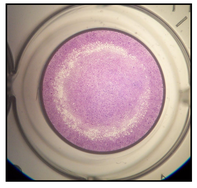
INDUCTION OF A ZONE OF CELL DEATH IN MULTI-WELL PLATES BY REFEEDING

H. Raabe, G. Moyer, G. Mun, M. Clear, and J.W. HARBELL.
Institute for In Vitro Sciences, Inc., Gaithersburg, MD 20878.

ABSTRACT

Multi-well plates provide an efficient format for cell-based bioassays. As the number of wells increases per plate, the surface area and number of cells per well decreases. Use of the small-well format can present some problems. We have observed cell cultures in 96-well plates where significant populations of cells begin to die shortly after refeeding the cell cultures (dumping the spent medium and adding fresh medium). The zones or areas of cell death appear to occur within a predictable ring around the edges of the multi-well plate wells, coinciding with the formation of a meniscus formed by the residual medium in the well following removal of the spent culture medium. This effect appears in larger well (e.g., 24-well plates) but has more impact in smaller well formats where the ratio of wall circumference to cell area is greater. The impact of these effects tend to be more pronounced when cultures are refed at relatively low confluence, resulting in areas devoid of cells. The zone is also more evident with cell types where cell migration is limited. Cells cultured in the presence or absence of serum show qualitatively similar results. When uniform, 30% confluent lawns of human keratinocytes in 96-well plates were refed, the cells within the zone rapidly lost the ability to take up neutral red and showed nuclear condensation. After 48 hours, the neutral red uptake (OD_{550}) was significantly reduced in the refed wells (0.830 ± 0.058, mean ± standard deviation, n=6 assays) compared to wells that were not refed (1.066 ± 0.034 where $p < 0.0001$). These results were confirmed in 3T3 cells cultured in serum-containing medium. These observations show the impact of the medium change in the 96-well plate format, and suggest that protocols should be designed to minimize this cell loss.

Figure 1. Crystal Violet-Stained NHEK Cultures Showing Circular Zone of Cell Death



INTRODUCTION

We have observed changes in cell cultures in 96-well plates where significant populations of cells begin to die shortly after refeeding the cell cultures (refeeding is performed by inverting the plate and dumping the spent medium, followed by gentle addition of fresh pre-warmed medium). The zones or areas of cell death appear to occur in a ring around the well inside of the periphery of the wells (Figure 1). The zone of cell death is indicated by nuclear condensation, lack of vital dye uptake and, for certain cell types, complete loss of cells. This effect has been reported by others as well (P.D. Bowman and W. Smith, personal communications). When cultures are refed at low cell densities, the effect manifests itself in large areas nearly devoid of cells, frequently with a confluent outer ring of cells, and small islets of cells near the center of the well. This loss of cells might adversely impact the quality of a variety of microtiter plate-based bioassays.

For example, the Neutral Red Uptake (NRU) cytotoxicity assay is a chemosensitivity assay typically conducted in healthy mammalian cells cultured in 96-well tissue culture plates (Triglia, 1989; NTP, 2003). The assay is based on the ability of viable cells to incorporate and bind neutral red (NR) dye. The total amount of dye bound reflects the number of viable cells present (Barstad, 1991). In a population of proliferating cells, a toxic chemical, regardless of site or mechanism of action, can slow cell proliferation or kill cells outright, which would lead to a reduced number of viable cells compared to the untreated culture. Cytotoxicity is thus expressed as a concentration-dependent reduction in the uptake of NR after chemical exposure thereby providing a sensitive measure of both cell integrity and growth inhibition. In the NRU cytotoxicity assay, cultures are typically treated by removing the spent culture medium and replacing it with treatment medium when the cultures are at relatively low densities. As a consequence, the resulting zone of cell death as described above can affect a relatively large portion of the cell culture surface within each well, thereby reducing the number of viable cells in the treated and control groups, and decreasing the ratio of viable cell signal to background.

It appears that the refeeding step induces the cell death as a result of the meniscus which is formed by the residual medium in the well following removal of the spent culture medium. A series of experiments were conducted to determine the following: 1) what event triggers the induction of cell death during the refeeding step, 2) what is the impact of the culture refeeding step upon the overall cell growth as measured by the relative neutral red uptake of control cultures, 3) what is the impact upon the sensitivity of the NHEK NRU cytotoxicity assay to the positive control, sodium lauryl sulfate, and 4) can the culture-refeeding step immediately prior to test article treatment be eliminated without adversely affecting the cell-based test system.

