# Development of an in vitro test method for irritation of medical devices used in the oral cavity

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## Abstract

Any medical device (MD) intended for use in oral cavity needs to be evaluated. This project focuses on development and validation of in vitro assay to assess the oral irritation of MDs. This assay is intended to replace historical in vivo assay performed on Syrian hamsters. The ISO 10993-23 standard requires in vitro irritation testing based on reconstructed human epidermis (RhE) for evaluation of MDs prior to animal or human patch testing is performed. However, RhE models are not appropriate for MDs designed for use in oral cavity, therefore ISO recommends use of other in vitro models produced from relevant cells. EpiOral tissue model consists of normal, human-derived oral epithelial cells cultured to form multilayered, highly differentiated model of the human buccal tissue. Produced commercially for more than 15 years, several methods have been developed to study oral penetration, drug delivery, and irritancy of oral care products such as toothpastes, mouthwashes, and orthodontic devices. To assess the feasibility of an in vitro method, initial experiments tested solutions of irritant chemicals contained in MDs designed for oral cavity. Increasing concentrations of ethanol, lactic acid, methyl methacrylate, sodium dodecyl sulfate (SDS), phosphoric acid, sodium hypochlorite, hydrogen peroxide, and chlorhexidine digluconate in NaCl or sesame oil were applied to the EpiOral model. The time required to reduce tissue viability by 50% (ET-50), was determined. The results showed a clear relationship between tissue viability and exposure time and between ET-50 and concentration of the irritant chemical. Compared to historical in vivo data, the in vitro method classified the samples containing an irritant at the expected concentration. In addition, the ET-50s allowed differentiation between strong and mild irritants. The data demonstrate that this in vitro assay has equivalent or superior performance to in vivo method. The next step of the project is to assess the irritation potential of several marketed medical devices, some of which are known to induce irritant responses in vivo. We welcome other stakeholders (producer of medical devices, regulators, and other interested parties) to join us as we further develop the assay method and move it into the validation process.

## Methods

**Tissue preparation:** EpiOral tissues consist of normal, human-derived oral epithelial cells. The cells have been cultured on specially prepared cell culture inserts (0.6 $cm^2$ surface) for up to 7 days in serum-free medium to form multilayered (8-11 cells layers), highly differentiated 3D models of the human buccal phenotype. The EpiOral tissue models exhibit in vivo-like morphological and growth characteristics which are uniform and highly reproducible (**Figure 1**).

**In vitro irritation testing:** Following overnight storage at 4ºC (to mimic standard delivery times), the tissues were pre-incubated for 1 hr in 6-well plates with 0.9 mL of fresh medium under standard culture conditions (5% $CO_2$, 37°C). On the day of the experiment, the tissues were transferred to fresh medium and 100 µL of the test solution diluted in a polar (saline, 0.9% NaCl) or non-polar solvent (sesame oil) were applied to the apical surface of N=2 tissues. The exposure was stopped by washing the tissues with PBS after 1, 4, or 18 hours. Cell viability of the tissues was determined by MTT viability assay.

**MTT viability assay:** Following treatment with the various chemicals, tissue viability was determined using the MTT assay. % viability was determined using the equation:

% viability = OD (treated tissue)/OD (control tissue)*100.

**ET-50 calculation:** Using a semi-log scale, the % viability (linear y axis) is plotted versus the dosing time (log x axis). Using mathematical interpolation, the time at which the viability dropped to 50% was determined (ET-50). If the 50% decrease was reached within the first hour, the ET-50 was scored "<1 hr“ and the test article is considered highly irritating. If cell viability is above 50% at 18 hours, the ET-50 was scored ">18 hr“ and the material is considered non-irritating.

