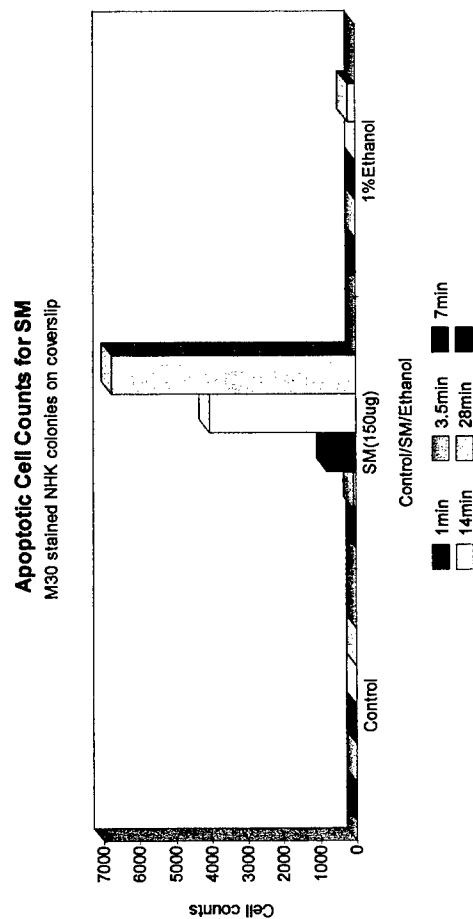


Fig. 6: M30 Cytodeath staining for 150 μ m SM and 1% Ethanol at two different intervals.

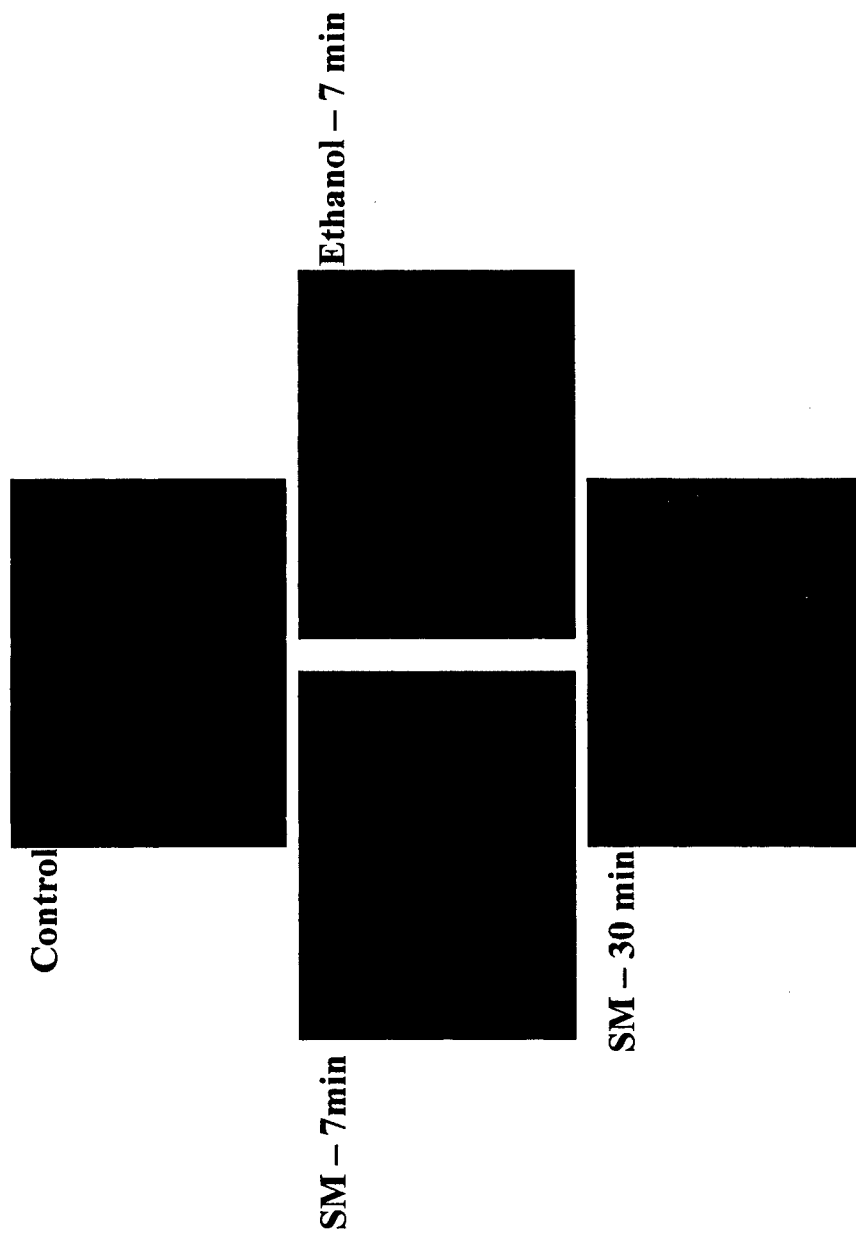


Fig.7: MTT Assay for NHK's on Plastic plates at diff. Cell density.

Plastic				
Chemical	50000	25000	10000	1000
SM	0.605	0.153	0.302	0.029
Ethanol	0.828	0.449	0.289	0.013

MTT Assay for NHKs on Plastic plate (24

SM (150µm) for 7min); 1% Ethanol for

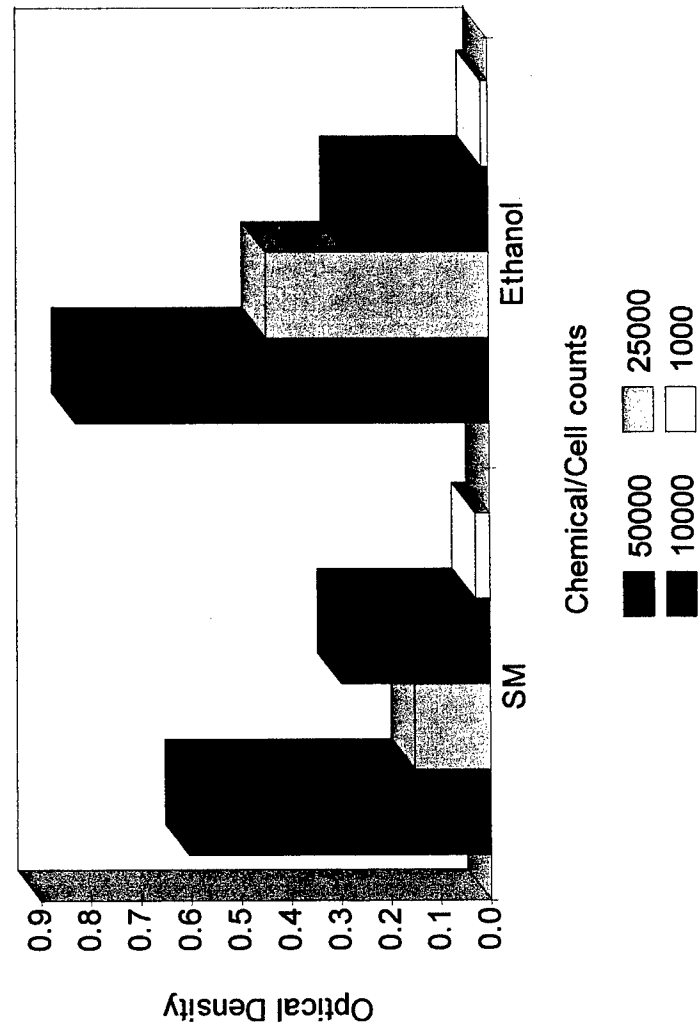


Fig. 8: MTT Assay for NHK's at varied Cell density on Type IV Collagen plates.

Chemical	50000	25000	10000	1000
SM	0.564	0.133	0.332	0.047
Ethanol	0.807	0.287	0.307	0.0413

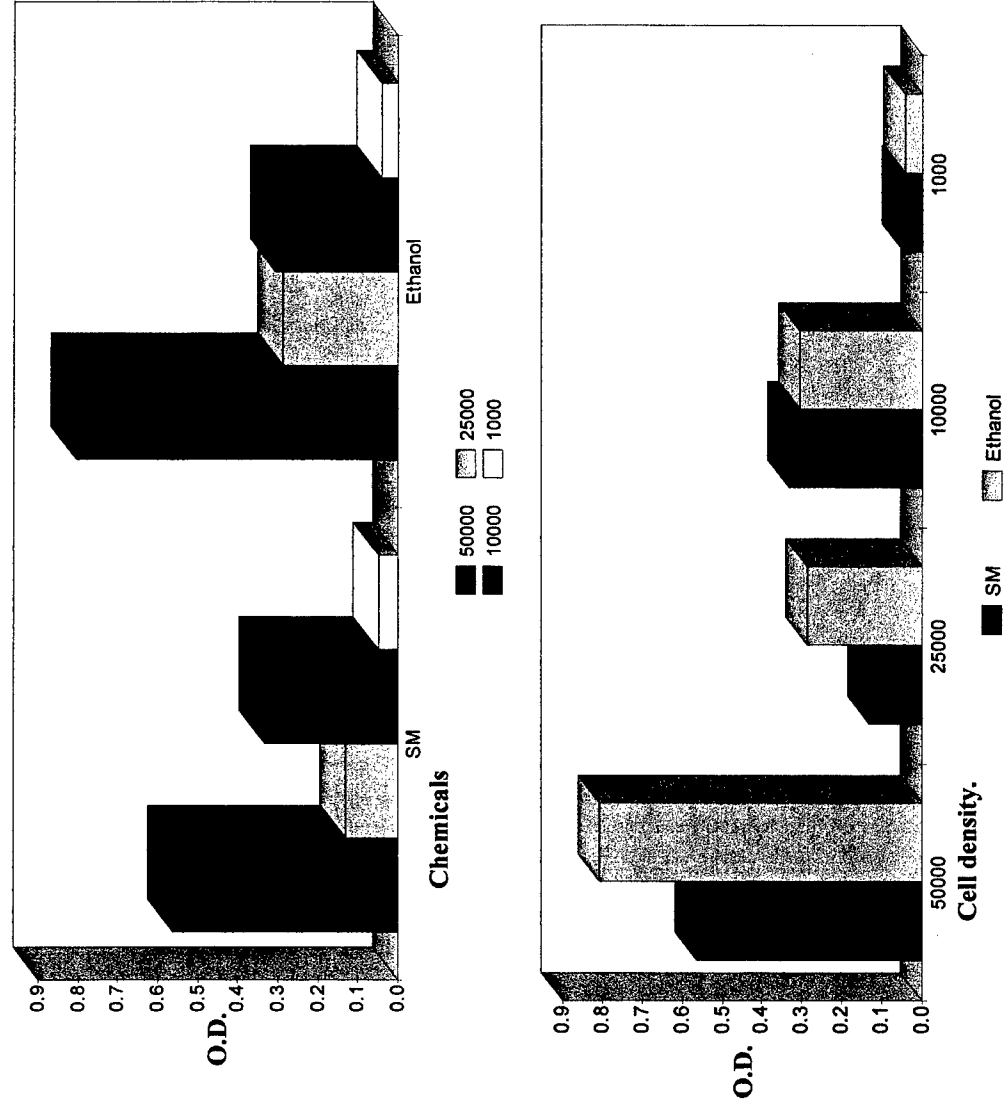


Fig. 9: MTT Assay at two different cell densities of NHK's on Col I

Chemica	50000	25000
SM	0.491	0.272
Ethanol	0.657	0.369

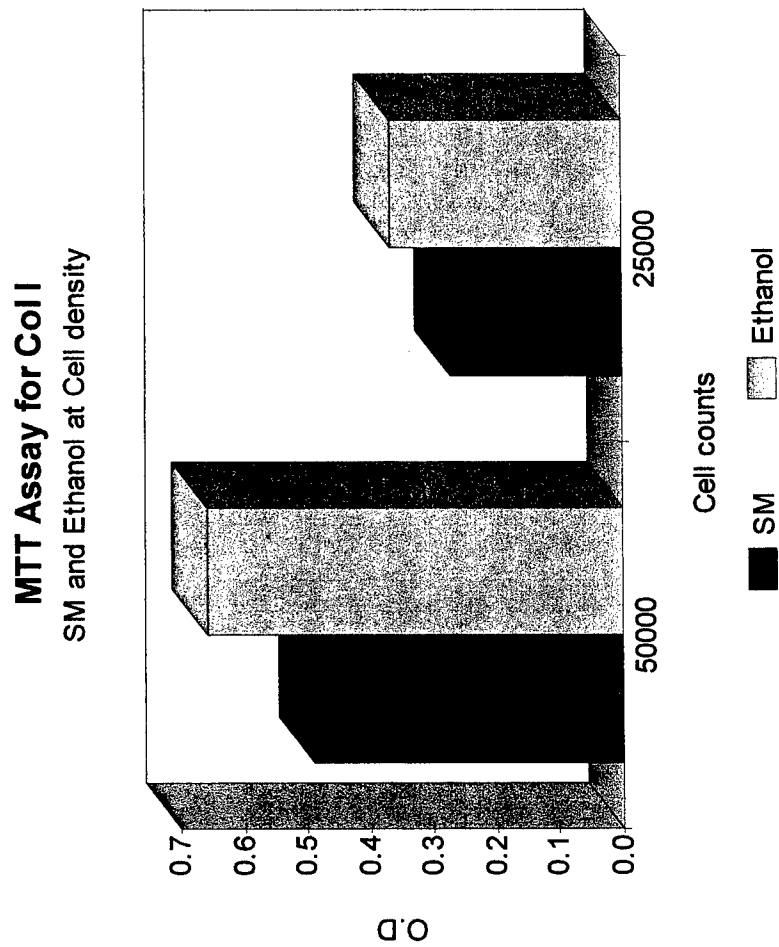


Fig.10: MTT Assay for 50000NHKs on diff. Plates.

