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Cytotoxicity testing of silver-containing burn treatments using primary and immortal skin cells

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ABSTRACT

A novel burn wound hydrogel dressing has been previously developed which is composed of 2-acrylamido-2-methylpropane sulfonic acid sodium salt with silver nanoparticles (silver AMPS). This study compared the cytotoxicity of this dressing to the commercially available silver products; Acticoat™, PolyMem Silver® and Flamazine™ cream. Human keratinocytes (HaCaT and primary HEK) and normal human fibroblasts (NHF) were exposed to dressings incubated on Nunc™ polycarbonate inserts for 24, 48 and 72 h. Four different cytotoxicity assays were performed including; Trypan Blue cell count, MTT, Celltiter-Blue™ and Toluidine Blue surface area assays. The results were expressed as relative cell viability compared to an untreated control. The cytotoxic effects of Acticoat™ and Flamazine™ cream were dependent on exposure time and cell type. After 24 h exposure, Acticoat™ and Flamazine™ cream were toxic to all tested cell lines. Surprisingly, HaCaTs treated with Acticoat™ and Flamazine™ had an improved ability to survive at 48 and 72 h while HEKs and NHFs had no improvement in survival with any treatment. The novel silver hydrogel and PolyMem Silver® showed low cytotoxicity to all tested cell lines at every time interval and these results support the possibility of using the novel silver hydrogel as a burn wound dressing. Researchers who rely on HaCaT cells as an accurate keratinocyte model should be aware that they can respond differently to primary skin cells.

Key words: cytotoxicity; hydrogel; dressing; silver nanoparticles; burn care

Introduction

The increase of antibiotic resistance in infected wounds has lead to the need to develop more agents that can be used to treat colonized wounds effectively. There is substantial evidence to support the use of silver containing products in infected wound management and silver has been used for infection treatment for centuries [1] More recently, silver sulphadiazine (e.g. Flamazine™) is commonly used to treat burn wounds [2]. In the last decade, a number of silver products have been introduced, which are available in different formulations and contain various forms of silver including: pure metallic silver and compounds such as silver phosphate, silver sulfadiazine, silver-sodium carboxymethyl cellulose and silver chloride [3]. Recent advancements in nano-technology have lead to the development of nanocrystalline silver, and a new dressing coated with silver nanoparticles for burn treatment (Acticoat™) [4].

Various research groups have studied the cytotoxicity of silver products using different cell lines and various cytotoxicity assays. In 2004, a cytotoxicity study using MTT assays to assess the effect of nanocrystalline silver dressing (Acticoat^{TM}) on primary human keratinocytes proposed that Acticoat^{TM} was not appropriate for use as a topical dressing for cultured skin grafts [5]. Another study used MTT assays on primary human keratinocytes and fibroblasts and found Acticoat^{TM} was likely to produce significant cytotoxic effects on both cell lines, whereas PolyMem Silver$^{®}$ showed the least toxicity compared to other silver-based dressings tested [6]. Our research group previously found that Silvazine^{TM} (which has ceased production) and its replacement Flamazine^{TM} cream, had cytotoxic effects on HaCaT cells demonstrated by a Toluidine Blue staining assay [7]. PolyMem Silver$^{®}$ was found to have low toxicity on HaCaT cells assessed by counting surviving cells after incubation with treatments [8].

Recently, a dressing containing silver nanoparticles (SNPs) has been developed by our research group [9], which is composed of 2-acrylamido-2-methylpropane sulfonic acid (AMPS) sodium salt hydrogel. The hydrogel acts to provide a moist environment to stimulate healing, while absorbing wound exudate during the healing process. It feels cool to touch, which may reduce the pain of wounds. The transparency of the hydrogel enables observation of the wound healing process. Silver has been incorporated into the dressing to help prevent wound infection. Although hydrogels have been used previously on burns to

keep them moist and silver-containing products have also been used in burn care, this novel treatment combines both advantages in the one dressing. It is also relatively economical to produce. The antibacterial activity of the novel silver dressing against MRSA and *P. aeruginosa* has been evaluated using bactericidal measurement (broth culture and plate count method) [9] and the results support the possibility of using 5 mM silver hydrogels as antimicrobial burn wound dressings.

In this study, the cytotoxicity of the novel silver hydrogel dressing (containing 5 mM silver) was compared to the commercially available silver products: Acticoat™, PolyMem Silver® and Flamazine™ cream, with neat AMPS hydrogel (containing no silver) used as a negative control. Three cell monolayer culture systems were compared: HaCaT (a human keratinocyte immortalised cell line), HEK (primary human epidermal keratinocytes) and NHF (primary normal human fibroblasts), to investigate the cytotoxicity of the silver agents.

Methods

Cytotoxicity assessment

Burn wound products

Three common silver-containing burn treatments were used in this experiment as a comparison for the silver hydrogel dressing: Acticoat™, PolyMem Silver® and Flamazine™ cream (Figure 1, Table 1). The neat hydrogel (containing no silver) served as a negative control.

