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Optimisation of Cell-seeding Density and Incubation Time for the MTS Assay in Human Oral Cell Models

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ABSTRACT

The MTS assay measures cellular metabolic activity, and its absorbance is influenced by the cell model, number of cells seeded, culture duration and incubation time after reagent addition. This study optimised cell-seeding density and MTS incubation time for human gingival fibroblasts (HGF), dental pulp stem cells (DPSC), human oral keratinocytes (HOK) and ORL-48 oral squamous cell carcinoma cells. Model-specific density series were cultured for 72 h before MTS reagent was added. Reference- and blank-corrected absorbance was measured at 490 nm, using 690 nm as the reference wavelength, at 0.5, 1, 2, 3 and 4 h after reagent addition. An investigator-defined absorbance range of 0.5-1.5 was used as a practical criterion for condition selection; it was not treated as a universal linear range. Each condition was tested in technical triplicate in three independent experiments. Absorbance increased with seeding density in all models. Descriptive linear regression yielded R^2 values of 0.9598 for HGF, 0.9773 for DPSC, 0.8889 for HOK and 0.8754 for ORL-48. At the 2-h measurement, 2×10^4 cells/well for HGF and DPSC and 8×10^3 cells/well for HOK and ORL-48 produced absorbance values within 0.5-1.5. Two hours was the earliest measurement at which all four models met this criterion. Subsequent mean values showed no marked increase through 4 h on descriptive visual assessment; no quantitative plateau threshold was specified. The selected conditions are specific to the tested 72-h culture and reader configuration and require separate verification at other endpoints.

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1. INTRODUCTION

Cell-based metabolic assays are commonly used to screen candidate drugs, plant extracts, bioactive compounds and biomaterials before selected concentrations proceed to functional or molecular studies. In oral wound-healing research, an unsuitable baseline signal may distort concentration selection for subsequent migration or wound-closure experiments. Assay conditions should therefore be established for the cell model and planned culture duration before treatment screening begins (Niles et al., 2009; Adan et al., 2016).

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay estimates cellular metabolic activity. Cellular reducing activity converts MTS, through an intermediate electron acceptor, into a water-soluble formazan product measured by absorbance. Unlike the conventional MTT assay, the soluble product does not require a crystal-solubilisation step (Cory et al., 1991; Riss et al., 2016; Ghasemi et al., 2021).

MTS absorbance is not a direct cell count or a specific measure of proliferation. Tetrazolium reduction varies with the cell model, culture condition, growth phase, passage, nutrient availability and treatment (Berridge et al., 2005; Stockert et al., 2018). Changes in MTS signal may reflect changes in the number of metabolically active cells, metabolic activity per cell, or both. When the biological interpretation is important, the result should therefore be confirmed using a complementary method that measures the relevant outcome directly (Riss et al., 2016; Kamiloglu et al., 2020).

The number of cells seeded and the incubation period after MTS addition strongly influence absorbance. Too few cells may produce a signal close to the blank, whereas too many cells may lead to high baseline absorbance after prolonged culture (Ghasemi et al., 2021). Similarly, a short reagent incubation may produce a weak signal, while a longer incubation may continue to increase formazan formation and reduce the capacity to detect further increases. Cell-seeding density, culture duration and reagent incubation time should therefore be evaluated together (Cory et al., 1991; Niles et al., 2009; Riss et al., 2016).

The present study examined four biologically distinct oral cell models. Human gingival fibroblasts (HGF) and dental pulp stem cells (DPSC) represent connective-tissue and regenerative contexts, respectively (Gronthos et al., 2000; Huynh et al., 2016). Human oral keratinocytes (HOK) represent the epithelial component relevant to re-epithelialisation (Hujiahemaiti et al., 2018). ORL-48 is an oral squamous cell carcinoma cell line and was included as a malignant comparator, not as a wound-healing model (Hamid et al., 2007; Ahmad et al., 2024). The comparison was intended to establish assay conditions for each cell model; MTS alone was not used to infer wound healing or cancer-cell proliferation.