Table:

	Plastic	Col I	col IV	FBN	Laminin	Poly-D
SM	0.27	0.261	0.226	0.165	0.189	0.096
Ethanol	0.317	0.349	0.436	0.301	0.285	0.107

Graph:

MTT assay for NHKs on different plates
Comparisons between SM and Ethanol

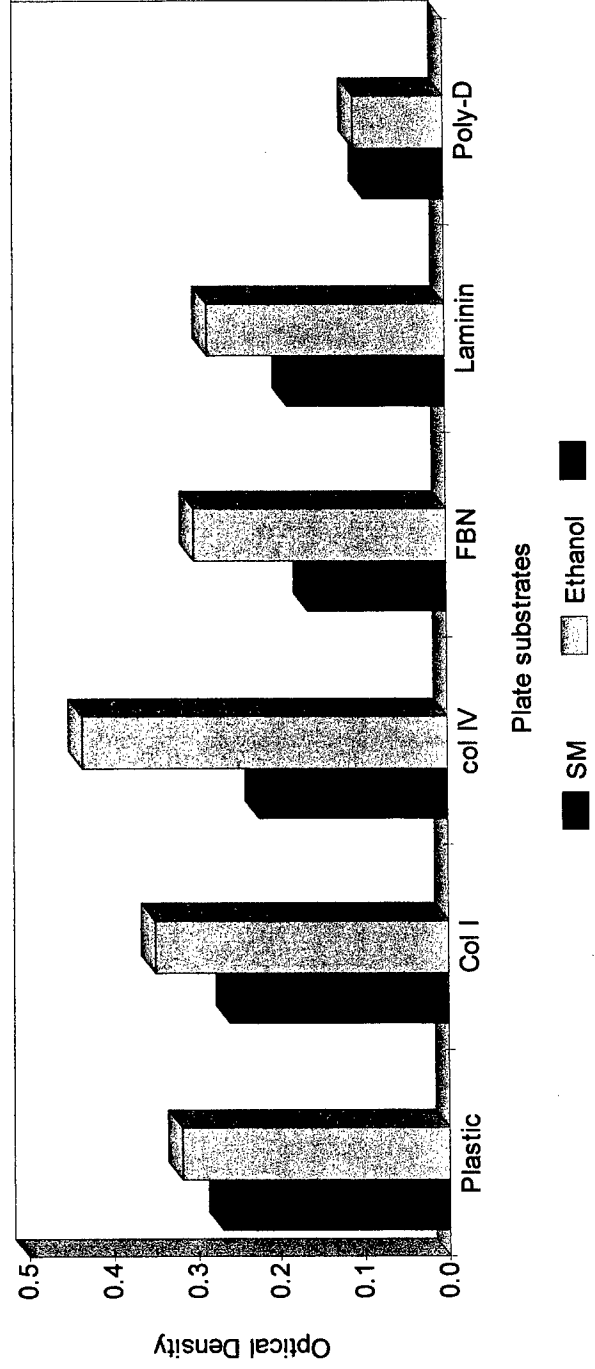


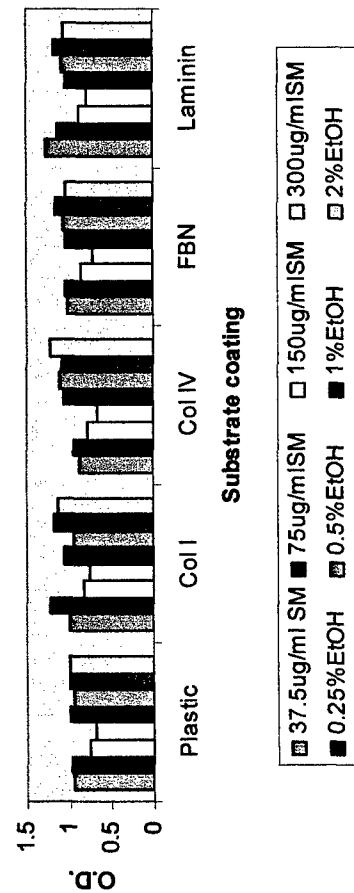
Fig. 11: MTT Assay for different doses of SM and ethanol on diff. substrates.

Table:

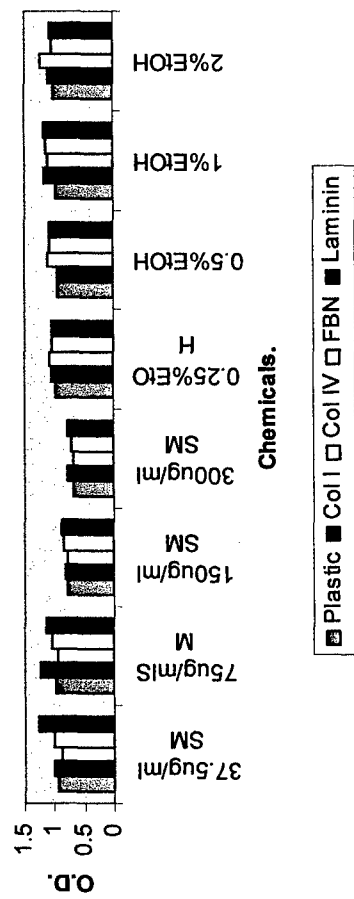
Doses/coating	Plastic	Col I	Col IV	FBN	Laminin
37.5ug/ml of SM	0.942	1.002	0.886	1.005	1.277
75ug/ml of SM	0.98	1.225	0.95	1.032	1.133
150ug/ml of SM	0.768	0.822	0.795	0.85	0.873
300ug/ml of SM	0.701	0.769	0.679	0.71	0.786
0.25% EtOH	0.991	1.059	1.067	1.038	1.044
0.5% EtOH	0.957	0.951	1.103	1.06	1.083
1% EtOH	0.992	1.178	1.096	1.155	1.173
2% EtOH	0.996	1.123	1.228	1.048	1.068

Graphs:

MTT assay for NHK's on different substrates for diff. Doses of SM and Ethanol.



MTT assay for different doses of SM and Ethanol on diff. substrates.



• •

• •

Table:

Gamma2wt. Delta C 115 Fgal. NHK

Graphs:

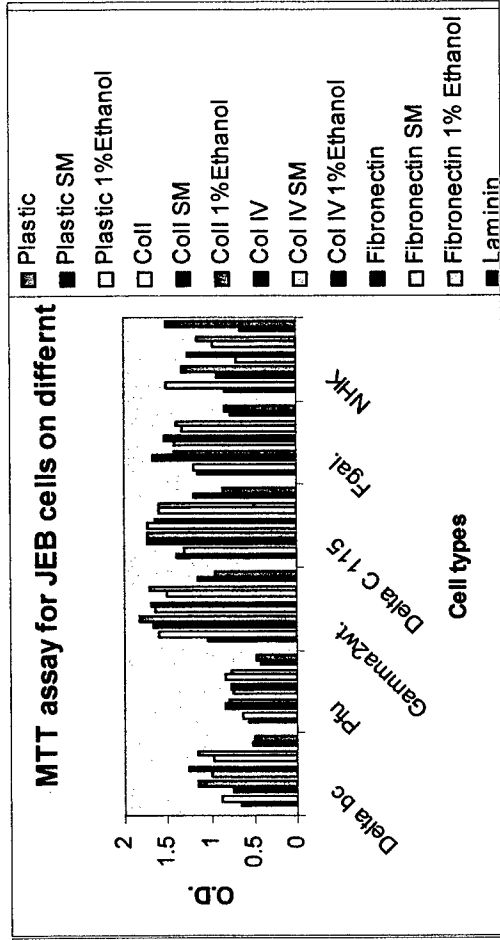
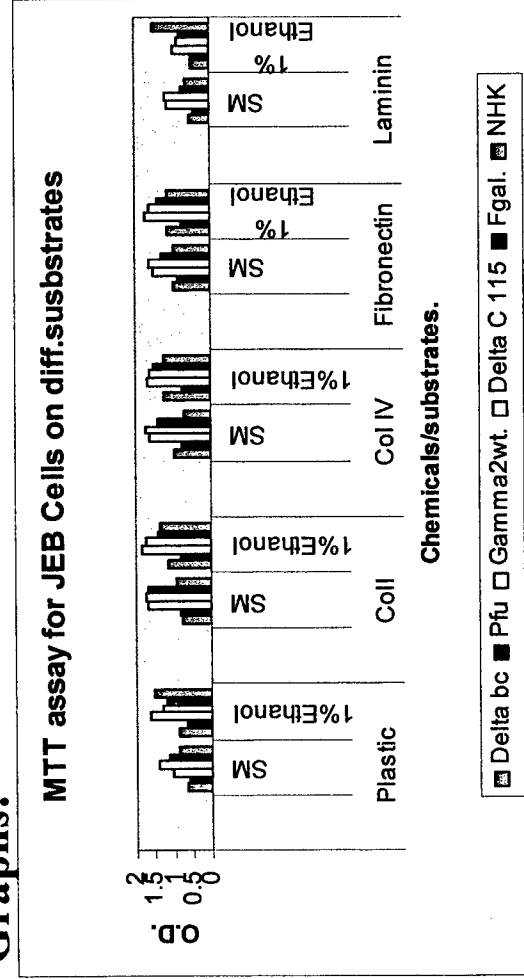


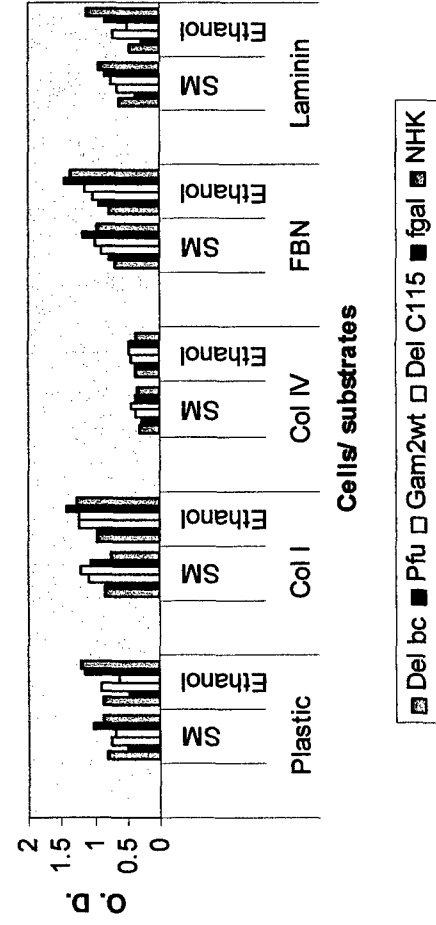
Fig. 13: MTT Assay for diff. Cell types on diff. Substrates.(300 μ m SM & 2% Ethanol.

Table: Plastic

	Del bc	Pfu	Gam2wt	Del C115	fgal	NHK
SM	0.804	0.489	0.744	0.682	1.008	0.875
Ethanol	0.875	0.447	0.885	0.63	1.151	1.187
Col I						
SM	0.838	0.82	1.064	1.196	1.034	0.751
Ethanol	0.956	0.968	1.219	1.224	1.429	1.263
Col IV						
SM	0.316	0.291	0.373	0.441	0.369	0.328
Ethanol	0.356	0.373	0.419	0.469	0.465	0.369
FBN						
SM	0.677	0.78	0.885	0.971	1.162	0.944
Ethanol	0.78	0.924	1.03	1.138	1.433	1.347
Laminin						
SM	0.606	0.359	0.658	0.738	0.823	0.935
Ethanol	0.474	0.285	0.697	0.479	0.831	1.114

Graph:

MTT assay for JEB cells on diff. substrates



MTT assay for JEB Cells on diff. substr

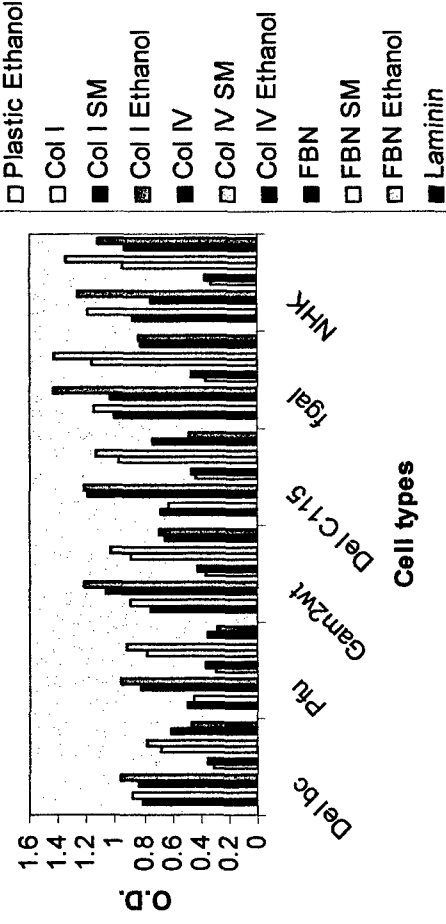


Fig. 14: MTT assay for 75 μ m SM on diff. Substrates.