MATERIALS AND METHODS

Cells and Reagents

- Normal Human Epidermal Keratinocytes (NHEK) (Clonetics, Cambrex Corp.)
- Keratinocyte Growth Medium (KGM) containing 0.1 mM $CaCl_2$ (Cambrex Corp.)
- Positive Control - Sodium Lauryl Sulfate (SLS)
- Neutral Red stock solution (100X) (Sigma): 3.3 mg/mL
- NR Desorb (50% ethanol, 1% glacial acetic acid, in water)

Seeding of NHEK Cells into 96-Well Plates

- NHEK cells were cultured in KGM in T25 flasks and sub-cultured directly into 96-well plates. Within each trial, 2 culture plates (1 plate per test group) were seeded with the standard number of NHEKs (2500 cells/well)
  - **Refeed Group**: 2500 cells seeded in the standard culture volume of 250 µL
  - **No-refeed Group**: 2500 cells seeded in half of the typical volume (125 µL)

- Cultures were incubated for 66 to 72 hours to attain approximately 30% confluence prior to use in the assay.

Positive Control Treatment Procedures

- SLS was prepared as a dilution series of eight 2X concentration dosing solutions in KGM. Final SLS concentrations ranged from 10 to 0.68 µg/mL.
- **Refeed Group**:
  - Medium in the wells was decanted (by inversion of the plates) and the cultures were refed with 125 µL of fresh, pre-warmed KGM.
  - The cultures were then treated by addition of 125 µL of each of the 2X dosing solutions to the appropriate wells. Negative control cultures received 125 µL of fresh KGM.
- **No-refeed Group**:
  - The cultures were treated by direct addition of 125 µL of each of the 2X dosing solutions to the appropriate wells, without refeeding. Negative control cultures received 125 µL of fresh KGM.

- The cultures were incubated in the dosing solutions for 48 hours.
- Prior to assay termination, the cultures were examined microscopically and cell morphology and growth characteristics were evaluated.

Neutral Red Uptake Assay Endpoint

- Filtered neutral red medium (33 µg NR/mL KGM) was prepared immediately before use.
- Wells were rinsed with DPBS, and 250 uL of the NR medium were added to all wells.
- Cultures were incubated with NR medium for 3 hours.
- NR medium was removed and the cultures were rinsed with DPBS. NR Desorb was added to each well, and the plates were agitated for 20 to 45 minutes to extract the NR taken up by viable cells.
- The OD_{550} was determined using a microtiter plate reader.

Additional Experiments

To illustrate the impact of the refeeding step on proliferating cell cultures, NHEK and BALB/c 3T3 cells were cultured in 96-well plates, and refed with NR medium (33 µg/mL) at select times throughout the culture period to highlight the circular zone of cell death. When the cultures reached approximately 30% confluence, one set of cultured NHEK and BALB/C 3T3 cells were refed with NR medium for 3 hours and photographed. A second set of NHEKs were refed with fresh medium and returned to the incubator, and a third set of NHEKs were cultured continuously without refeeding. After an additional 48-hour incubation, the NHEK cultures in both the refed and continuously-cultured groups were refed with NR medium for 3 hours and photographed. Finally, as a control group, one set of NHEK cultures that had been continuously cultured without refeeding was treated by direct addition of a 10X NR medium (for a final concentration of approximately 33 µg/mL), incubated for 3 hours, and photographed.

REPRESENTATION OF MENISCUS EFFECT

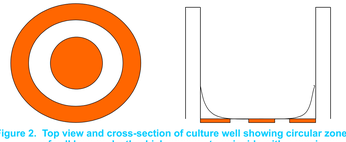


Figure 2. Top view and cross-section of culture well showing circular zone of cell loss or death which appears to coincide with a meniscus of residual medium formed during refeeding.