Cell culture systems

HaCaT cells were a gift from Dr N. Fusenig (German Cancer Research Centre, Heidelberg, Germany) [10]. The primary keratinocytes and fibroblasts were obtained from foreskin surgical discards obtained with institutional ethics approval. Both keratinocyte cell lines were cultured on 35 cm diameter tissue culture plates at a seeding density of 5,000 cells/cm^2 in 2 mL of growth medium. HaCaTs grew in Roswell Park Memorial Institute **(**RPMI) media (Gibco, Australia) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco, Australia). HEKs were grown in serum-free medium (SFM) supplemented with 0.15 ng/mL Epidermal Growth Factor (EGF), Bovine Pituitary Extract (BPE) and antibiotic/antimycotic (AA). The seeding density of NHF was 3,500 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Australia) containing 30 mM Hepes, 10% FBS and AA. Cells were grown for 5 days at 37^oC in 5% CO_2 to achieve 95-100% confluency, with medium changed at day 1 and day 4.

Experimental design

A cytotoxicity testing method using polycarbonate cell culture inserts developed by our research group [8] was applied to a monolayer tissue culture system of cells. In brief, the novel dressings and silver agents were individually placed on top of a permeable Nunc™ polycarbonate cell culture insert and were incubated on the cell monolayer in 2.5 mL of culture media for different time intervals (24, 48 and 72 h). The original size of each dressing varied in order to obtain a 1.0 x 1.0 cm^2 swelled dressing after 24 h of incubation (Figure 1). Flamazine™ cream (200 mg) was spread on top of the membrane to an area of 1.0 x 1.0 cm^2 and the control contained only the polycarbonate insert, with no dressing. After the incubation times (24, 48 and 72 h), the inserts carrying the dressings were taken

off and cell morphology images were taken using a SONY, SLT-A55V digital camera (Sony Corporation of America, USA) attached to an OLYMPUS CKX41 light microscope (Olympus America Inc, USA).

Cytotoxicity assays

Initially, four different cytotoxicity assays were conducted using HaCaT cells which are easy to handle and have an unlimited supply, in order to choose the best assay for further studies with HEK, NHF and HaCaT at different exposure time intervals. After 24 h treatment of HaCaT cells, the four different cytotoxicity assays conducted were; Trypan Blue cell count, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, Sigma, USA), CellTiter-Blue™ (Promega, USA) and Toluidine Blue surface area assessment. On the basis of these results, MTT and surviving cell count assays were carried out for the 48 h and 72 h exposures of HaCaT and 24, 48 and 72 h exposures of treatment to HEK and NHF cells. Additionally, we used cell counts from light microscope (LM) photos to assay the cell density of the monolayer cell lines; HaCaT and HEK to verify the MTT assay results as MTT assays measure cell metabolism, not necessarily cell number.

Trypan Blue cell count

After the treatments were incubated with the HaCaT, HEK or NHF cells, any dead cells on the culture plates were washed off and photos were taken to observe the morphology of the cells. The cells were then trypsinized, collected, spun down and resuspended in the culture medium. After mixing 1:1 with Trypan Blue, dead blue cells were excluded from the haemocytometer count. The number of cells from the untreated control represented optimal cell survival (100%) and the relative surviving cells of each treatment were calculated using the following equation:

$$\text{Relative cell viability (\%)} = \frac{\text{No. surviving cells in sample}}{\text{No. surviving cells in control}} \times 100\%$$

MTT assay

After treatment, the culture medium was removed and cells were gently washed. A 1.5 mL aliquot of 1.0 mg/ml of MTT in DMEM without phenol red was added and the plate was incubated at 37^{o}C for 5 min for HaCaT and HEK cells and 10 min for NHF cells. MTT solution was then removed and replaced with 2.5 ml of dimethylsulfoxide solution (10:1.25

of DMSO:glycine buffer, pH 10.5). A 200 µl aliquot of the solution was added to a 96-well plate and the A_{570nm} of the solution was measured using a FLUOstar Omega microplate reader (BMG LABTECH, Germany). The relative cell viability is proportional to the absorbance and was calculated using the equation below with the untreated control used to approximate 100% cell viability:

$$\text{Relative cell viability (\%)} = \frac{A_{570}\text{nm of sample}}{A_{570}\text{nm of control}} \times 100\%$$

CellTiter-Blue™ Assay

The culture medium was removed and replaced with 2.0 ml of fresh medium. A 400 µl aliquot of the CellTiter-Blue™ reagent was added and the plate was incubated for 20 min at 37°C. Aliquots of 100 µl were added to a 96-well plate and the cellular fluorescence excitation at 544 nm and emission at 590 nm was measured using a FLUOstar Omega microplate reader. The background fluorescence was measured and the untreated control fluorescence was taken to be 100% cell survival. The relative cell viability of each treatment was proportional to the fluorescence values and was calculated using the following equation:

$$\text{Relative cell viability (\%)} = \frac{\text{Fluorescence of sample} - \text{Background}}{\text{Fluorescence of control} - \text{Background}} \times 100\%$$