Table:

Substrates	Chemicals	Delta bc	Pfu	Gam. Wt	Delta C115	Fgal	NHK
Col I	SM(75 μ m)	1.433	1.219	2.021	1.376	1.421	1.265
	0.5% EtOH	1.549	1.216	2.064	1.474	1.664	1.527
Col IV	SM(75 μ m)	1.372	0.992	1.851	1.414	1.459	1.392
	0.5%EtOH	1.33	1.039	1.99	1.318	1.448	1.512
FBN	SM(75 μ m)	0.979	0.828	1.606	1.089	1.257	0.968
	0.5%EtOH	1.201	0.997	1.898	1.184	1.306	1.193

Graph:

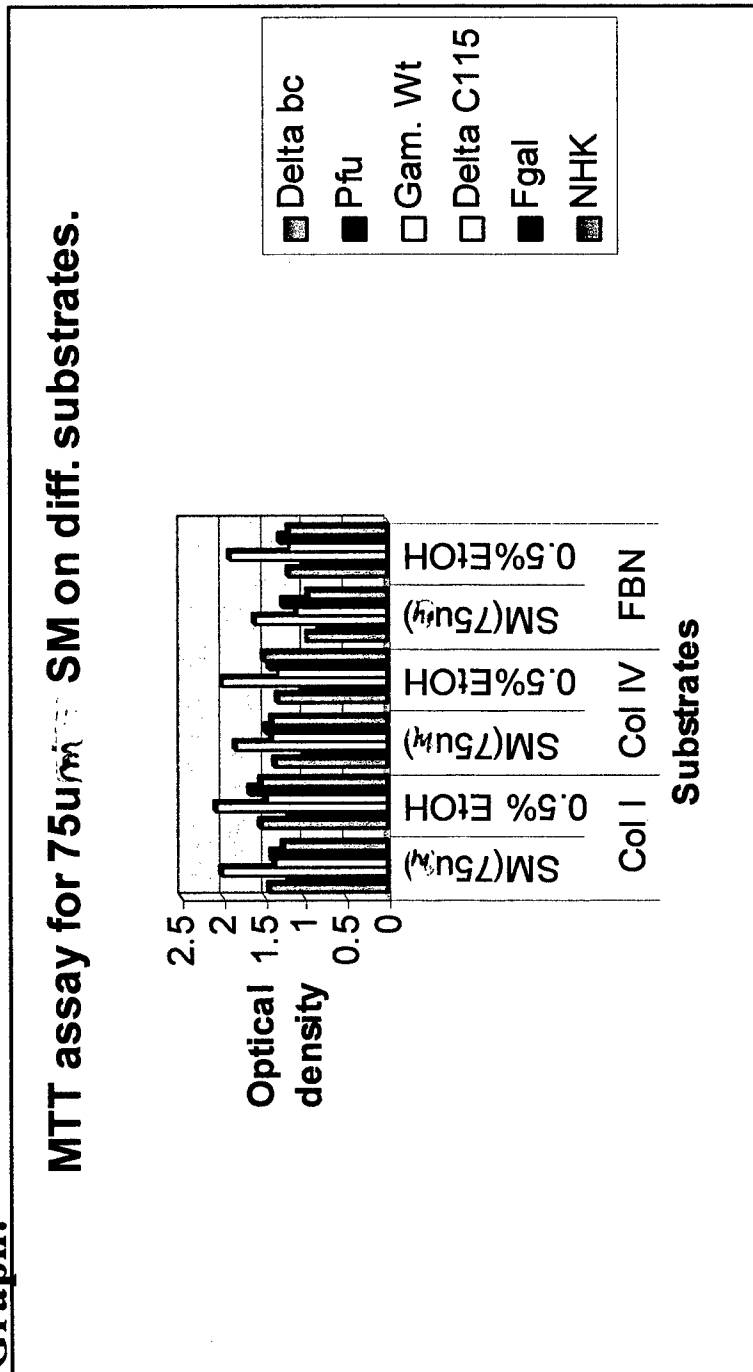


Fig. 15: MTT assay for 150 μ m SM on diff. Substrates.

Table:

Substrates	Chem.	Delta bc	Pfu	Gam. Wt	Delta C115	Fgal	NHK
Coll	SM (150 μ m)	1.192	0.954	1.633	1.349	1.115	1.116
	1% EtOH	1.288	1.156	2.095	1.315	1.455	1.309
Col IV	SM (150 μ m)	1.126	1.094	1.59	1.134	1.258	1.446
	1%EtOH	1.145	0.98	1.836	1.238	1.264	1.568
FBN	SM (150 μ m)	0.907	0.739	1.627	1.158	1.068	1.03
	1% EtOH	1.169	0.985	1.816	1.074	1.041	1.189

Graph:

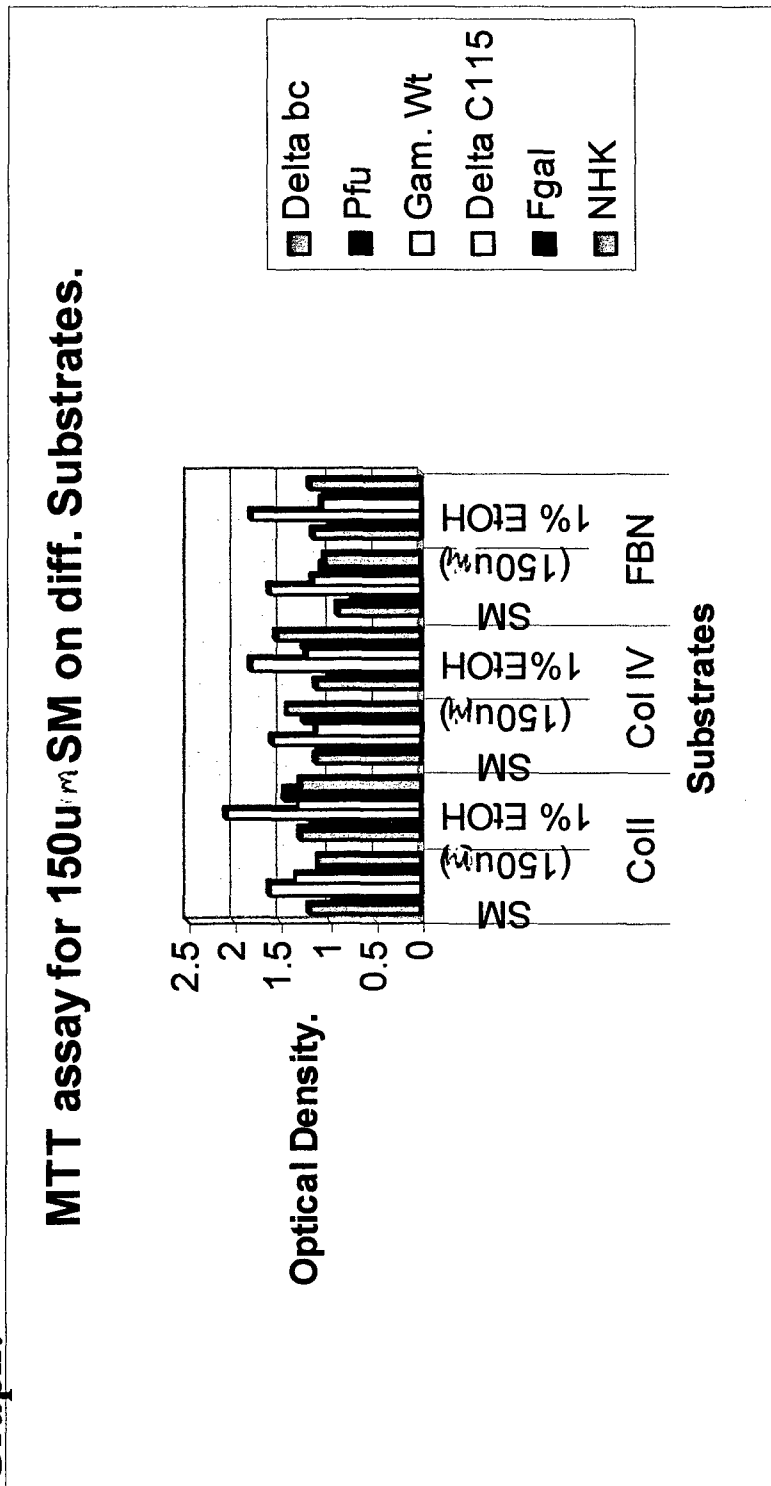


Fig. 16: MTT Assay for 300 μ m SM on diff. Substrates.

Table:

Sustrates	Chemicals	Delta BC	Pfu	Gamm Wt	Delta C115	Fgal	NHK
Coll	SM (300 μ m)	2.115	1.943	1.666	1.038	1.594	1.122
	2% EtOH	2.277	0.828	2.178	1.514	1.433	1.637
Col IV	SM (300 μ m)	0.998	0.933	1.833	1.317	1.141	1.03
	2%EtOH	1.478	1.112	2.002	1.273	1.295	1.326
FBN	SM (300 μ m)	0.876	0.594	1.538	0.892	1.007	0.846
	2% EtOH	1.103	0.912	1.64	1.003	1.176	1.341

Graph:

MTT assay for 300 μ m SM on diff. substrates.

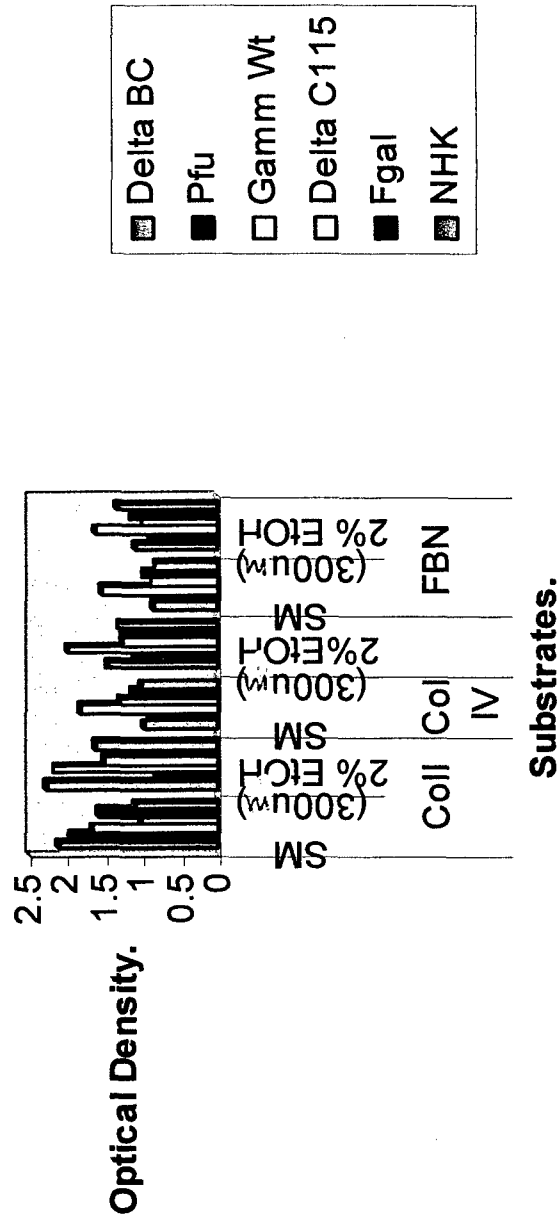


Fig. 17: MTT Assay for Delta bc Cells on Col I Plate.

Table:

Chemicals	O.D
750 μ M SM	1.745
0.5% EtOH	1.491
150 μ M SM	1.433
1% EtOH	1.592
300 μ M SM	1.119
2% EtOH	1.978
450 μ M SM	1.01
3% EtOH	1.28
600 μ M SM	0.82
4% EtOH	1.352
750 μ M SM	0.801
5% EtOH	1.399
900 μ M SM	0.767
6% EtOH	1.394
1200 μ M SM	0.593
8% EtOH	1.428

Graph:

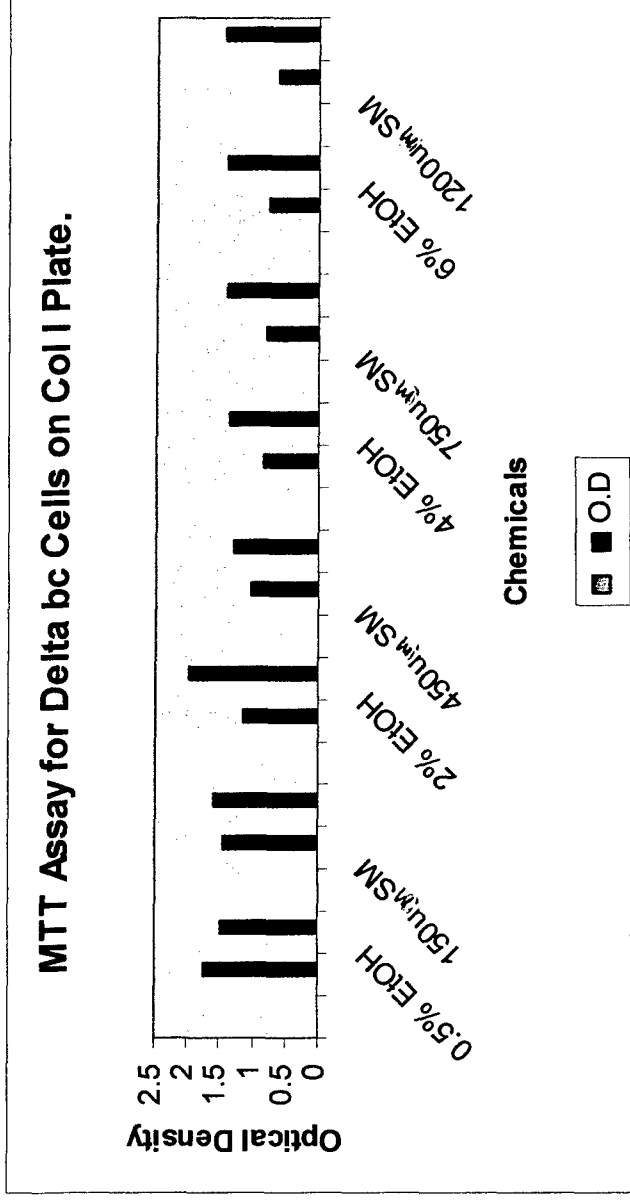


Fig. 18: MTT Assay for Delta bc Cells on Col IV Plate.

Table:

Chemicals	O.D
75u _M SM	1.292
0.5% EtOH	1.628
150u _M SM	1.32
1% EtOH	1.539
300u _M SM	1.103
2% EtOH	1.168
450u _M SM	1.112
3% EtOH	1.124
600u _M SM	0.909
4% EtOH	1.234
750u _M SM	0.729
5% EtOH	1.193
900u _M SM	0.743
6% EtOH	1.267
1200u _M SM	0.701
8% EtOH	1.01

Graph:

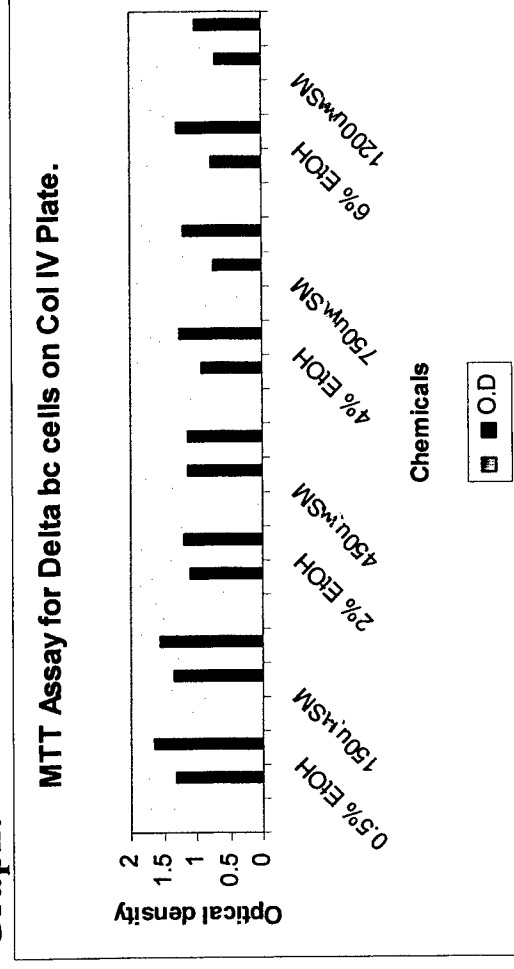


Fig 19: MTT Assay for Fgal Cells on Col I Plate.