## Results

| Chemicals | C° | Saline 1h | Saline 4h | Saline 18h | Sesame Oil 1h | Sesame Oil 4h | Sesame Oil 18h |
|---|---|---|---|---|---|---|---|
| **Hydrogen peroxide** | 1% | 75.8 | 73.5 | 68.0 | 111.0 | 103.7 | 132.5 |
| | 3% | 60.0 | 52.5 | **29.7** | 103.0 | 95.6 | 119.8 |
| | 10% | **11.1** | **5.7** | **6.4** | **10.1** | **5.5** | **9.7** |
| **Sodium hypochlorite** | 0.10% | 108.8 | 99.2 | 84.0 | | | |
| | 0.20% | 88.7 | 99.6 | 101.3 | 76.1 | 108.3 | 67.2 |
| | 1% | **4.9** | **4.2** | **6.2** | **45.0** | 94.6 | **9.5** |
| | 2% | **4.9** | **4.2** | **7.8** | **34.2** | **24.8** | **8.5** |
| | 10% | **0.8** | **1.9** | **2.8** | | | |
| **SDS** | 0.10% | 107.2 | 110.8 | 109.2 | | | |
| | 1% | 80.6 | **11.1** | **4.9** | 109.6 | 52.0 | **10.8** |
| | 3% | 50.2 | **7.6** | **3.3** | 90.5 | 24.2 | **3.4** |
| | 5% | **22.2** | **4.7** | **2.8** | 79.9 | 18.3 | **3.0** |
| **Lactic acid** | 0.10% | 93.1 | 90.2 | 101.2 | | | |
| | 0.50% | 88.2 | 81.1 | 81.6 | | | |
| | 1% | 90.7 | **38.8** | **18.7** | | | |
| | 4% | **11.1** | **9.9** | **9.0** | | | |
| | 5% | **14.7** | **8.9** | **12.3** | | | |
| **Phosphoric acid** | 0.10% | 105.1 | 100.9 | 93.6 | | | |
| | 1% | 96.6 | **15.5** | **12.3** | | | |
| | 5% | **15.1** | **14.4** | **15.0** | | | |
| | 10% | **14.7** | **17.6** | **19.8** | **14.7** | **14.1** | **16.7** |
| | 25% | **10.9** | **14.0** | **16.6** | **13.4** | **14.6** | **17.2** |
| | 50% | **1.4** | **4.7** | **9.3** | **4.1** | **7.1** | **11.8** |
| **Methyl methacrylate** | 0.10% | 103.1 | 92.4 | 109.5 | | | |
| | 0.50% | 101.0 | 105.8 | 118.2 | | | |
| | 1% | 95.1 | 90.8 | 100.3 | | | |
| | 5% | 102.4 | 101.3 | 94.1 | | | |
| | 25% | 108.9 | 104.7 | 91.6 | 95.9 | **49.3** | **39.9** |
| | 50% | 108.9 | **8.2** | **22.6** | 93.3 | **30.5** | **6.6** |
| **Methyl laurate** | 0.10% | 90.9 | 90.2 | 101.5 | | | |
| | 0.50% | 101.4 | 103.7 | 111.8 | | | |
| | 1% | 100.6 | 101.9 | 114.2 | | | |
| | 5% | 96.5 | 88.0 | 103.4 | | | |
| **1-Decanol** | 1% | 100.4 | 90.5 | 108.9 | 99.1 | 91.4 | 121.1 |
| | 5% | 99.9 | 96.3 | 121.7 | 107.9 | 111.3 | 102.1 |
| | 10% | 98.8 | 98.9 | 113.1 | 118.0 | 95.6 | **15.6** |
| | 100% | 131.8 | 96.3 | **8.1** | | | |
| **Ethanol** | 25.0% | 84.2 | 101.9 | 87.9 | 81.3 | 89.4 | 85.3 |
| | 50% | 71.1 | 70.0 | **39.1** | 83.4 | 75.9 | 68.8 |
| | 100% | 55.6 | **23.9** | **7.6** | | | |
| **Chlorhexidine digluconate** | 0.2% | 104.2 | 102.2 | 87.6 | 125.5 | 131.1 | **24.9** |
| | 1% | 103.2 | 105.4 | 89.7 | | | |
| | 2% | 106.3 | 109.7 | 95.7 | **37.0** | **13.2** | **7.3** |
| | 10% | **15.9** | **9.3** | **5.5** | **16.1** | **10.7** | **8.6** |

**Table 1:** Tissue viability (%) of the 10 chemicals tested at different concentrations in saline (NaCl) or sesame oil (SO). Toxic exposure conditions (<50% viability) highlighted in **bold**.