Toluidine Blue surface area assessment

The assessment of Toluidine Blue stained surface area of cell growth is a method previously developed by our research group [7]. In brief, after treatment, the culture medium was removed and the dead cells were washed off. Cells were stained with 400 µl of 1% Toluidine Blue in 1% borax buffer for 10 min on a shaker at 100 rpm. The dye was removed and the cells were thoroughly washed with PBS and then air dried. Digital photos of the plates were taken and the stained surviving cell areas were measured using Image Pro Plus v5.1 software (Media Cybernetics Inc., Silver Spring, USA). The relative cell viability (%) of each treatment was calculated using the equation below:

$$\text{Relative cell viability (\%)} = \frac{\text{Area of control} - \text{Dead area of sample}}{\text{Area of control}} \times 100\%$$

Cell count from light microscope (LM) photos

Counting cells from LM photos was used to estimate the relative cell density (%) of surviving cells compared to the control. This was an extra control to verify the results from the MTT assay, which assesses cell metabolism as an indicator of cell number. The ImageJ program (version 1.45s) was used for the creation of grids on the morphology photos of cells. Single cells were then counted manually via the software. The relative cell density (%) was calculated using the equation:

$$\text{Relative cell density (\%)} = \frac{\text{Cells in sample}}{\text{Cells in control}} \text{ x } 100\%$$

Statistical analysis

Three independent experiments were performed for each cytotoxicity assay. Differences between samples and the control were evaluated with One-way analysis of variance (ANOVA) and the Tukey's Multiple Comparison Test using GraphPad Prism version 5.03. Statistically significant differences were set at $p < 0.01$ (99% confidence).

Results

Comparison of four different cytotoxicity assays

Of the four different cytotoxicity assays, the MTT assay is considered the fastest assay and has relatively low cost, with good sensitivity and low variability. Manual cell counts are time-consuming and can be prone to human error, the Cell Titer-Blue assay is easy but expensive and has low sensitivity and the Toluidine Blue staining assay has low sensitivity and accuracy for cell viability >90%. It is for these reasons that the MTT assay was selected as the best assay to conduct further work in this study.

After HaCaT cells were exposed to treatment for 24 h, all assays indicated significant decreases in cell viability for Acticoat™ and Flamazine™ treatments (Figure 2, $p < 0.01$). The neat hydrogel, silver hydrogel and PolyMem silver® were less toxic with an average cell viability of 89.9-91.7 % compared to Acticoat™ and Flamazine™ (81.7 and 75.8 % respectively ($p < 0.01$) (Figure 2)). There was no significant difference in toxicity between Acticoat™ and Flamazine™ treatments ($p > 0.01$) or between PolyMem Silver®, neat hydrogel and silver hydrogel ($p > 0.01$). In addition, no dead cell area was observed in photos of Toluidine Blue staining of cells exposed to PolyMem Silver® and the two hydrogel treatments, indicating they had no or only slight cytotoxicity. However, photos of Toluidine Blue staining of Acticoat™ and Flamazine™ treatments showed obvious dead cell areas (15.3% and 24.2% % dead cell areas, respectively).

Cytotoxicity of burn products on three human skin cell lines (using MTT and Trypan Blue cell count assays)

The morphology of the HaCaT, HEK and NHF cells after 24 h treatment is shown in Figure 3. After Acticoat™ (Figure 3b, e, h) and Flamazine™ treatments, dead cells lost contact with their neighbors, appeared rounded, and floated on top of the living cell monolayer. Normal morphology was observed after exposure to PolyMem Silver®, neat hydrogel and silver hydrogel (Figure 3c, f, i), similar to the untreated control for all cells (Figure 3a, d, g).

After 24 h incubation, MTT and Trypan Blue cell count assays indicated that HaCaTs exposed to Acticoat™ and Flamazine™ had significantly lower cell viability than the untreated control (73.9-83.0% cell viability, $p < 0.01$, Figure 4A, a), indicating the relatively high toxicity of the two burn products. In comparison, the two assays indicated PolyMem

Silver® and silver hydrogel treatment had no significant difference in cell viability compared the control ($p > 0.01$) while neat hydrogel showed slight toxicity (88.2-89.2% cell viability, $p < 0.01$). Similar results were found for 24 h incubation of HEK and NHF cells with treatments, with Acticoat™ and Flamazine™ having statistically significant toxicity compared to the control (62.8-71.1% and 75.8-82.2% respectively, $p < 0.01$) and HEK and NHF cells showing no toxicity with PolyMem Silver®, neat hydrogel and silver hydrogel (Figure 4B, b, C, c).

However, after 48 and 72 h incubation, MTT and Trypan Blue cell count assays demonstrated that HaCaT cells exposed to Acticoat™ (Figure 4A, a) and Flamazine™ had significantly increased cell viability compared to 24 h exposure and the untreated control ($p < 0.01$) with a cell viability of 113.8-123.6% (48 h) and 132.0-133.5% (72 h), respectively. This result was verified by cell counts of LM photos of HaCaTs exposed to Acticoat™ and Flamazine™ which also showed an increased cell density for the 48 h and 72 h treatments compared to their 24 h exposures (cell densities of 102.5-109.3% (48 h) and 119.5-127.7% (72 h), respectively ($p < 0.01$)), data not shown. For the other dressings, after 48 h and 72 h exposure of HaCaT cells, PolyMem Silver® and neat hydrogel had increased toxicity (70.7-87.3% cell viability) and this was significantly different from the untreated control ($p < 0.01$). The silver hydrogel showed slight toxicity at 48 h (79.9-85.2%, $p > 0.01$).