Table: Chemicals	O.D
75u _m SM	1.49
0.5% EtOH	1.427
150u _m SM	1.317
1% EtOH	1.521
300u _m SM	1.202
2% EtOH	1.164
450u _m SM	0.979
3% EtOH	1.472
600u _m SM	1.078
4% EtOH	1.334
750u _m SM	0.884
5% EtOH	1.228
900u _m SM	1.003
6% EtOH	1.284
1200u _m SM	0.82
8% EtOH	1.436

Graph:

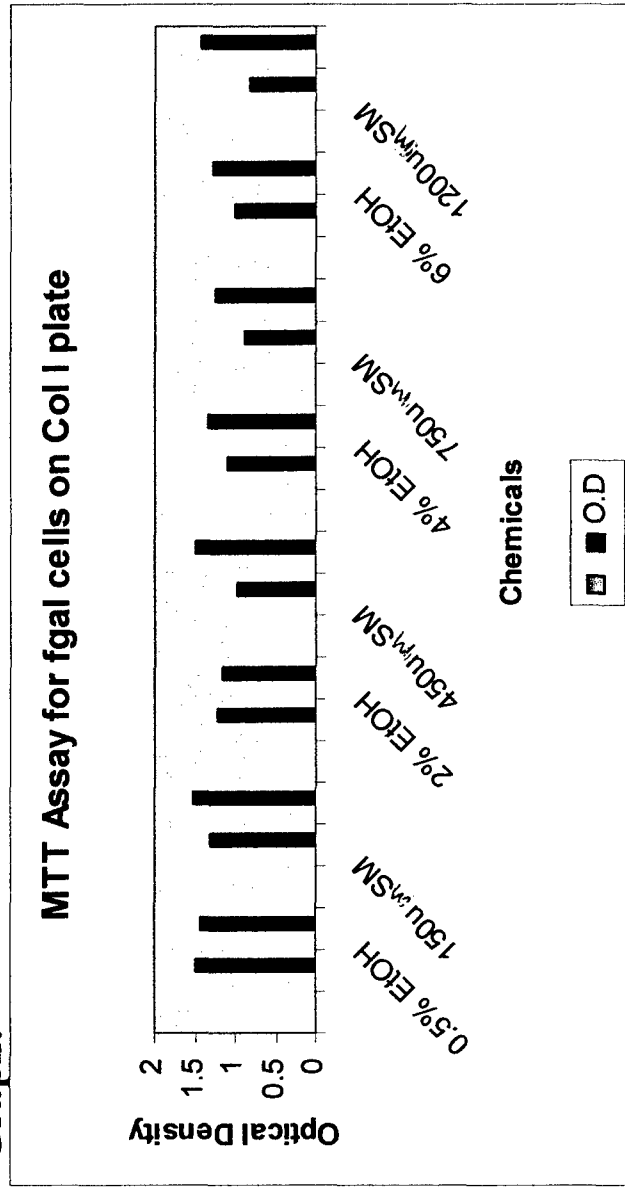


Fig. 20: MTT Assay for Fgal cells on Col IV Plate.

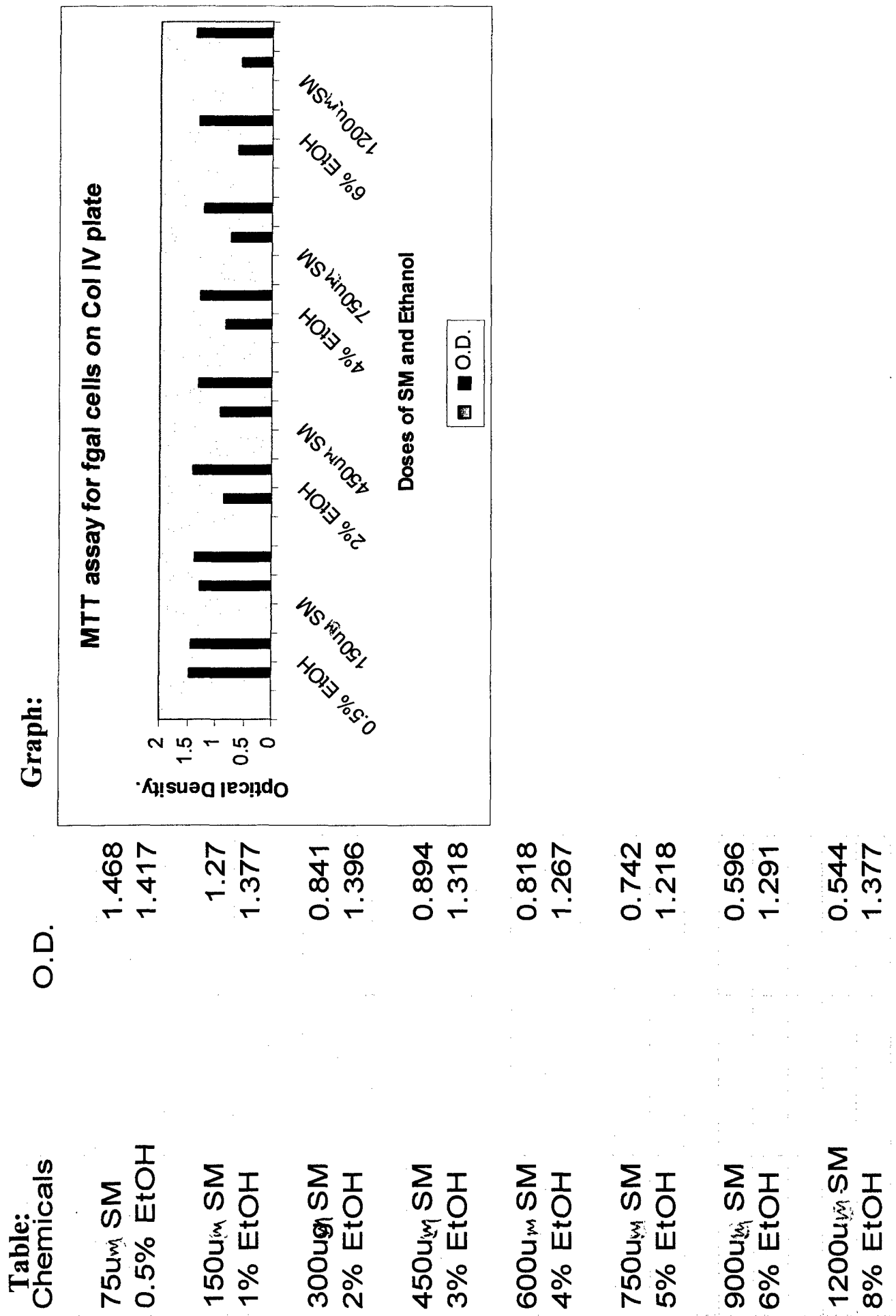


Fig. 21: H & E Staining for Rafts exposed to SM and Ethanol.

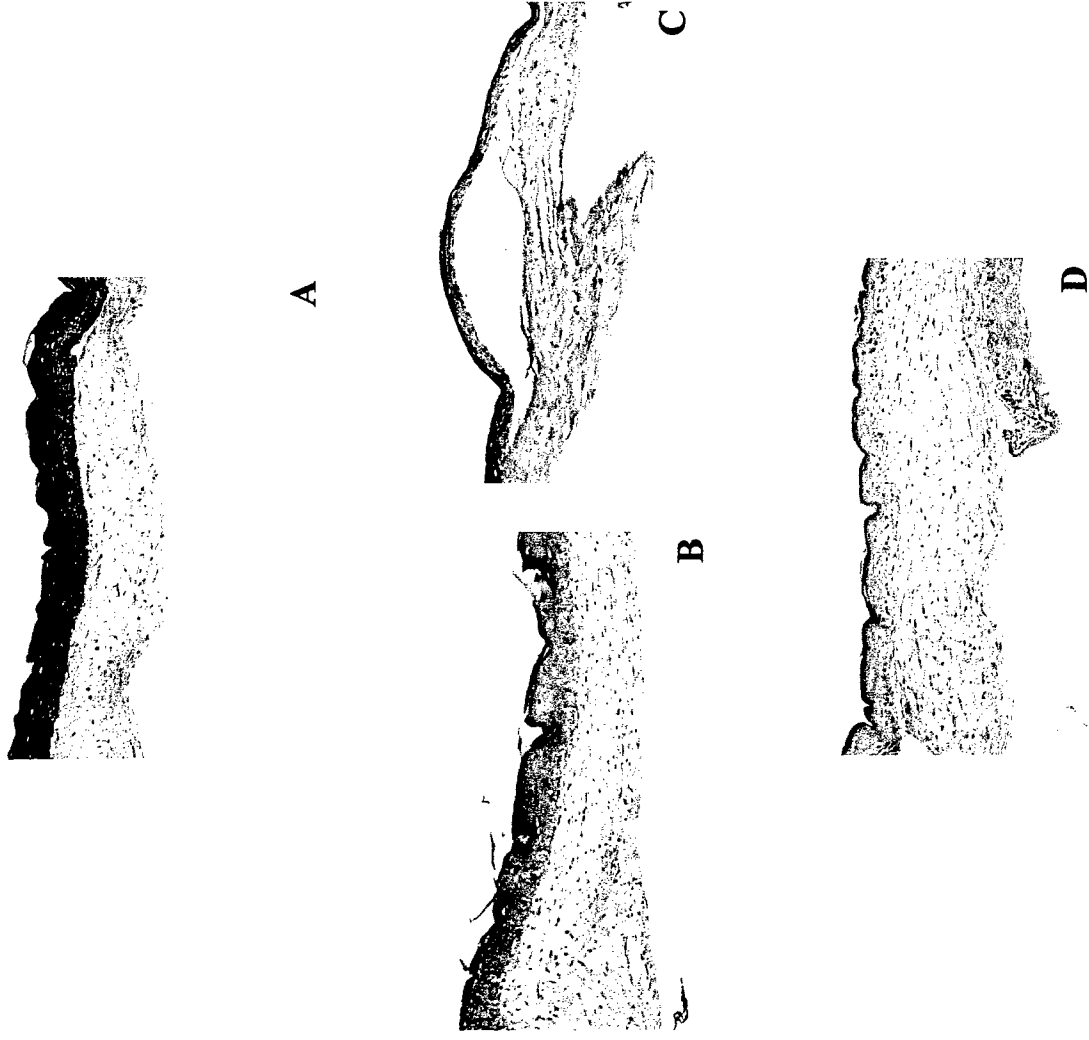


Fig: A- Control; B & C – 75 & 150μm SM; D- 1% Ethanol.

Fig. 22: H&E Staining for Rafts exposed to diff. Doses of SM along with Controls



Fig: A - Control; B & C - 75 μ m SM; D - 1% Ethanol.

Fig. 23: M30 Staining for Rafts exposed to SM and Ethanol.

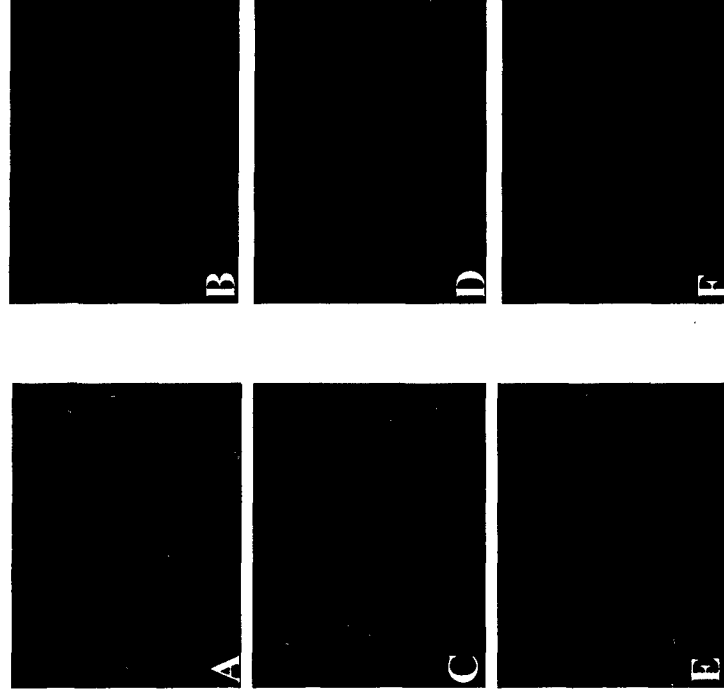


Fig: A& B - Raft exposed to 1% Ethanol; C&D - Raft exposed to 75um SM; E& F - Raft exposed to 150um SM.

Fig. 24: H&E Staining for diff. doses of SM and Ethanol controls.

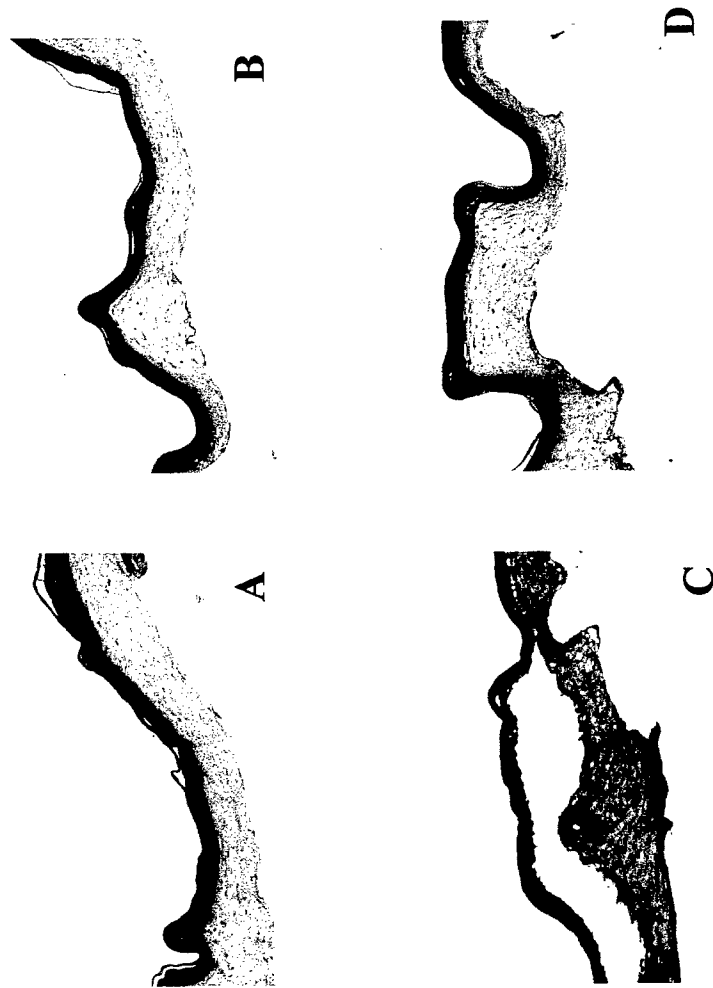


Fig : A & C – 150 & 300 μ m of Sulfur Mustard.

