EYE AND SKIN IRRITATION IN 3-D TISSUE CONSTRUCTS USING MTT AND ATP ENDPOINTS

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Abstract

The irritation potential of formulations and ingredients for industrial screening and product development is often conducted using *in vitro* 3-D human ocular and epidermal tissue constructs. To predict irritation potential after chemical exposure, tissue viability is typically determined by the ability of live cells to reduce MTT. Toxic exposures result in decreases in relative MTT reduction. However, two issues may contribute to inaccurate viability assessment: subtoxic exposures that induce higher metabolic rates typically greater than controls (i.e., hormesis) and chemicals that directly reduce MTT causing an overestimation of tissue viability (e.g., NaOH, α-tocopherol (α-t), ascorbic acid). For such chemicals, residues left on the tissues may increase the total MTT signal, so freeze-killed tissues are used to estimate chemical-mediated reduction of MTT. However, alternative methods of measuring tissue viability, such as amount of adenosine triphosphate (ATP) may be used. We compared these two methods by testing a series of model mild skin care formulations in 3-D human eye and skin constructs. The formulations were spiked with various concentrations of Triton® to induce a range of toxic effects, and were prepared with and without α-t, a MTT reducer. For formulations with α-t, freeze-killed tissues were tested in parallel in both the MTT and ATP assays. The results showed the same irritancy predictions for the 4 formulations containing α-t as for the 4 control formulations without α-t (e.g., formula with highest Triton conc.: ET_{50} eye = 172 and 157 min, ET_{50} skin = 778 and 772 min, with and w/o α-t). The ATP assay provided the same rank order of irritancy as did the MTT assay although the relative viability values from the ATP assay at each exposure were overall lower (e.g., formula with highest Triton conc.: ET_{50} eye = 14.5 and 12.9 min, ET_{50} skin = 202 and 231 min, with and w/o α-t). In summary, the MTT assay of formulas capable of MTT reduction should include freeze-killed tissues, and the ATP assay can confirm the relative rank order of the irritancy predictions.

Introduction

In vitro eye and skin model assays are typically used to assure safety prior to consumer use by employing them in product development to support the creation of products with minimal irritation potential. As part of a high quality program, the test systems are constantly monitored for applicability, investigated for potential limitations, and continuously improved to ensure accurate and reliable data and information to support safety assessments. The applied research presented here evaluates an additional endpoint of consideration in special cases where potential confounders may skew interpretation of results in core standard model systems.

MTT Assay

Viability assessments in 3-D *in vitro* eye and skin constructs have historically been assessed using the MTT assay. The MTT conversion assay measures the NAD(P)H-dependent microsomal enzyme and succinate dehydrogenase reduction of MTT to a blue formazan precipitate in viable cells (Berridge, 1996). Decreases in tissue viability resulting from exposures to toxic chemicals result in decreases in MTT reduction. Two factors can affect the accuracy of the MTT assay. First, since the MTT assay measures the mean metabolic rate of a cell population, subtoxic exposures may induce hormesis, where increased metabolism in response to cell damage incorrectly suggests high viability. In practice, the impact of hormesis on the cytotoxicity endpoint is minimized by selecting test chemical exposures which result in a full range of cytotoxic responses. Second, chemicals that directly reduce MTT (e.g., NaOH, α-tocopherol, ascorbic acid) may overestimate tissue viability if these chemicals persist in the tissue model after rinsing. Freeze-killed tissue controls (KC) are tested in parallel to the viable tissues to determine the extent, if any, of the direct reduction by the test chemical.

Assessment of Direct MTT Reduction

- 100 µL of the test chemical are added into 1 mL of a 1.0 mg/mL MTT solution
- The mixture is incubated in the dark at 37±1ºC in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for at least one hour
- If the MTT solution color turns blue/purple, the test chemical is presumed to directly reduce MTT. Aqueous-insoluble chemicals often show direct reduction (darkening) only at the interface between the test chemical and the MTT solution

Freeze-killed Tissue Controls (KC)

- Freeze-killed tissue controls (KC) are prepared by freezing 3-D tissue constructs at -20±5ºC at least overnight, thawing at room temperature, and then refreezing the tissues
- To evaluate the impact of the MTT reducing test chemicals, KC tissues are treated with the test chemicals
- All MTT viability assay procedures are performed as for the viable tissues
- A negative control KC treated with sterile deionized water is tested since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue

If little or no MTT reduction is observed in the test chemical KC, the MTT reduction in the test chemical-treated viable tissue is ascribed to the viable cells. If there is appreciable MTT reduction in the test chemical KC, additional calculations are performed to account for the chemical reduction.

ATP Assay

The adenosine triphosphate (ATP) endpoint is an alternative to MTT reduction which would not be affected by hormesis since the endpoint measures cellular ATP content, rather than metabolic rate. The ATP endpoint may also be more appropriate for chemicals that are strong reducers of MTT, including many that are commonly used in personal care products (α-tocopherol, ascorbic acid).