In contrast, for 48 and 72 h exposures of HEK, MTT and Trypan Blue cell count (Figure 4B, b) results showed that Acticoat™ and Flamazine™ treatments were toxic to cells with a relative cell viability of 58.0-73.3% ($p < 0.01$). No toxicity was found for other treatments. MTT assays of the silver hydrogel with double silver content (10 mM rather than 5mM) exposed to HEK for 24, 48 and 72 h were also conducted and showed no difference in relative cell viability and cell density compared to the untreated control (data not shown).
For 48 and 72 h exposures of NHF, MTT and Trypan Blue cell count (Figure 4C, c) results showed that Acticoat™ treatment slightly increased the cell viability compared to the 24 h treatment but this difference was not statistically significant compared to the untreated control ($p > 0.01$). Flamazine™ treatment gave slightly increased toxicity after 48 and 72 h exposure compared to the 24 h treatment but no significant difference was observed ($p > 0.01$). Trypan Blue cell counts indicated no significant difference in cell viability of PolyMem Silver®, neat hydrogel and silver hydrogel treatments compared to the control at every time interval ($p > 0.01$). MTT assay results indicated PolyMem Silver® and silver

hydrogel had no toxicity ($p > 0.01$), while neat hydrogel showed toxicity at 72 h incubation (77.47% cell viability, $p > 0.01$).

Figure 5 shows the morphology of HaCaT cells after 72 h exposure to treatments. Dead cell areas were observed with Acticoat™ (Figure 5b) and Flamazine™ treatments. HaCaT cells treated with Acticoat™ (Figure 5b) and Flamazine™ appeared longer, smaller and denser compared to the control (Figure 5a). In contrast, the cells treated with PolyMem Silver®, neat hydrogel and silver hydrogel (Figure 5c) seemed to be less dense than the control. There was an increase in small vacuoles after treatment with Acticoat™ (Figure 5b) and Flamazine™ in HaCaT cells at longer incubation times compared to at 24 h. For treatments of HEK and NHF cells at 72 h, obvious dead cells areas were observed for exposures to Acticoat™ (Figure 5e, h) and Flamazine™ and the morphologies were similar to results of the treatments at 24 h except the dead cell areas were larger. Normal morphology was observed for both primary cell lines after exposure to PolyMem Silver®, neat hydrogel and silver hydrogel (Figure 5f, i), however the cells were slightly less dense compared to the controls (Figure 5d, g). NHF cells showed signs of oxidative stress (small vacuoles) after every silver treatment at 72 h (Figure 5h, i). The higher the silver content in the agents, the more small vacuoles were observed.

Discussion

The MTT and Trypan Blue cell count assays showed that HaCaT cells had improved ability to survive exposure to the more toxic Acticoat™ and Flamazine™ treatments after 48 h and 72 h, whereas there was significant cell death at 24 h. This surprising result was confirmed by the cell counts at 48 and 72 h, in which the cell densities after Acticoat™ and Flamazine™ treatments had increased (not just the metabolism of the remaining cells, as measured by MTT). Similar increases in HaCaT cell survival were found using a clonogenic assay after an 8 day exposure to silver nanoparticles [11]. The growth enhancement of the HaCaT cells appeared to be less after Flamazine™ treatment compared to Acticoat™ and this may have been due to Flamazine™ inhibiting the available growth area on the plate as seen with the Toluidine Blue staining. No improvements in survival were observed from the treatments of the low toxicity agents PolyMem Silver®, neat hydrogel and silver hydrogel in HaCaT cells.

The reason for this improvement of survival of HaCaT cells under toxic conditions is unclear. HaCaTs possibly have developed a more altered phenotype which may improve their survival ability under stressful conditions, whereas NHF and HEK seem to be more sensitive to toxic agents and showed no improvement of survival after any treatment. HEK cells especially seemed to be the most sensitive to Acticoat™ and Flamazine™, with higher toxicity seen at every time interval. High variability was also seen for most HEK experiments, due to the difficulty in differentiating live and dead HEK cells stained with Trypan Blue. Importantly, the HEK toxicity results differed between cells from different passage numbers (e.g. from passage number 2 versus 4) and this may suggest that immortal cell lines (like HaCaTs) which are able to survive for an extended time have developed an altered phenotype with enhanced growth ability, enabling improved survival after exposure to toxic agents. One possibility is that these cells are better able to produce stimulating factors to enhance the growth of neighboring cells in response to reactive oxygen species which are produced by silver nanoparticles [12]. HaCaT cells have been shown to contain relatively high natural antioxidant (e.g. GSH) levels which help them to survive nanoparticle generated oxidative stress [11]. More studies on other immortal cell lines are required to prove this hypothesis.

In this study, PolyMem Silver®, neat hydrogel and silver hydrogel treatments showed no significant NHF or HEK toxicity for all time intervals. The only exception to this was the neat hydrogel, which appeared to cause significant toxicity to NHF cells at 72 h for an unknown reason. NHF cells also had an increased number of vacuoles after exposure to silver hydrogel at 72 h, confirming that they were under some stress. However, significant toxicity to HaCaT cells was seen at most time points for the PolyMem Silver®, neat hydrogel and silver hydrogel treatments.