B & D – 1 & 2 % Concurrent Ethanol controls.

Fig. 25: H & E Staining for diff. Doses of SM and Ethanol.



Fig: A & C – 150 and 300 μ m of SM;

B & C – 1 and 2 % Ethanol controls.

**Fig. 26: M30 Staining for the rafts exposed to diff .
doses of SM and Ethanol**

SM— 150um/7min.



1% Ethanol/7min.



SM—300um/7min.



2% Ethanol/ 7min.



Fig. 27: H&E Staining for NHK's on different substrates (3-D culture)

SM (150um)

1 % Ethanol



A



D



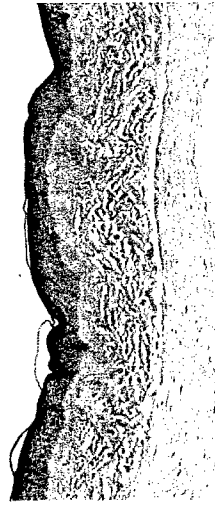
B



E



C



F

Fig: A&D- Plastic; B&E- Raft; C&F- Alloderm.

Fig. 28: H & E Staining for NHK's on diff. substrates.

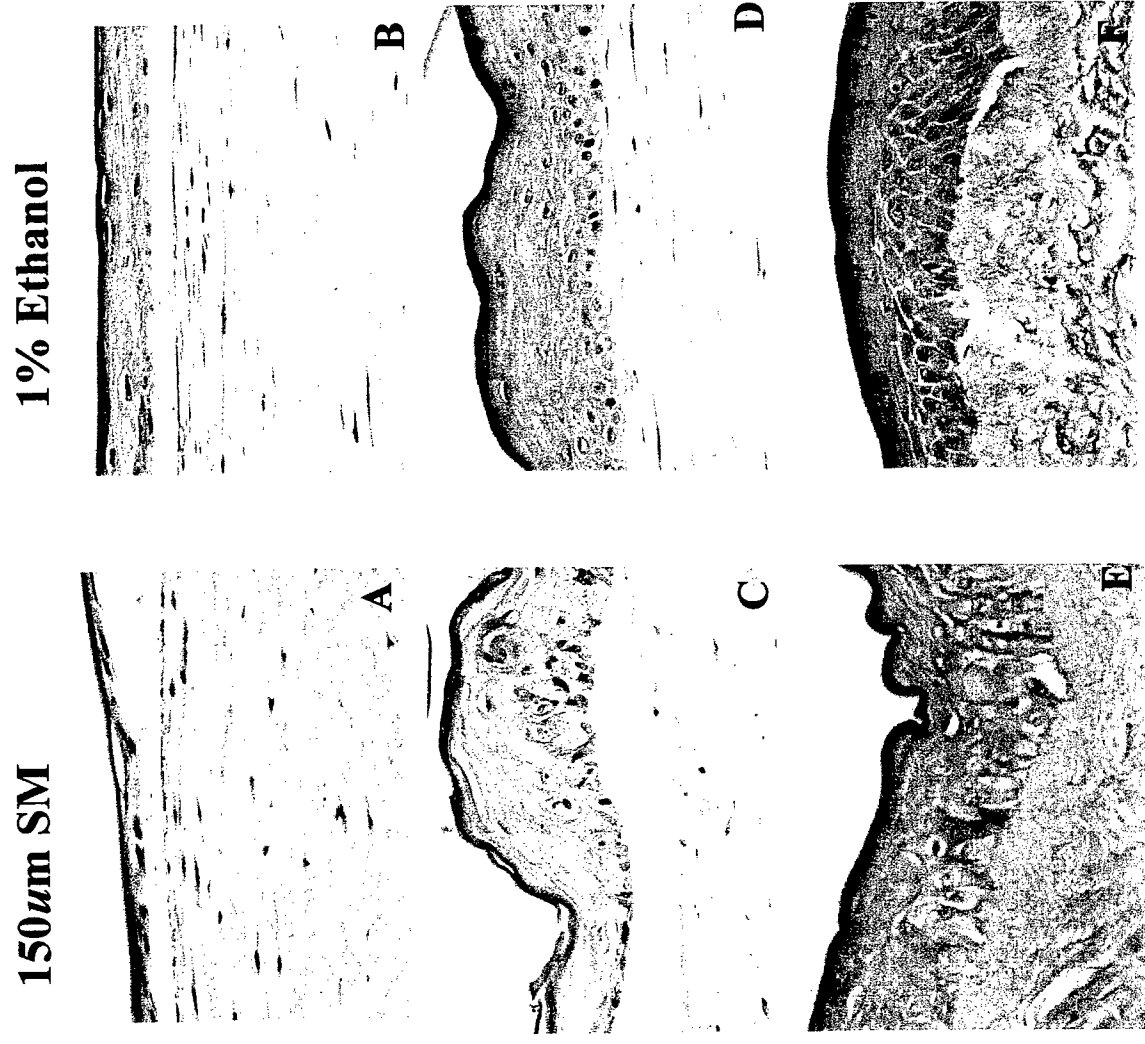


Fig: A&B – Plastic; C&D – Raft; E&F – Alloderm.

Fig. 29: H & E Staining for NHK's on diff. Substrates (3- D cultures)

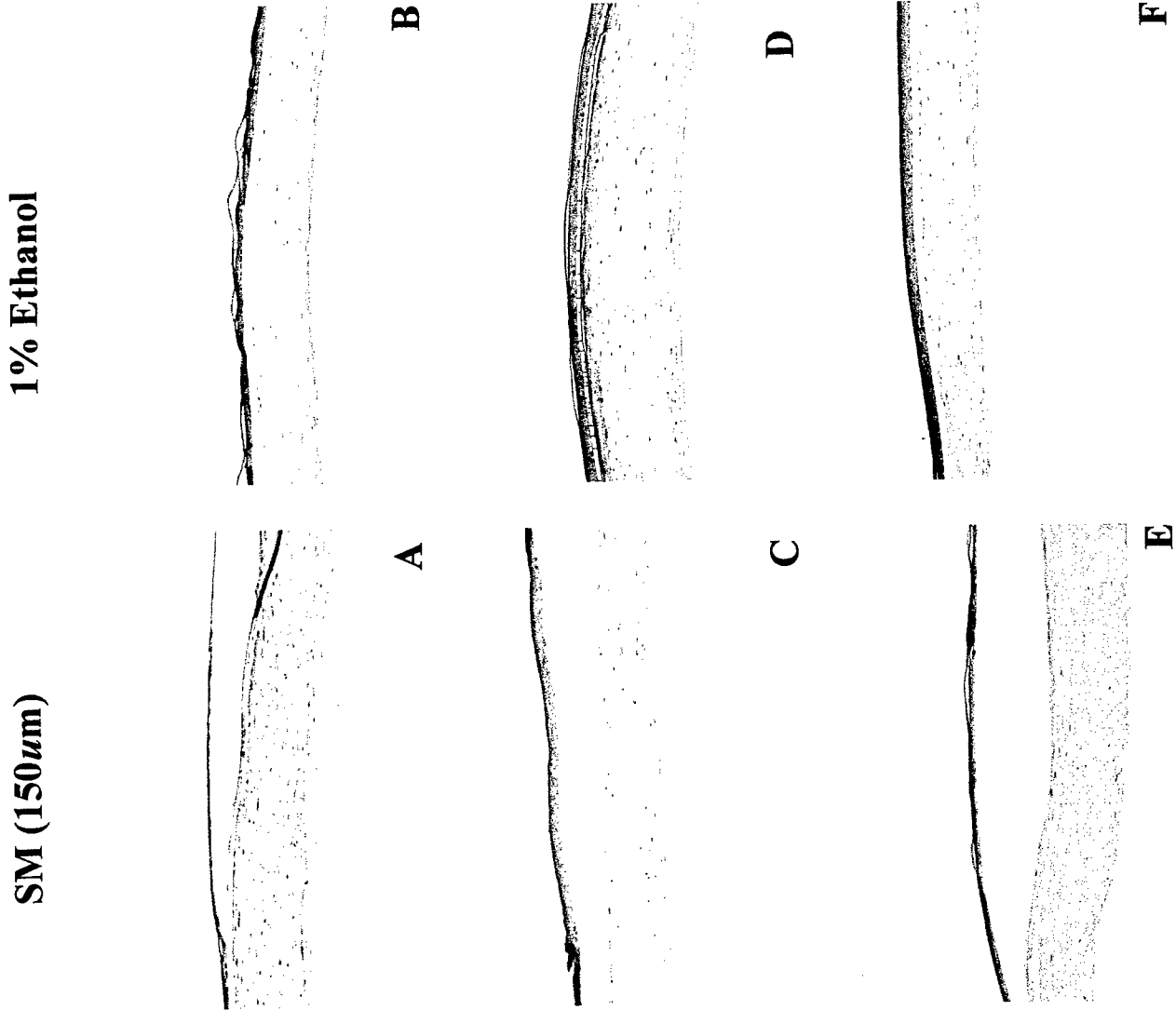


Fig: A&B- Col I; C&D- Col IV; E& F- Fibronectin.

Fig. 30; Effects of SM and Ethanol on NHK's on diff. Substrates (3-D)

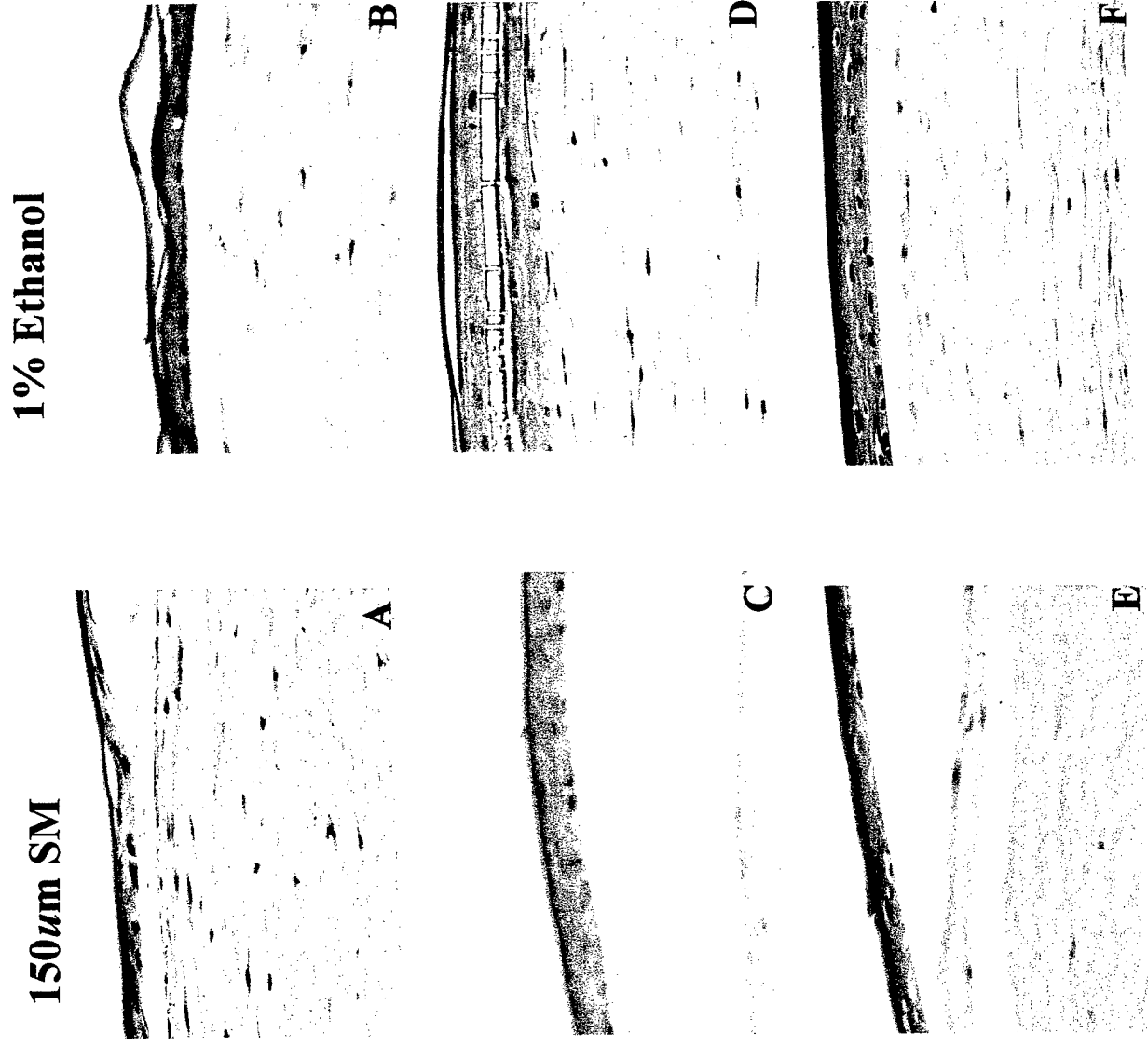


Fig: A&B – Col I; C&D – Col IV; E&F – Fibronectin.

Fig. 31: Effects of SM on Alloderm and Raft along with controls.