RESULTS

Table 1 presents the results of the experiment. The neutral red uptake (mean corrected OD_{550} values) for the vehicle control in each trial are presented for the refed and non-refed cultures. Neutral red uptake was significantly reduced ($p < 0.0001$) in the refed culture wells (OD_{550} = 0.830 ± 0.058, mean ± standard deviation, c.v. = 7.0%, n=6 assays) compared to wells that were not refed (OD_{550} = 1.066 ± 0.034, c.v. = 3.2%). The mean IC_{20}, IC_{50} and IC_{80} values for SLS for all of the trials conducted by refeeding the culture wells prior to treatment are 1.40, 3.41, and 8.37 µg SLS/mL, respectively. The 95% confidence interval around the IC_{50} of SLS of the 6 trials is 2.24 to 4.58 µg SLS/mL. The mean IC_{20}, IC_{50} and IC_{80} values for SLS for all of the trials conducted without refeeding the culture wells are 1.42, 3.49, and 8.64 µg SLS/mL, respectively. The 95% confidence interval around the IC_{50} of SLS of the 6 trials is 2.71 to 4.28 µg SLS/mL. Based upon a non-statistical comparison of the two experimental groups, no appreciable change in the sensitivity of the test system to SLS was noted. In these trials, the relative cell coverage within the wells was quite high in both experimental groups. However, the impact upon the sensitivity of the assay system could be quite significant in cases where the zone of cell death affects 30 to 50% of the cell culture surface.

Table 1. A Comparison of Six Replicate Trials of 96 Well Plates With or Without Refeeding

Trial Number and Test Condition	Vehicle Control [3] Mean Corrected OD_{550}	SLS IC_{20} (µg/ml)	SLS IC_{50} (µg/ml)	SLS IC_{80} (µg/ml)
1 Refed[1]	0.824	1.58	3.46	7.58
1 Not Refed[2]	1.084	1.51	3.54	8.30
2 Refed[1]	0.784	1.44	3.72	9.59
2 Not Refed[2]	1.120	1.61	3.89	9.42
3 Refed[1]	0.938	1.59	4.19	11.0
3 Not Refed[2]	1.078	1.56	3.92	9.87
4 Refed[1]	0.775	1.64	3.64	8.07
4 Not Refed[2]	1.037	1.52	3.44	7.79
5 Refed[1]	0.841	0.984	2.68	7.31
5 Not Refed[2]	1.050	0.982	2.87	8.38
6 Refed[1]	0.819	1.16	2.77	6.66
6 Not Refed[2]	1.029	1.35	3.30	8.08

[1] The plate was refed with 125 µL KGM, prior to SLS treatment.
[2] The plate was not refed prior to SLS treatment.
[3] Average of 12 control wells/plate/trial

Microscopic Observations

- At the completion of assays where cultures were refed prior to treatment, large areas within the negative control wells were devoid of viable cells, while the periphery of the wells were nearly confluent (see Figures 7 and 8).
- In BALB/C 3T3 cells, the zone of cell death induced during the initial refeeding step became re-populated during the 48-hour treatment phase. This suggests that the effect may be less detectable in cells that are more migratory in culture.
- The circular zone of cell death coincides with the observed formation of a meniscus of residual medium following medium removal (see Figures 3 through 6).
- Microscopic observations of cultures within the circular zone of cell death revealed nuclear changes (condensing nuclei) within 1 hour after refeeding with fresh medium.
- In cultures allowed to grow to a confluent lawn, the impact of removing the spent culture medium and refeeding with NR medium is clearly evident in the induction of a circular zone of cells lacking the ability to retain NR, (see Figures 9 and 10)
- In a set of control cultures allowed to grow to a confluent lawn, no zone of cell death was induced in cells after the direct addition of a 10X NR to the culture medium. Since the cultures were never refed by emptying the wells, this suggests that the induction of the zone of cell death is due to the removal of medium (see Figures 11 and 12).

BALB/C 3T3 cells - Cultures were refed when approximately 30% confluent, and stained with NR to highlight induced zone of cell death and cell loss. Circular zone of cell loss coincides with the meniscus of residual medium (shaded region in Figure 3).

BALB/c 3T3

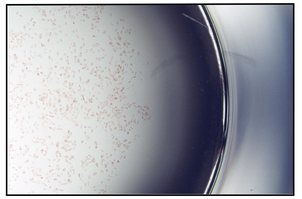


Figure 3. 37X Magnification

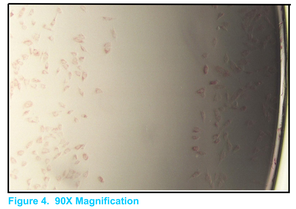


Figure 4. 90X Magnification

NHEK cells - Cultures were refed when approximately 30% confluent, and stained with NR to highlight induced zone of cell death. Note the absence of neutral red uptake in cells within the circular zone of cell death. Circular zone coincides with the meniscus of residual medium (shaded region in Figure 5).