The significantly different results found between immortal keratinocytes (HaCaTs) and primary keratinocytes (HEK) is an important warning for many researchers who use HaCaTs as a keratinocyte model and suggests cell survival study results obtained using solely HaCaT cells may not be indicative of the true skin response to treatment. Given that the primary keratinocyte and fibroblast cell lines showed little or no toxicity with the silver hydrogel treatment, this indicates that it may be a beneficial dressing for the management of burn injuries in the future.

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Table 1: Burn wound products tested

Product	Manufacturer	Basic composition	Silver form	Silver content
Acticoat™	Smith & Nephew (Hull, UK)	An absorbent polyester core laminated between two outer layers of silver coated polyethylene mesh	nanocrystalline silver	0.84-1.34 mg/cm^2
PolyMem Silver®	Ferris MFG Corp. (Burr Ridge, IL, US)	Polyurethane foam containing F68, superabsorbent starch	silver particles	minimum 0.124 mg/ cm^2
Flamazine™	Smith & Nephew (Hull, UK)	Cetyl alcohol, distilled water, glycerol stearate, liquid paraffin, polysorbate 60, polysorbate 80, and propylene glycol.	Silver sulfadiazine	maximum 0.60 mg /200 mg cream
neat AMPS hydrogel	developed by our lab	AMPS sodium salt hydrogel	None	none
silver AMPS hydrogel	developed by our lab	AMPS sodium salt hydrogel	Silver nanoparticle	maximum 0.054 mg/cm^2

Figure 1 Photos of burn products tested in this study (top) and of the Nunc™ Polycarbonate inserts with dressings or cream on top incubated with HaCaT cell cultures for 24 hours (below); **a**: Acticoat™, **b**: PolyMem Silver®, **c**: Flamazine™ cream, **d**: neat hydrogel, **e**: silver hydrogel.

Figure 2 Relative cell viability (%) of HaCaTs treated with burn wound products for 24 h compared to untreated cells assessed by four different cytotoxicity assays including: Trypan Blue cell count, MTT, Celltiter-Blue™, and Toluidine Blue surface area. *denotes a statistically significant ($p < 0.01$) difference from the untreated control.

Figure 3 Morphology images of 20X HaCaT (**a**-**c**), 20X HEK (**d**-**f**) and 10X NHF (**g**-**i**) cells after 24 h exposure to treatment: control (**a**, **d**, **g**), Acticoat™ (**b**, **e**, **h**), silver hydrogel (**c**, **f**, **i**).

Figure 4 HaCaT, HEK and NHF cells exposed to burn treatments for 24, 48 and 72 h determined by: MTT (**A**-**C**); Trypan Blue cell count (**a**-**c**). Data are expressed as relative cell viability (%) ± SD of three independent experiments. *denotes a statistically significant ($p < 0.01$) difference from the untreated control.

Figure 5 Morphology images of 20X HaCaT (**a**-**c**), 20X HEK (**d**-**f**) and 10X NHF (**g**-**i**) cells after 72 h exposure to treatments: control (**a**, **d**, **g**), Acticoat™ (**b**, **e**, **h**), silver hydrogel (**c**, **f**, **i**).

Figure

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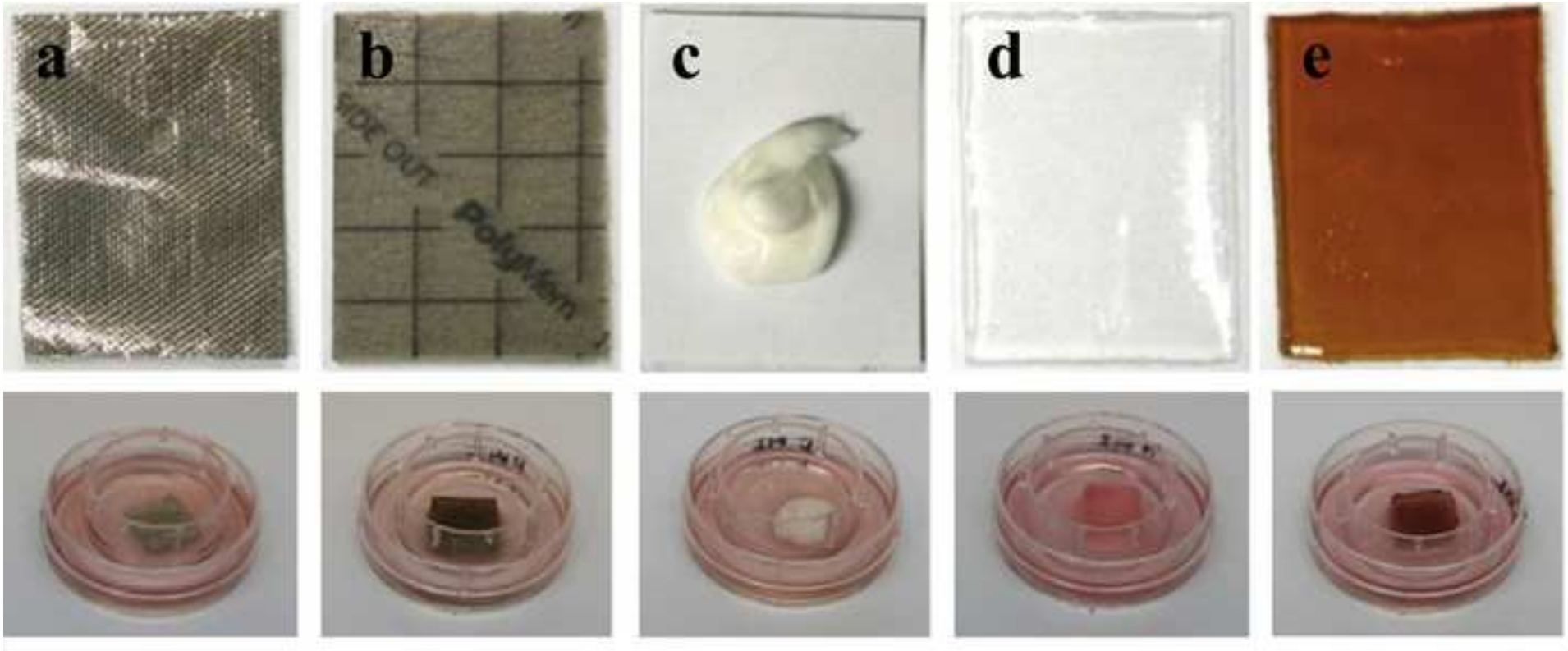