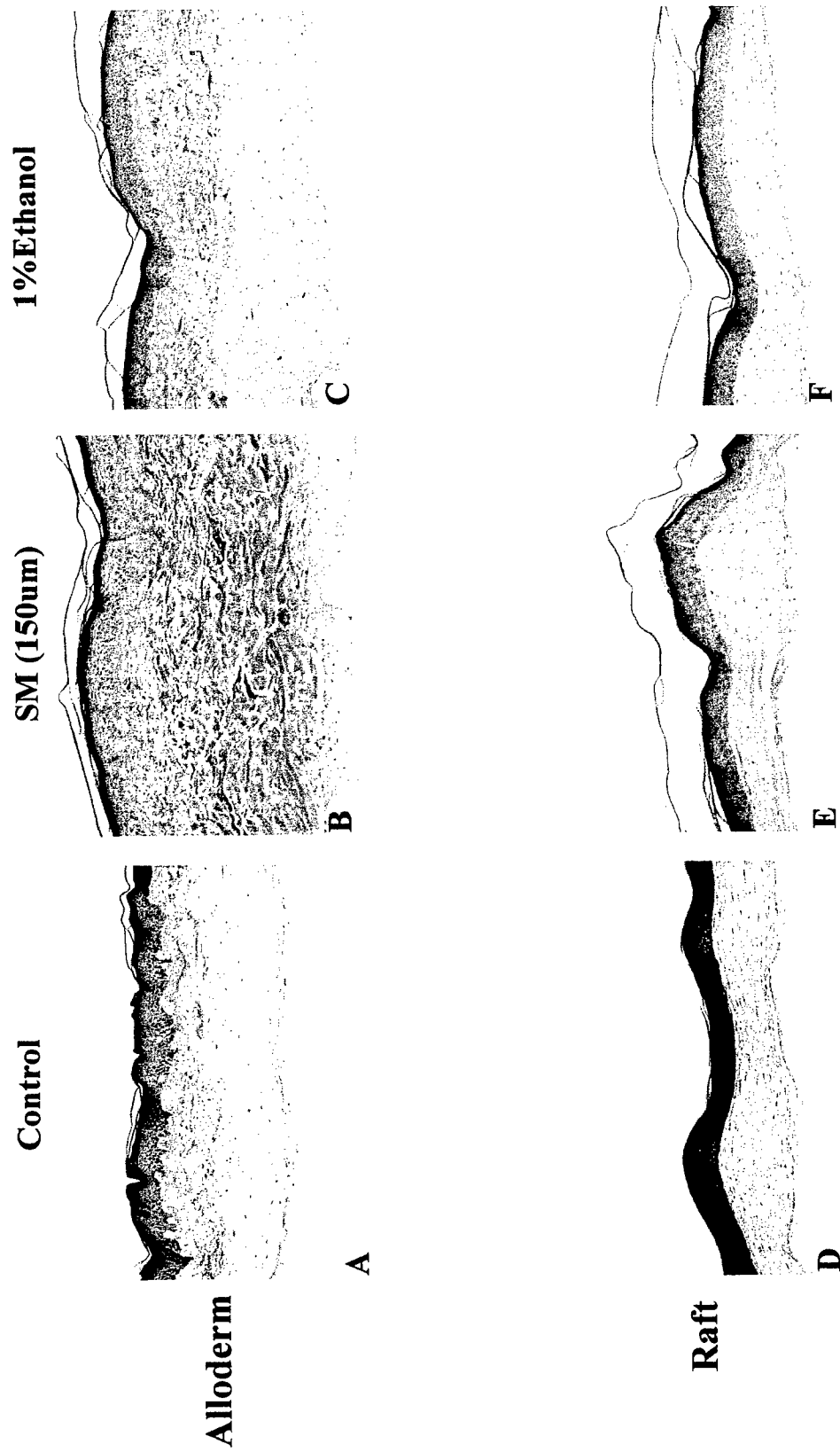


Fig: A& D – Control; B& E – 150um SM; C&F – 1% Ethanol.

Fig. 32: Effects of SM on Alloderm and Rafts along with controls.

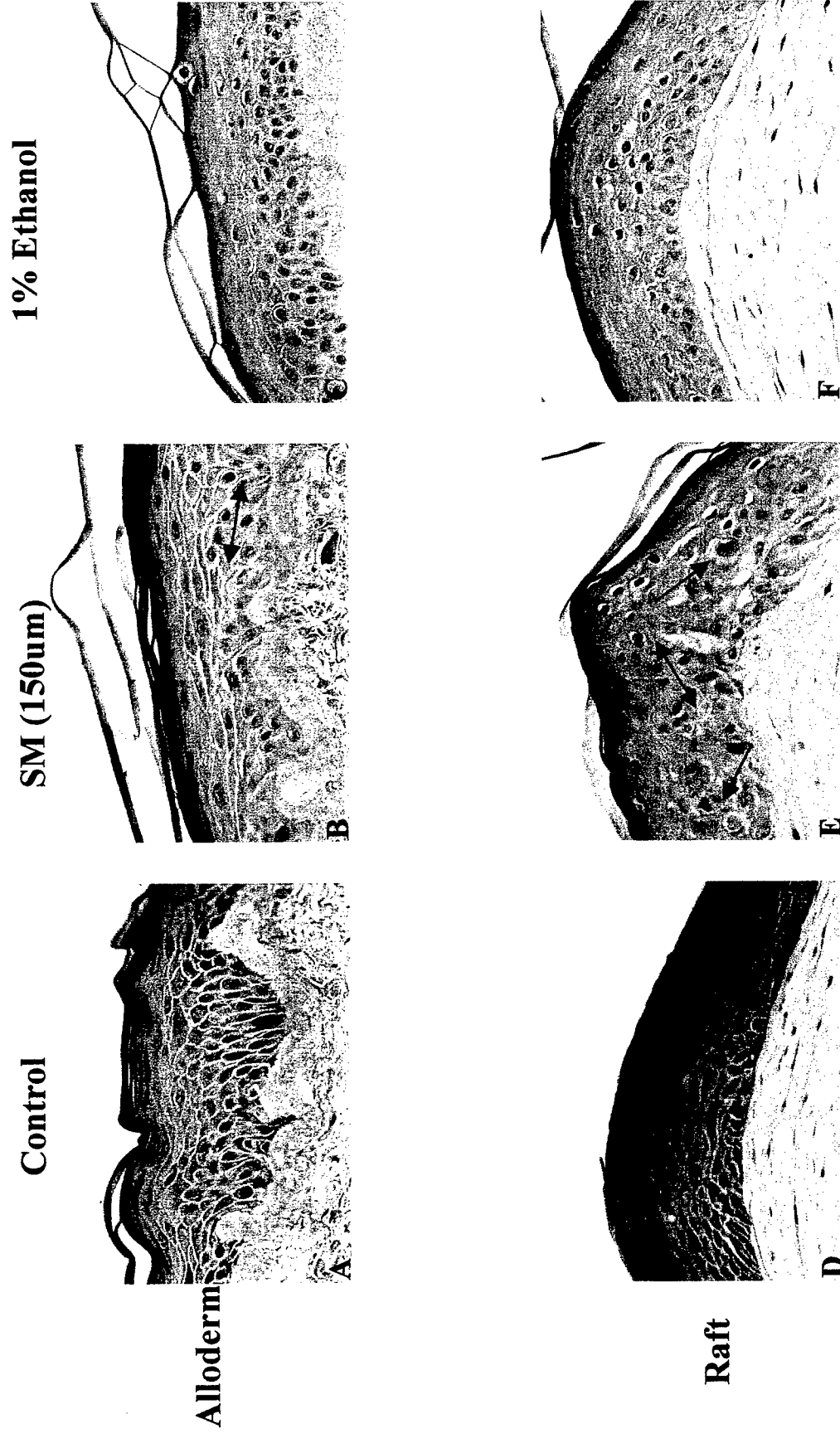
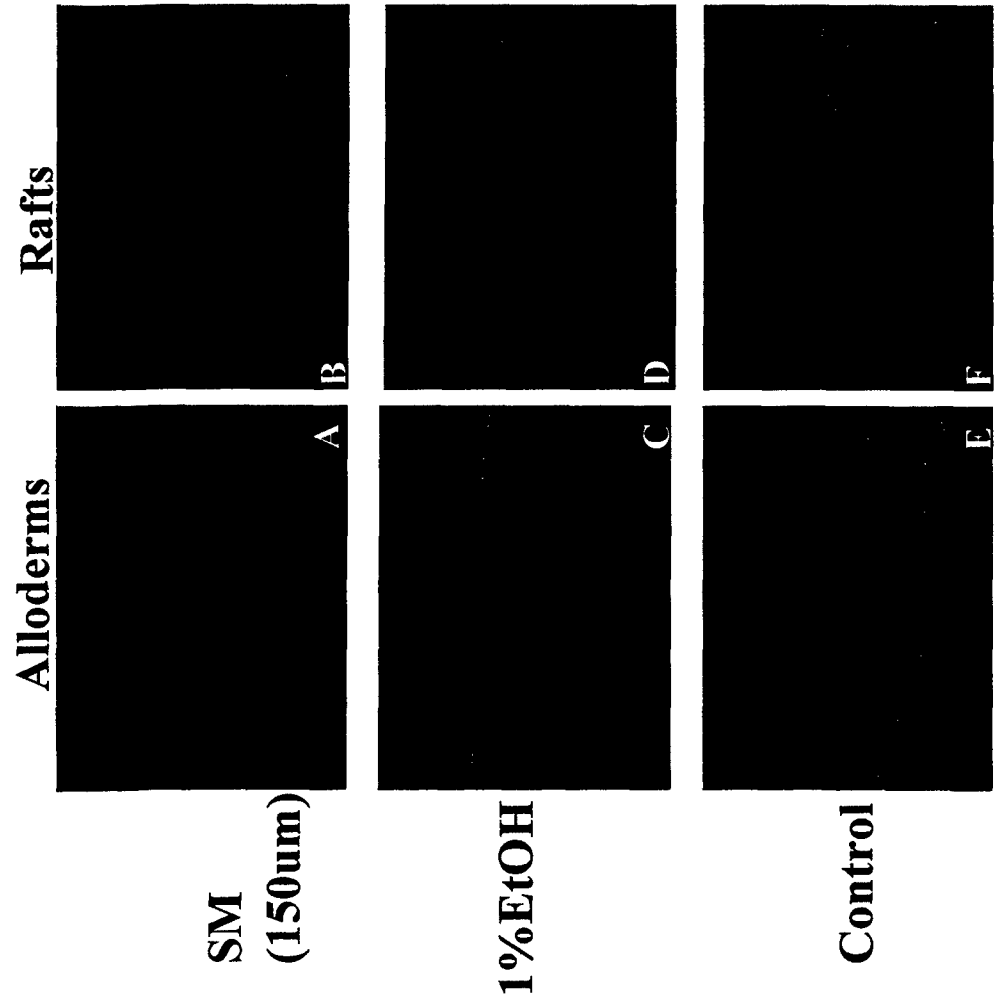


Fig: A&D – Controls; B&E – 150um of SM; C&F – 1% Ethanol.

Fig 33: M30 Staining for Alloderms and Rafts exposed to SM and Controls.



A,C, E –Alloderms; B, D, F- Rafts.

Fig. 34: Apoptotic cell counts for keratinocytes on rafts and alloderms.

Table:		
Chemicals	No. of apoptotic cells on alloderm	No. of apoptotic cells on raft.
Control	0	3
1% Ethanol	6	9
SM (150u _m)	22	265

Graph:

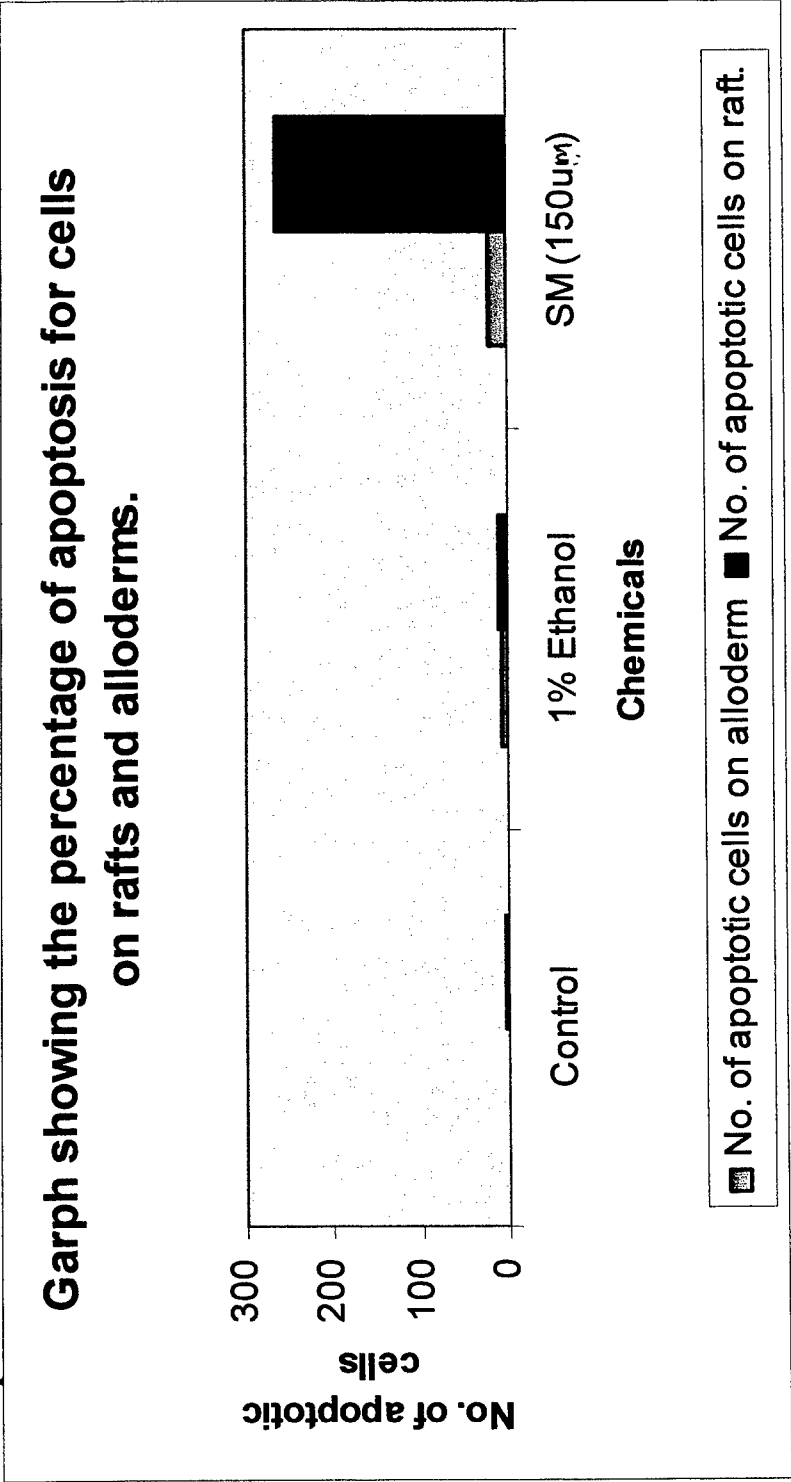


Fig. 35: Alloderm exposed to diff.doses of SM and Controls.