Published oral cell studies have used different cell sources, seeding densities, tetrazolium reagents, reagent exposure periods and culture endpoints (Nizam et al., 2014; Tabatabaei & Torshabi, 2016; Hujiahemaiti et al., 2018; Kamaruddin et al., 2019). These differences indicate that a condition used for one model should be verified before it is applied to another. For the present study, the 72-h culture period was used as the primary decision endpoint because it was the longest planned duration and therefore carried the greatest risk of excessive baseline absorbance.

This study optimised cell-seeding density and post-reagent MTS incubation time for HGF, DPSC, HOK and ORL-48 cells after 72 h of culture. An investigator-defined absorbance range of 0.5-1.5 was used as a practical working range to exclude signals close to background and high baseline signals that could

limit detection of further increases. This range was a study-specific selection criterion, not a universal linear range for the reader or the MTS assay. The aim was to determine whether the four models required different starting densities and whether one MTS incubation time was suitable for all four models under the tested conditions.

2. MATERIALS AND METHODS

2.1 Study design

This *in vitro* methodological study optimised cell-seeding density and MTS reagent incubation time for HGF, DPSC, HOK and ORL-48 cells. The 72-h culture period was the primary decision endpoint because it was the longest intended treatment duration and therefore presented the greatest risk of high baseline absorbance. Conditions selected at 72 h were regarded as candidate starting conditions for later experiments at shorter endpoints; they were not considered validated at 24 or 48 h in the present report.

2.2 Cell models and culture conditions

HGF cells were obtained from the American Type Culture Collection (ATCC; CRL-2014, Manassas, VA, USA) and used between passages 3-8. DPSC cells were obtained from Lonza Walkersville, Inc. (catalogue no. PT-5025, Walkersville, MD, USA) and used between passages 2-10. HOKs were obtained from ScienCell Research Laboratories (catalogue no. 2610, Carlsbad, CA, USA) and used between passages 3-9. ORL-48 cells were provided by Cancer Research Malaysia and used between passages 15-35.

HGF, DPSC and ORL-48 cells were maintained in Dulbecco's modified Eagle medium/Ham's F-12 (DMEM/F-12; Gibco, USA) supplemented with 10% foetal bovine serum FBS; Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA). HOK cells were maintained in complete Oral Keratinocyte Medium (OKM; catalogue no. 2611, ScienCell Research Laboratories, Carlsbad, CA, USA), supplemented with Oral Keratinocyte Growth Supplement (OKGS; catalogue no. 2652, ScienCell Research Laboratories, Carlsbad, CA, USA) and penicillin–streptomycin solution (catalogue no. 0503, ScienCell Research Laboratories, Carlsbad, CA, USA), following the culture method described by Hujiahemaiti et al. (2018). HOKs were cultured in poly-L-lysine-coated T25 or T75 culture flasks (Thermo Fisher Scientific Nunc, USA). All cells were cultured as adherent monolayers in a humidified CO_2 incubator (CelCulture®, Esco Micro Pte. Ltd., Singapore) at 37 °C with 5% CO_2. Cultures were monitored routinely and sub-cultured before reaching full confluence; cells at approximately 75–80% confluence were used for the optimisation experiments.

2.3 Optimisation of cell-seeding density and MTS reagent incubation time

Cells were seeded into 96-well plates (Thermo Fisher Scientific Nunc, USA) in 100 µL of the corresponding complete culture medium per well. HGF and DPSC were each tested at 3×10^3, 8×10^3, 1×10^4, 2×10^4, 3×10^4, 4×10^4 and 5×10^4 cells/well. HOK and ORL-48 were each tested at 2×10^3, 4×10^3, 6×10^3, 8×10^3, 1×10^4, 2×10^4 and 3×10^4 cells/well. The use of different density series was informed by routine culture observations and prior laboratory experience with each cell model. HOK and ORL-48 tended to reach high culture density at lower starting cell numbers, whereas HGF and DPSC generally required higher starting densities to produce readily measurable MTS absorbance after prolonged culture. Accordingly, the lower density series for HOK and ORL-48 was intended to reduce the likelihood of

excessive cell accumulation and high baseline absorbance at 72 h, while the higher series for HGF and DPSC allowed a usable response range to be captured. Each model-specific series was used to identify densities that produced absorbance values below, within and above the study-defined working range after 72 h. The experiment was not designed to compare identical cell numbers across biologically distinct models. Plates were incubated for 72 h under the culture conditions described above.