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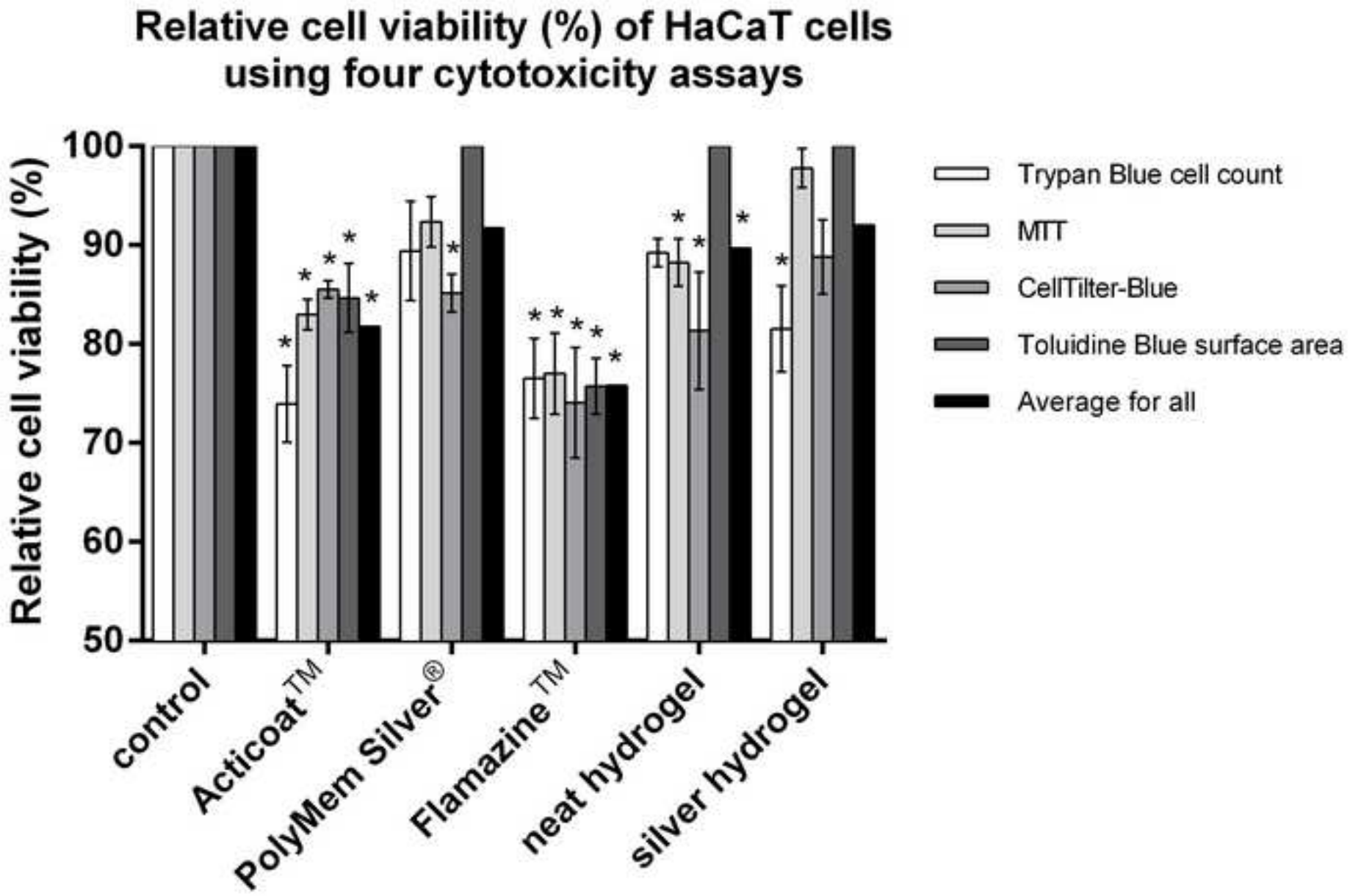