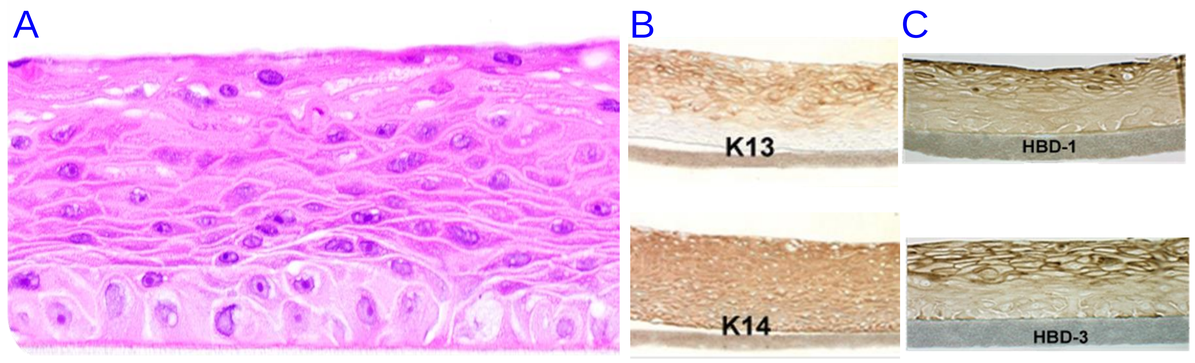



**Figure 1:** **A**) H&E staining of the EpiOral tissue model. **B**) The tissues express cytokeratins 13 and 14 similar to their corresponding native oral tissues. **C**) The tissues also produce the antimicrobial peptides called human beta defensins (HBDs). EpiOral constitutively expresses HBD-1 and HBD-3.

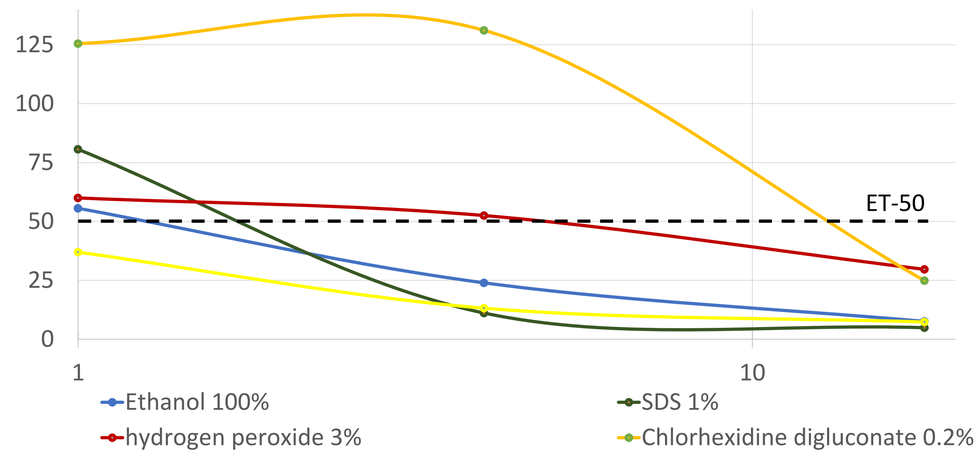
ET-50 for several concentrations in **Saline**