At the end of the 72-h culture period, 10 µL of MTS reagent (MTS Assay Kit; catalogue no. ab197010, Abcam, UK) was added directly to each well, giving a final volume of 110 µL. Absorbance was measured 0.5, 1, 2, 3 and 4 h after reagent addition. These time points covered the 0.5-4-h incubation period specified in the manufacturer's protocol. Between measurements, plates were returned to the incubator at 37 °C and 5% CO_2. The density-absorbance relationship was assessed using the 2-h measurement. The full series of measurements from 0.5 to 4 h was then examined at the selected density for each cell model.

2.4 Absorbance measurement and blank correction

Absorbance was measured using an Infinite 200 PRO microplate reader (Tecan, Switzerland) at 490 nm with 690 nm as the reference wavelength. Cell-free blank wells contained 100 µL of the corresponding complete culture medium and 10 µL of MTS reagent. Reference- and blank-corrected absorbance was calculated as:

$$\text{Corrected absorbance} = (A_{490} - A_{690})\ \text{sample} - (A_{490} - A_{690})\ \text{blank} \quad (1)$$

An investigator-defined absorbance range of 0.5-1.5 was used as a practical criterion for selecting assay conditions. The lower boundary was intended to exclude weak signals close to the blank, and the upper boundary was intended to exclude high baseline signals that could limit detection of a further increase. These boundaries were not derived from a universal linear range of the microplate reader or the MTS assay.

2.5 Selection of assay conditions

The seeding density for each model was selected using three criteria: the 2-h MTS absorbance after 72 h of culture was within 0.5-1.5; the response was consistent across the three independent experiments; and higher densities did not provide a more useful baseline signal. Because only the 72-h profiles are reported, the selected densities should be verified independently before use at 24- or 48-h endpoints.

MTS reagent incubation time was selected as the earliest measurement at which the mean absorbance for every model was within 0.5-1.5. Subsequent measurements were inspected descriptively for any marked further increase. No percentage-change threshold for a plateau was defined prospectively; therefore, the time-course assessment was visual and descriptive. MTS absorbance was interpreted as an indirect measure of cellular metabolic activity, not as a direct cell count or a standalone measure of proliferation.

2.6 Replication and data analysis

Each condition was assessed in technical triplicate in three independent experiments performed on separate days. Technical triplicates were averaged within each independent experiment, and the

independent experiment was treated as the unit of analysis (n = 3). Data are presented as mean ± standard deviation.

Analyses were performed using GraphPad Prism version 10.6.1 (GraphPad Software, Boston, MA, USA). Simple unweighted linear regression was used descriptively to summarise the association between numerical seeding density and mean blank-corrected absorbance at the 2-h MTS reading. The coefficients of determination (R^2) describe overall fit across the complete tested density series and were not used as evidence that the response remained linear at every density. The time-course profiles were assessed visually and descriptively; no quantitative plateau threshold was specified. No inferential group-comparison tests were applied because the objective was assay-parameter selection rather than hypothesis testing between treatment groups.

3. RESULTS

3.1 Cell-seeding density produced model-specific MTS responses at 72 h

MTS absorbance increased with cell-seeding density in all four cell models after 72 h of culture (Fig. 1). The density that produced a 2-h absorbance value within 0.5-1.5 differed among the models. Descriptive linear regression yielded R^2 values of 0.9773 for DPSC, 0.9598 for HGF, 0.8889 for HOK and 0.8754 for ORL-48.