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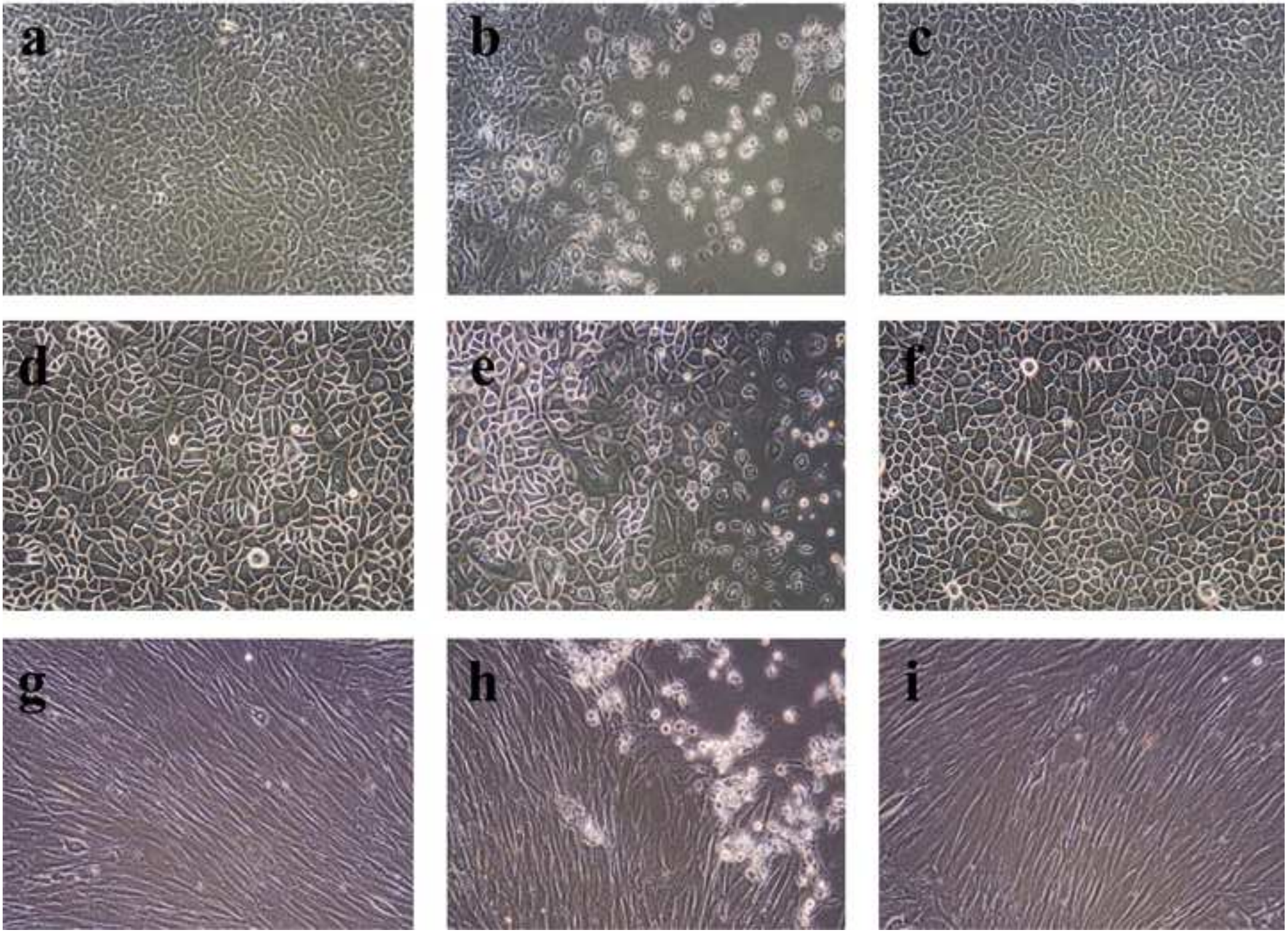

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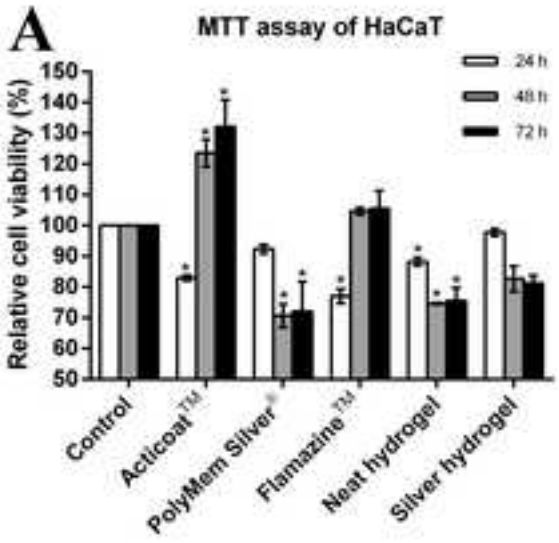