The ViaLight® Plus ATP assay kit utilizes the bioluminescent measurement of ATP (Crouch, *et al.*, 1993). Viable cells maintain a constant quantity of ATP, although some small variations would be expected depending upon the metabolic status and function of the cell (Beis and Newsholme, 1975). Upon cell stress or cell death, the amount of ATP is rapidly depleted or hydrolyzed. Since cytotoxicity is expressed as a reduction in the bioluminescent measurement of ATP, the assay provides a direct measure of the number of viable cells present.

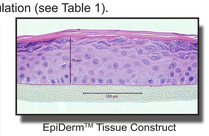
Materials and Methods

This poster presents the results of a comparison of the MTT and ATP endpoints. A series of model skin care formulations containing a range of concentrations of Triton® (to induce a range of cytotoxic effects) were tested, with and without the MTT reducer α-tocopherol (α-t), in the EpiOcular™ and EpiDerm™ 3-D *in vitro* eye and skin constructs. The MTT reducer, α-t, was not expected to add or reduce any cytotoxic effects in the formulation (see Table 1).

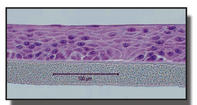
TABLE 1
Test Material Formulations

Designation	Base*	2% α-t (w/v)	Triton® X-100 (w/v)	EpiOcular™	EpiDerm™
0%Tx	X		0 %	X	X
0.03%Tx	X		0.03 %	X	X
0.3%Tx	X		0.3 %	X	
3%Tx	X		3.0 %	X	X
10%Tx	X		10 %		X
0%Tx w/ α-t	X	X	0 %	X	X
0.03%Tx w/ α-t	X	X	0.03 %	X	X
0.3%Tx w/ α-t	X	X	0.3 %	X	
3%Tx w/ α-t	X	X	3.0 %	X	X
10%Tx w/ α-t	X	X	10 %		X

* – Base is a mild personal care formulation. The formulation is described as a white semi-viscous lotion. Previous testing in 3-D tissues showed the formulation to be very mild. Formulation residues frequently persisted on the tissues after rinsing.
α-t – α-tocopherol
Tx – Triton® X-100



EpiDerm™ Tissue Construct



EpiOcular™ Tissue Construct

Test System Treatment

- The EpiOcular™ and EpiDerm™ cultures were tested in duplicate tissues at four to six exposure times close to the putative ET_{50}
- Exposure times ranged from 15 minutes to 24 hours
- 100 µL of the test formulations were applied topically on the tissues
- 100 µL of the negative control (sterile, deionized water) were tested concurrently
- Treated tissues were incubated at standard culture conditions

Treatment Termination

- After the exposures, the 3-D cultures were extensively rinsed with Calcium and Magnesium-Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS)
- EpiOcular™ tissues were transferred to 5 mL of Assay Medium for a 10 to 20 minute soak at RT after rinsing
- EpiDerm™ tissues were not soaked and were assayed for viability immediately after treatment termination

MTT Assay Procedures

- After treatment termination, cultures were transferred into 24-well plates containing 0.3 mL of a 1.0 mg/mL MTT solution for 3 hours at standard culture conditions.
- Tissues were transferred into 2 mL isopropanol to extract the reduced MTT
- The absorbance of the extraction solution was measured at 550nm (OD_{550})

Presentation of Data

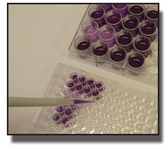
- Corrected OD_{550} values for each tissue were calculated by subtracting the mean blank OD_{550} value from the individual tissue OD_{550} values
- Corrected OD_{550} values for the test formulation-treated killed controls (KC) were calculated by the following equation to determine if there was significant MTT reduction from test chemical residues

$$\text{NET } OD_{550} \text{ Test Chemical KC} = \text{Raw } OD_{550} \text{ Test Chemical KC} - \text{Raw } OD_{550} \text{ negative control KC}$$

- If the net OD_{550} value was greater than 0.150, the net OD_{550} value was subtracted from the corrected OD_{550} values of the viable treated tissues to obtain a final corrected OD_{550} value
- % of Control values were calculated for each treatment group by the following equation:

$$\% \text{ of Control} = \frac{\text{corrected test formulation } OD_{550}}{\text{corrected exposure time control } OD_{550}} \times 100$$

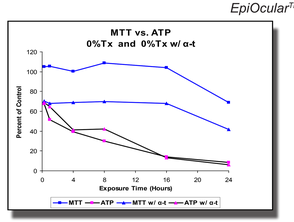
- Exposure time response curves were plotted presenting % of Control vs. formulation exposure time
- ET_{50} values were interpolated from the response curves