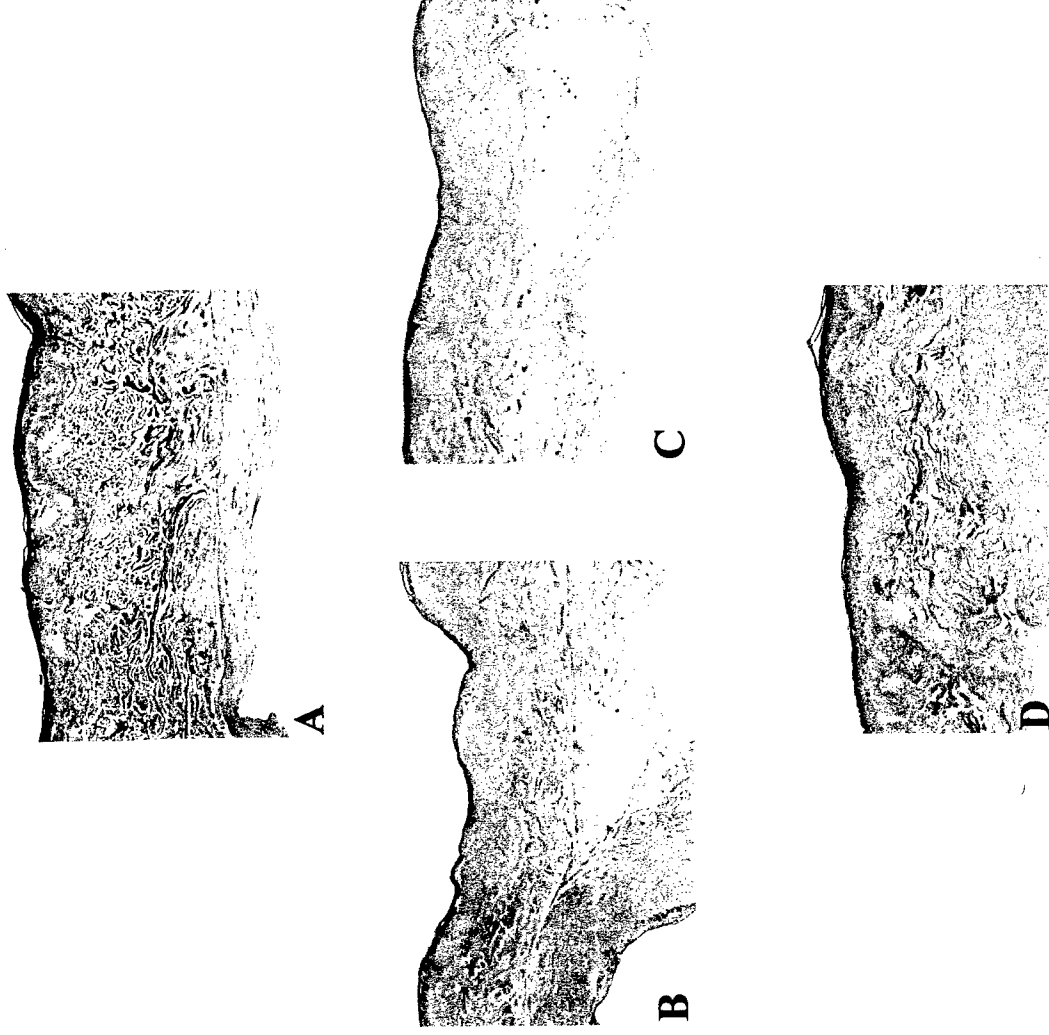


Fig: A-Control; B&C- 75& 150um SM; D- 1% Ethanol.

Fig. 36: Alloderm exposed to diff. Doses of SM and Controls



Fig: A- Control; B& C – 75 & 150um SM; D- 1% Ethanol.

Fig. 37: M 30 Staining for Alloderm exposed to SM and Controls.

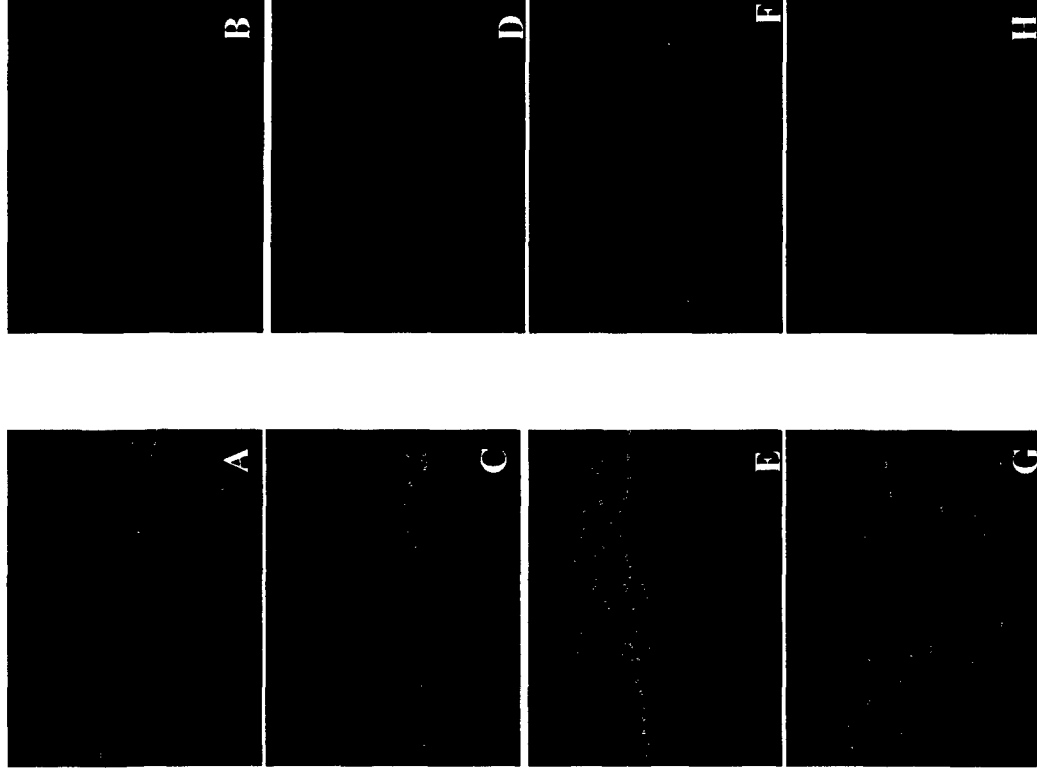


Fig: A, B- Control; C, D- 75 um SM; E, F-150um SM; G, H- 1% Ethanol.

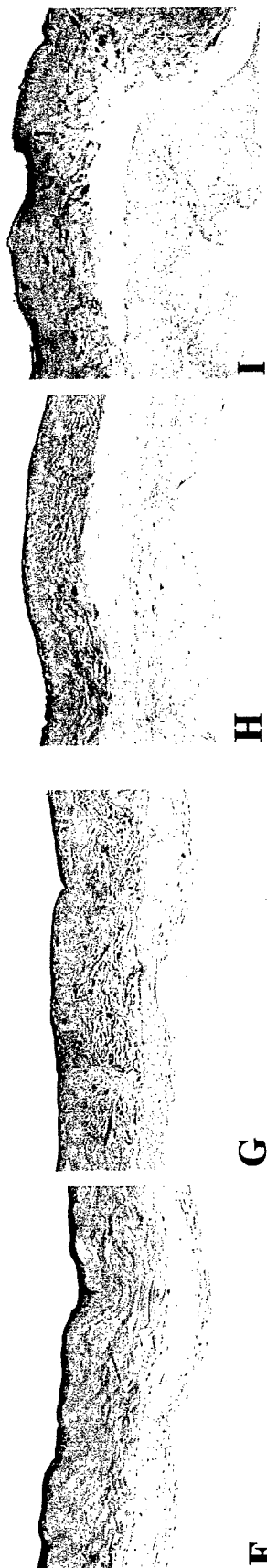
Fig. 38: Dose Response to SM shown by NHK's on Allos along with controls



**Fig: A-F - 75, 150, 300,
600, 1200um SM**



E



**Fig: F-J- 0.5, 1, 2, 4, 8%
Ethanol**



J

Fig.39: Dose Response to SM shown by NHK's on Alloderm along with controls



Fig: A-E – 75, 150, 300, 600, 1200um SM

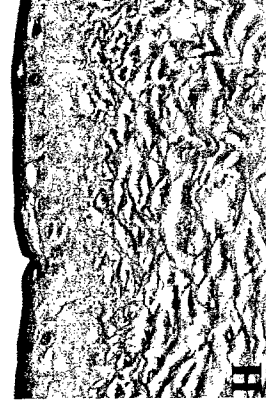
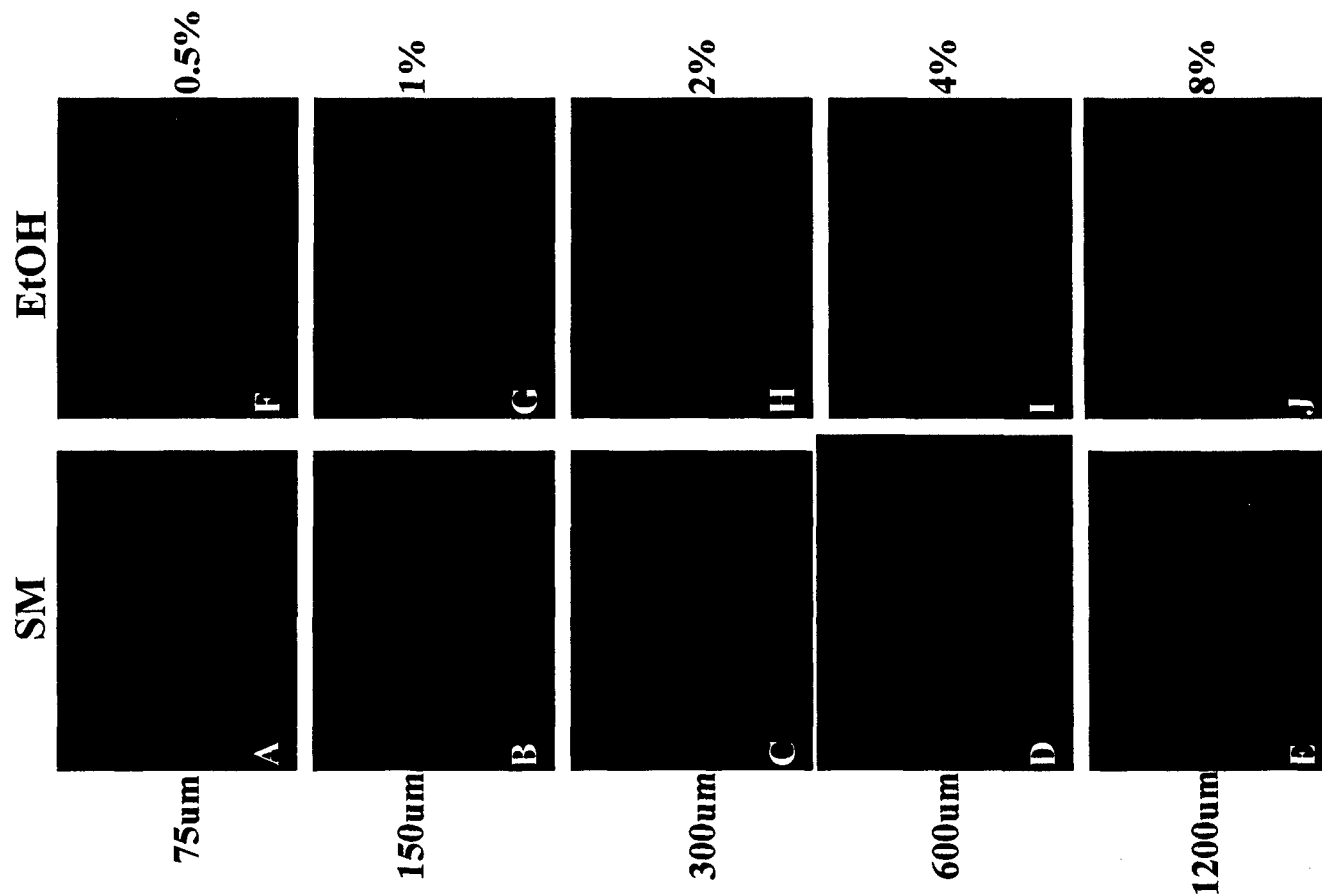


Fig: F-J – 0.5, 1, 2, 4, 8% Ethanol

Fig. 40: M30 Staining for dose-response shown by NHKs on Alloderm



**Fig: A-E – Diff. Doses of
SM: F-J – Concurrent
Ethanol controls.**

Fig. 41: Apoptotic Counts Alloderm Exposed to diff. Doses of SM along with concurrent controls

Table:

Chemical	Apoptotic counts.
75u _m SM	19
0.5% EOH	0
150u _m SM	24
1% EtOH	5
300u _m SM	61
2%EtOH	6
600u _m SM	102
4%EtOH	17
1200u _m SM	286
8%EtOH	18

Graph:

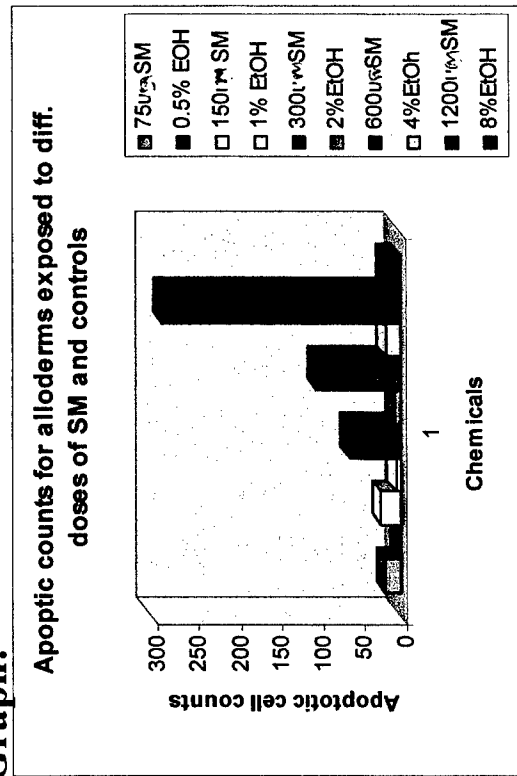


Fig. 42: Effects of SM on diff. Cell types seeded on alloderm along with controls.