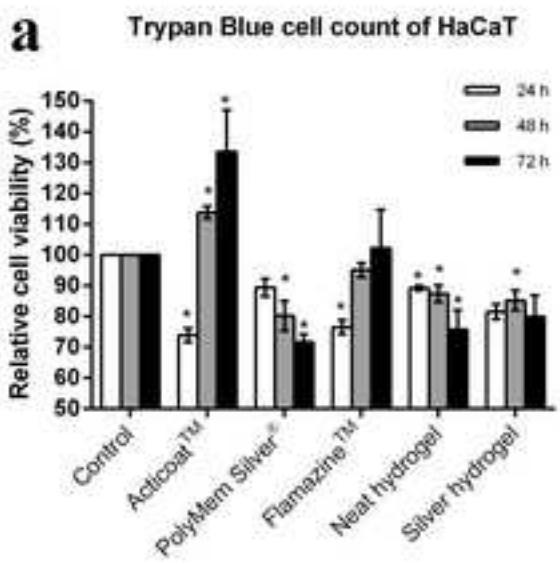

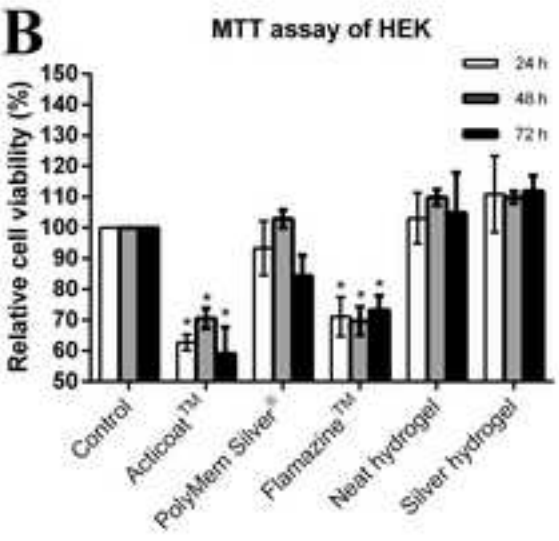

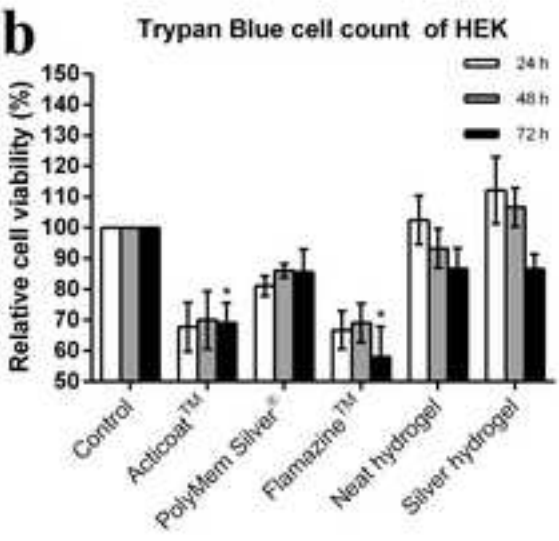

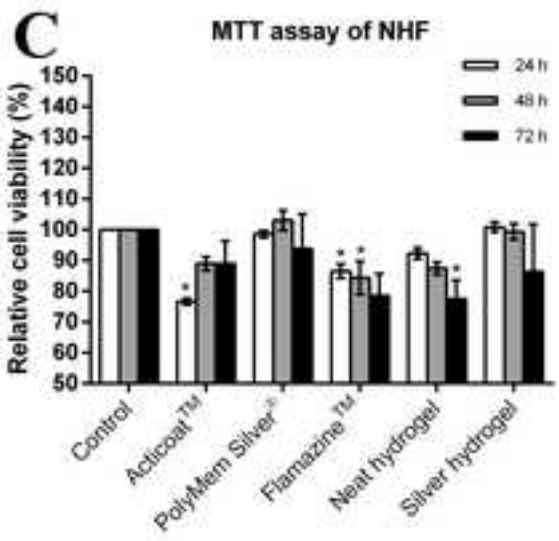

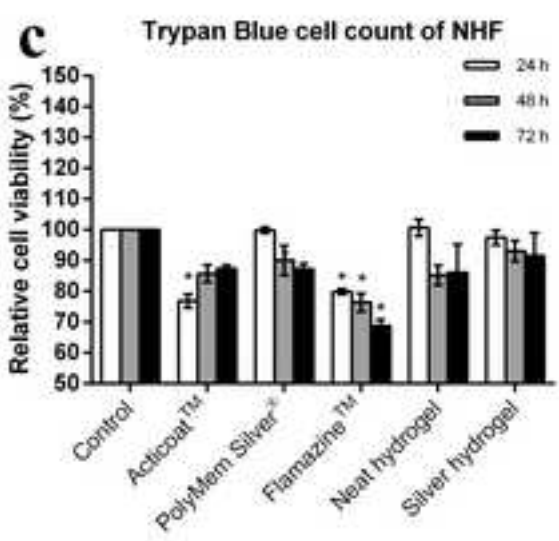

Figure

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