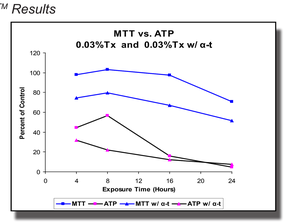


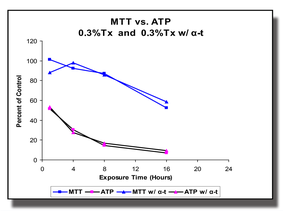
Results

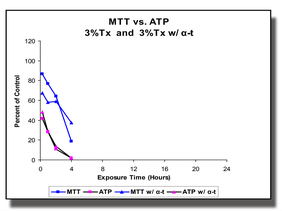
- Test formulation residues on freeze killed tissues frequently showed >0.150 net OD_{550} values using the MTT endpoint (not shown in figures).
- After correction for direct MTT reduction in killed control tissues, exposure time responses of formulation with α-t were similar to, or less than, exposure time responses of formulation without α-t using the MTT endpoint.
- Test formulation residues on freeze killed tissues showed no ATP signals using the ATP endpoint.
- Exposure time responses of formulation with α-t were similar to the exposure time responses of formulation without α-t using the ATP endpoint.
- Both MTT and ATP endpoints predicted progressively shorter exposure time responses in formulations with increasing concentrations of Triton®.
- Relative viability values using the ATP endpoint were notably lower than those from the MTT endpoint. Relative viability by the ATP endpoint was less than 100% of control for the Base formulation in both tissue models.

EpiOcular™ Results

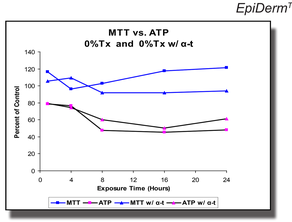


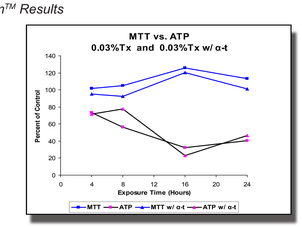


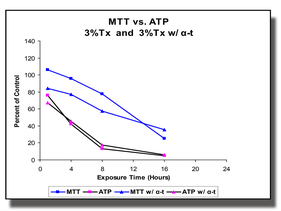


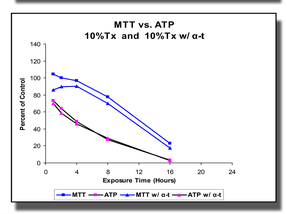


EpiDerm™ Results









ATP Assay Procedures

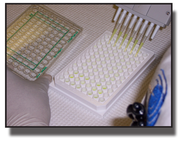
- After treatment termination, cultures were transferred into 6-well plates containing 1.3 mL of a CMF-HBSS/Cell Lysis Reagent mix and incubated at RT for 60 minutes to assure complete lysis of the 3-D tissues.
- 100 µL of a 1:4 dilution of each tissue lysate were added to a clear-bottom white-walled 96-well plate.
- A dilution series of an ATP standard and a blank were prepared and loaded onto the 96-well plate in parallel.
- 100 µL of AMR PLUS reagent were added to each well and the plates were incubated at RT for at least 2 min.
- The light emission (565 nm) of each well was measured using a Berthold Detection Systems Orion II luminometer. Data were recorded as relative light units (RLUs).

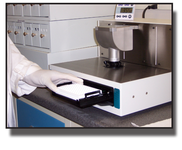
Presentation of Data

- Corrected RLU_{565} values for each tissue were calculated by subtracting the mean blank RLU_{565} value from the individual tissue RLU_{565} values
- % of Control values were calculated for each treatment group by the following equation:

$$\% \text{ of Control} = \frac{\text{corrected test formulation } RLU_{565}}{\text{corrected exposure time control } RLU_{565}} \times 100$$

- Exposure time response curves were plotted presenting % of Control vs. formulation exposure time
- ET_{50} values were interpolated from the response curves





Conclusions

- Freeze-killed control tissues demonstrated that test chemical (α-tocopherol) residues on treated tissues could directly reduce MTT in addition to the MTT reduction from viable cells.
- Relative viability values from the MTT endpoint can be corrected for the amount of MTT reduced directly by test chemical residues in freeze-killed tissue controls. In some cases, the correction for direct MTT reducers gave a more conservative irritancy prediction.
- Test chemical (α-tocopherol) residues on killed control tissues had no impact upon the ATP endpoint.
- Both the MTT and ATP endpoint assays provided the same rank order of irritancies for the model formulations.
- Since the relative viability values from the ATP assay were lower than those from the MTT assay, the ET_{50} values from the ATP endpoint may not be directly substituted for those from the MTT endpoint.
- No evidence of hormesis was observed in the ATP endpoint data.

Recommendations

Reference materials and benchmarks should ideally be included in all studies

- Provide basis for interpreting test material results even when using alternate endpoints
- Should be from same product class, with *in vivo* or market history
- May be used to establish the acceptability thresh-holds for evaluating test formulations

References

Beis I., and Newsholme E. A. (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. Biochem J 152: 23–32.

Berridge, M. V., Tan, A. S., McCoy, K. D., and Wang, R. (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts, Biochemica, 4, 14-19

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Lonza Walkersville research products were used for this study. Jeffrey Kelly, Lonza Walkersville, USA.