NHEK

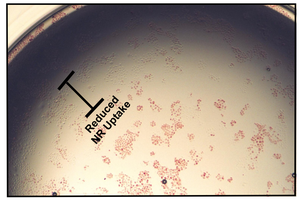


Figure 5. 37X Magnification

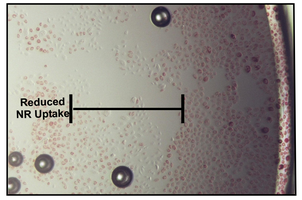


Figure 6. 90X Magnification

NHEK cells - Cultures refed at 30% confluency, and cultured for an additional 48 hours before evaluation. Cultures were refed and stained with NR. Note the large areas within the wells devoid of viable cells, while periphery is nearly confluent.

NHEK

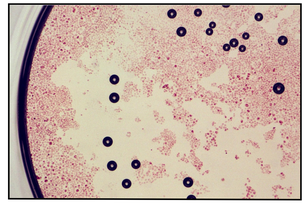


Figure 7. 37X Magnification

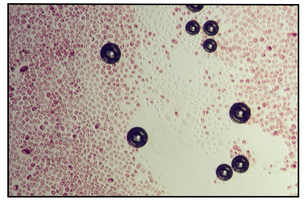


Figure 8. 90X Magnification

NHEK cells - Cells were not refed, but cultured continuously to a confluent lawn. Cultures were refed and stained with NR to highlight induced zone of cell death and cell loss. Note the absence of NRU in cells within the circular zone.

CONFLUENT NHEK CULTURES REFED ONLY WITH NR

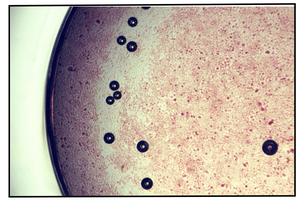


Figure 9. 37X Magnification

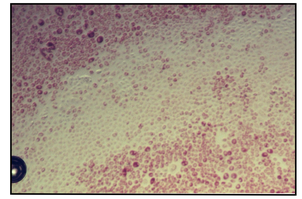


Figure 10. 90X Magnification

Control group of NHEK cells - Cells were not refed, but cultured continuously to a confluent lawn. Cultures were stained with NR by direct addition of a 10X NR medium to eliminate refeed step. No evidence of reduced NRU or zone of cell death.

CONFLUENT NHEK CULTURES NOT REFED

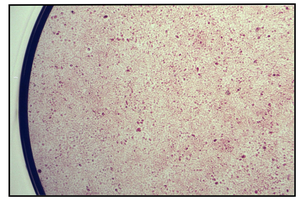


Figure 11. 37X Magnification

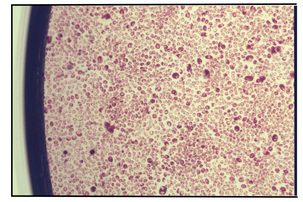


Figure 12. 90X Magnification

CONCLUSIONS

- The removal of spent culture medium during the culture refeeding step induces a circular zone of cell death or cell loss in micro-titer plates. The zone of cell death occurs within a predictable ring around the edges of the multi-well plate wells, coinciding with the formation of a meniscus from the residual medium following removal of the spent culture medium.
- This effect appears in larger well formats (e.g., 24-well plates, data not shown) but has a greater impact in smaller well formats (96-well plates) where the ratio of wall circumference to cell area is greater.
- The impact of these effects tend to be more pronounced when cultures are refed at relatively low confluence, resulting in areas devoid of cells. The zone is also more evident with cell types where cell migration is limited.
- The induction of cell death by removing the spent culture medium may have deleterious effects upon the endpoints of other bioassays (gene expression, live vs. dead cell staining, etc.).
- Protocols may be modified to eliminate culture refeeding steps in micro-titer plate bioassays to prevent the induction of the zone of cell death and cell loss.

REFERENCES

Barstad, R., Cortesi, J. And Janus, J. (1991) Use of Clonetics' neutral red bioassay to optimize components of serum-free medium for normal human anchorage-dependent cells. *In Vitro Cellular and Developmental Biology* 27(3), 160.

Triglia, D., P.T. Wegener, J. Harbell, K. Wallace, D. Matheson, and C. Shopsis. 1989. Interlaboratory validation study of the keratinocyte neutral red bioassay from Clonetics Corporation. In *Alternative Methods in Toxicology*, Volume 7. A.M. Goldberg, ed., pp. 357-365. Mary Ann Liebert, Inc., New York.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) Phase II Validation Test Method Protocol for the Normal Human Keratinocyte (NHK) Neutral Red Uptake Cytotoxicity Test, A Test for Basal Cytotoxicity