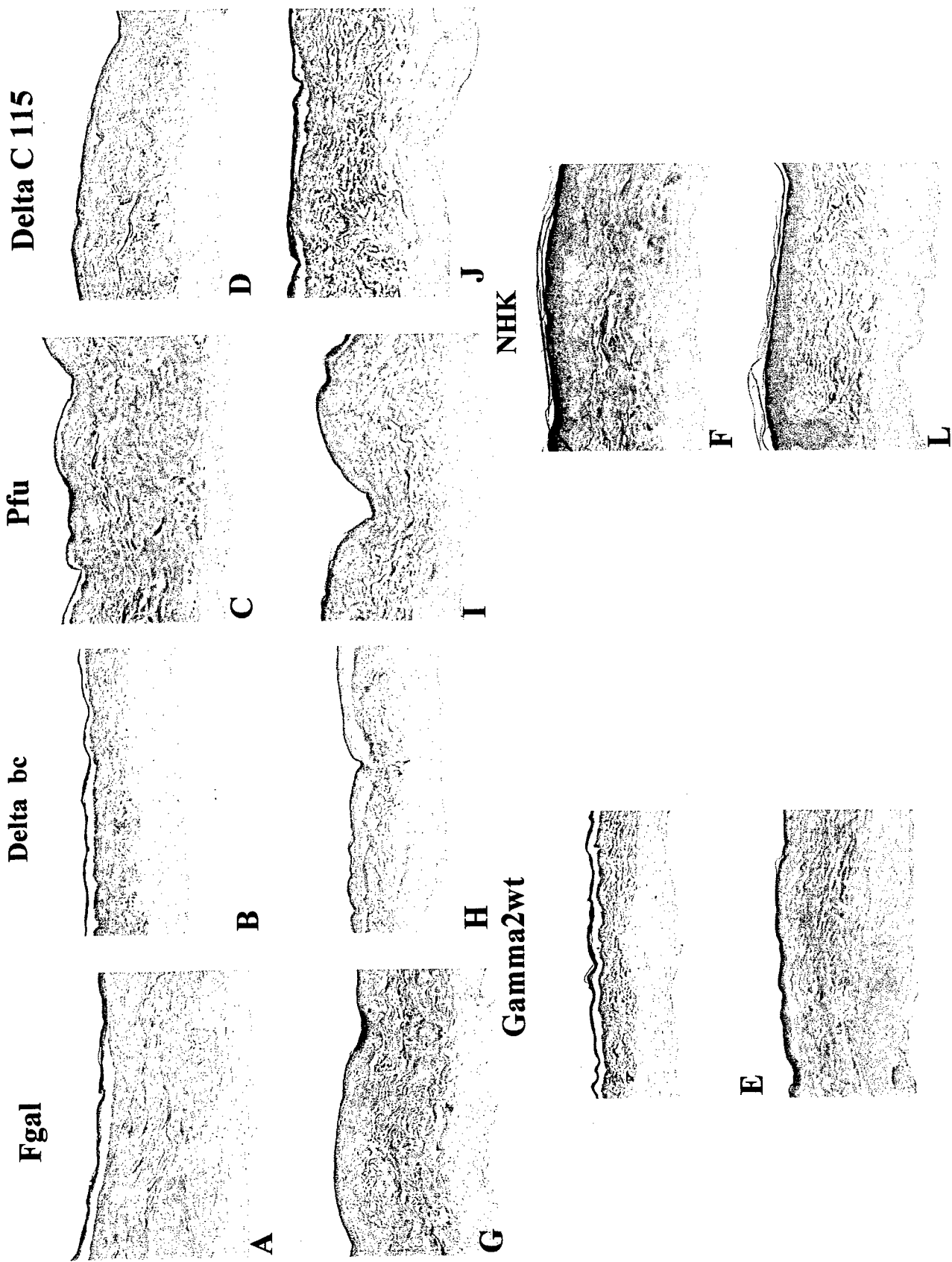


Fig: A-F – 150um of SM: G-I – 1% Ethanol.

Fig. 43: Effects of SM on diff. Cell types on alloderm along with controls.

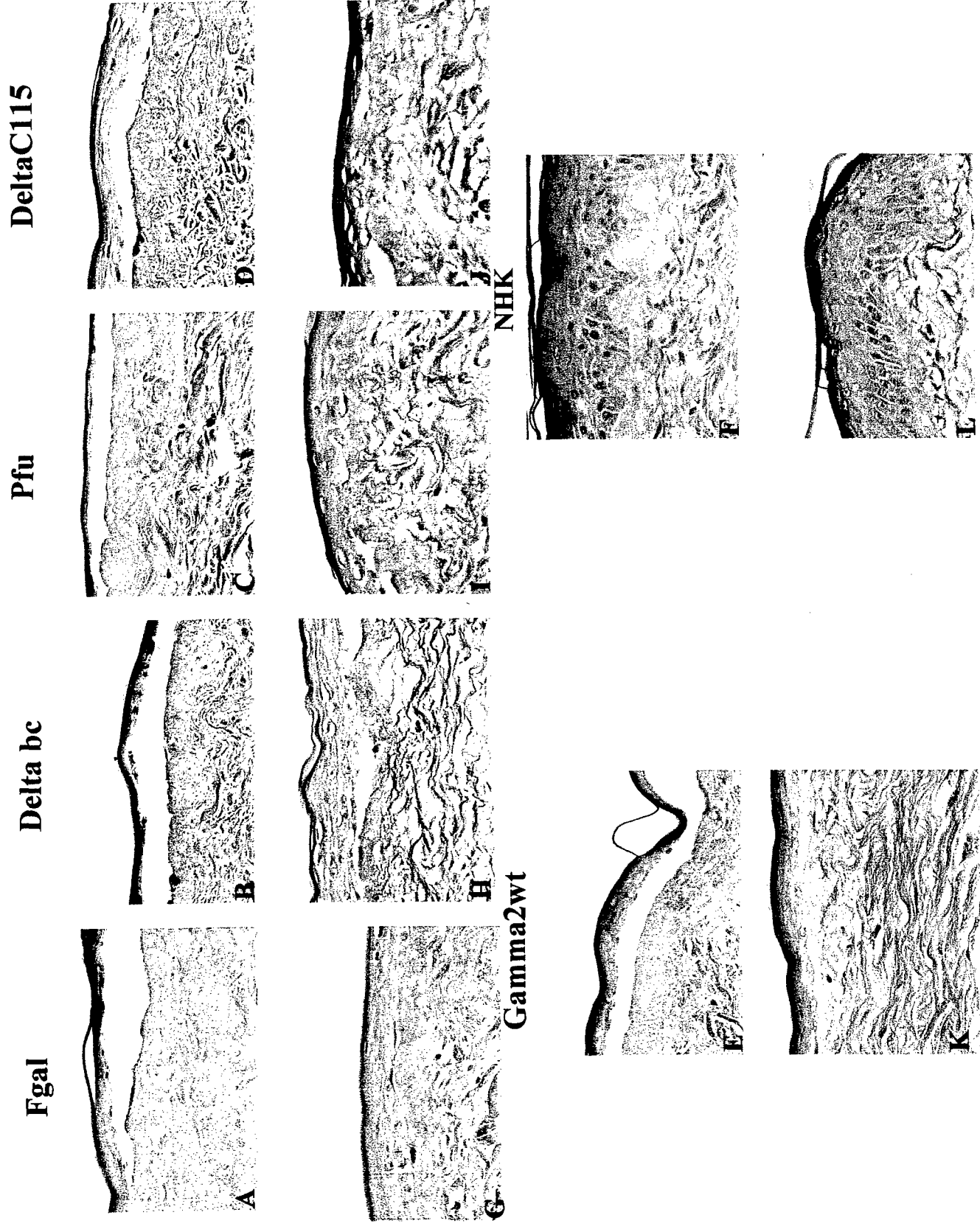
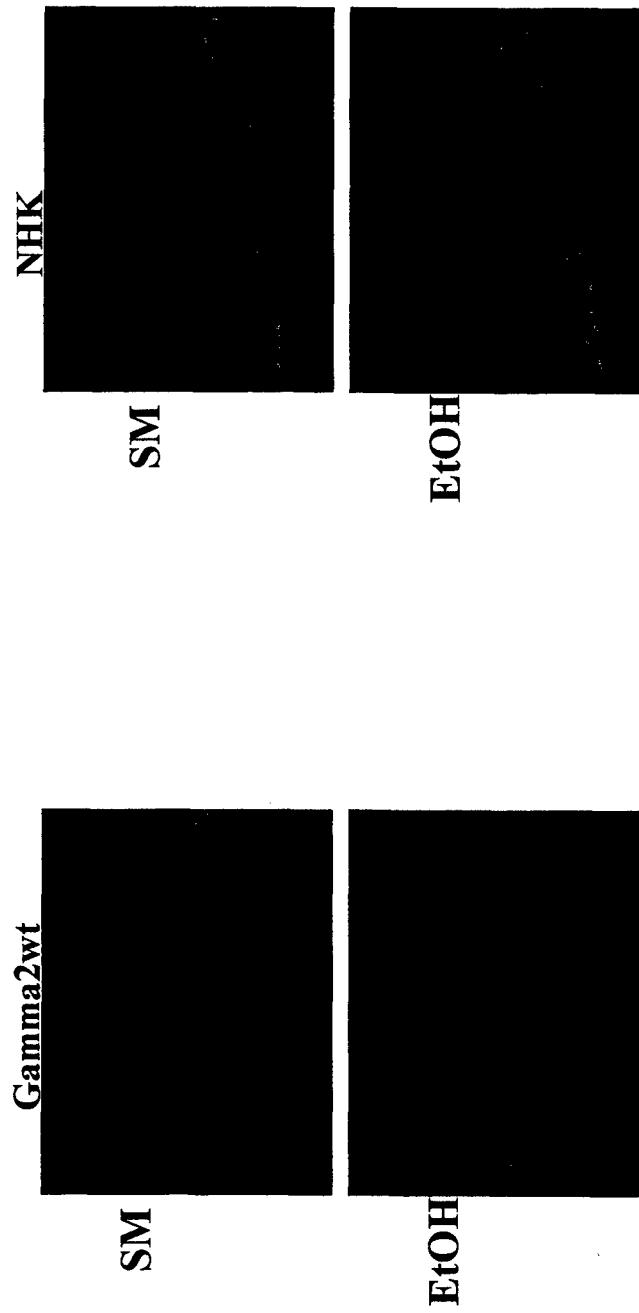
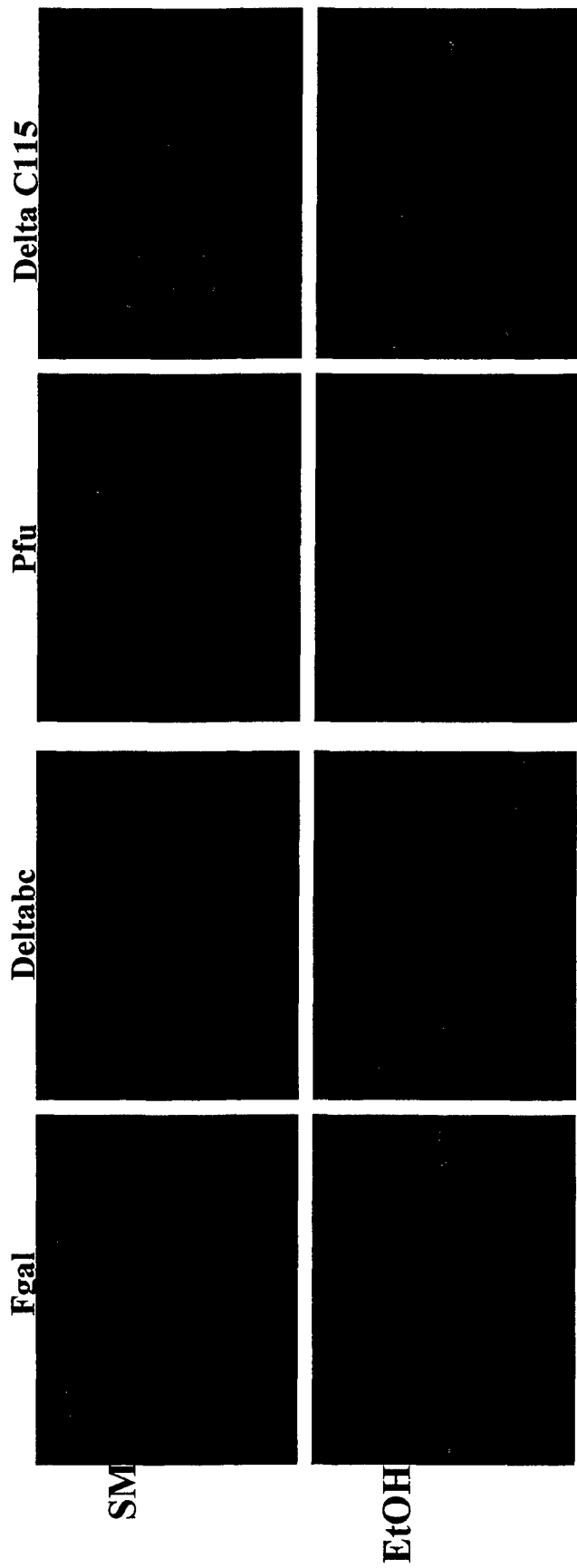


Fig: A-F – 150um SM; G-L- 1% Ethanol.

Fig. 44: M30Staining for diff.cell types on alloderm exposed to SM and Ethanol.



SM—150um; EtOH— 1%

Fig. 45: Apoptosis for diff. Cells on alloderm exposed to SM and Ethanol

Table: Percentage of apoptosis for diff.cell types on alloderm

Chemicals	fgal	Deltabc	pfu	DeltaC115	Gamma2wt	NHK
SM(150u _M)	20		4	17	7	19
1%EtOH	2		3	4	4	5
						2