| | 0.10% | 0.20% | 0.50% | 1% | 2% | 3% | 4% | 5% | 10% | 25% | 50% | 100% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sodium Hypochlorite** | > 18 | > 18 | | < 1 | < 1 | | | | < 1 | | | |
| **SDS** | > 18 | | | 1.8 | | 1.0 | | < 1 | | | | |
| **Phosphoric acid** | > 18 | | | 2.2 | | | | < 1 | < 1 | < 1 | < 1 | |
| **Lactic Acid** | > 18 | | > 18 | 3.0 | | | < 1 | < 1 | | | | |
| **Hydrogen peroxide** | | | | > 18 | | 4.7 | | | < 1 | | | |
| **Chlorhexidine digluconate** | | > 18 | | > 18 | > 18 | | | | < 1 | | | |
| **Methyl methacrylate** | > 18 | | > 18 | > 18 | | | | > 18 | | > 18 | 2.2 | |
| **Ethanol** | | | | | | | | | | > 18 | 10.6 | 1.3 |
| **1-Decanol** | | | | > 18 | | | | > 18 | > 18 | | | 8.8 |
| **Methyl laurate** | > 18 | | > 18 | > 18 | | | | > 18 | | | | |

ET-50 for several concentrations in **Sesame Oil**

| | 0.10% | 0.20% | 0.50 % | 1% | 2% | 3% | 4% | 5% | 10% | 25% | 50% | 100% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sodium Hypochlorite** | | | | | | | | | | | | |
| **SDS** | | | | 6.2 | | 1.9 | | 3.0 | | | | |
| **Phosphoric acid** | | | | | | | | | < 1 | < 1 | < 1 | |
| **Lactic Acid** | | | | | | | | | | | | |
| **Hydrogen peroxide** | | | | > 18 | | > 18 | | | < 1 | | | |
| **Chlorhexidine digluconate** | | 12.6 | | | < 1 | | | | < 1 | | | |
| **Methyl methacrylate** | | | | | | | | | | 3.9 | 2.6 | |
| **Ethanol** | | | | | | | | | | > 18 | > 18 | |
| **1-Decanol** | | | | > 18 | | | | > 18 | 9.4 | | | |

**Table 2:** ET-50 calculated from tissue viability (data in **Table 1**). Using the ET-50, materials can be classified over a broad range of irritancy, from strong irritants, such as sodium hypochlorite (GHS cat.1B) and phosphoric acid, to very mild/ non-irritants such as ethanol (used in mouthwashes) and 1-decanol (classified non-irritant or mild irritant GHS cat. 3 in skin).




| | Conc. | In Vivo Park et al., 2015 | | In Vitro ET-50 (hrs) | |
|---|---|---|---|---|---|
| **Hydrogen peroxide** | 3% | I | moderate | 4.7 | in Saline |
| **SDS** | 1% | I | moderate | 1.8 | in Saline |
| **Ethanol** | 100% | I | moderate | 1.3 | in Saline |
| **Chlorhexidine** | 2% | I | mild | <1 | in SO |
| **Chlorhexidine** | 0.2% | I | mild | 12.6 | in SO |

**Figure 2:** In vivo/in vitro comparison for 5 items between the in vivo Syrian Hamster test (oral irritation according to ISO 10993-23 on irritation of medical devices) and the in vitro method ET-50 with the EpiOral model. The 5 products were classified as irritants by the in vitro test that was able to distinguish between moderate and mild. Chlorhexidine digluconate at 2%, classified mild irritant in vivo, was classified strong in vitro.

## Conclusions

- The in vitro method can be used to identify and rank the irritancy of chemicals/solutions used in the oral cavity.
- Calculation of ET-50 allows this method to be applied to products ranging from non-irritating/mildly irritating to highly irritating.
- The in vitro method gave results that matched the historical in vivo animal tests for the 5 products (for which in vivo data were available).
- The next step will be to evaluate the EpiOral method with marketed medical devices, some of which are known to induce irritant responses in vivo.

## References

Park, K., Kim, K. & Kim, B., 2015. Condition Setting for Oral Mucosal Irritation Evaluation using Hamster Cheek Pouch. J Environ Health Sci., 41(6), pp. 405-411.