For HGF and DPSC, the lower tested densities produced absorbance values below 0.5, whereas densities of 3×10^4 cells/well and above produced values above 1.5. A density of 2×10^4 cells/well was therefore selected for both models. HOK and ORL-48 produced higher absorbance at lower numerical densities. For these models, 8×10^3 cells/well produced values within 0.5-1.5, whereas densities of 1×10^4 cells/well and above exceeded 1.5. Therefore, 8×10^3 cells/well was selected for HOK and ORL-48.

Thus, the same numerical seeding density could not be applied to all four models under the tested conditions. HGF and DPSC required 2×10^4 cells/well, whereas HOK and ORL-48 required 8×10^3 cells/well to produce a 2-h MTS absorbance within the study-defined range after 72 h of culture.

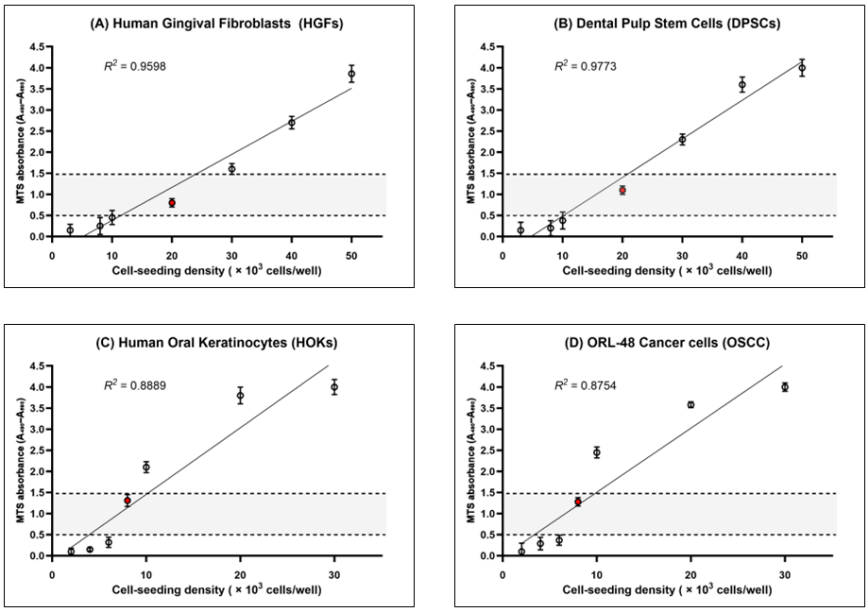


Fig. 1. Cell-seeding-density-dependent MTS absorbance across four oral cell models. Cells were cultured for 72 h, after which MTS absorbance was measured 2 h after reagent addition. Density–response relationships are shown for (A) human gingival fibroblasts, (B) dental pulp stem cells, (C) human oral keratinocytes and (D) ORL-48 oral squamous cell carcinoma cells. Simple linear regression lines and corresponding coefficients of determination are shown. The grey-shaded region represents the investigator-defined absorbance range of 0.5–1.5, while red-filled symbols indicate the selected densities: 2×10^4 cells/well for HGF and DPSC and 8×10^3 cells/well for HOK and ORL-48. Data are presented as mean ± SD from three independent experiments; technical triplicates were averaged within each experiment

Source: Authors' own data

3.2 A 2-h MTS incubation produced suitable absorbance values across the four models

At the selected seeding densities, mean MTS absorbance increased between 0.5 and 2 h after reagent addition in all four models (Fig. 2). The 0.5-h values were below 0.5. At 1 h, the HGF value remained below 0.5, whereas the other models were at or above this boundary. By 2 h, all four models had mean absorbance values within 0.5-1.5.

After 2 h, visual inspection showed no marked further increase in the mean absorbance values through 4 h. No quantitative plateau threshold was specified. Thus, this pattern is described only as a broadly stable response. Two hours was the earliest measurement at which every model produced a mean value within the study-defined range; a 2-h MTS reagent incubation was therefore selected.

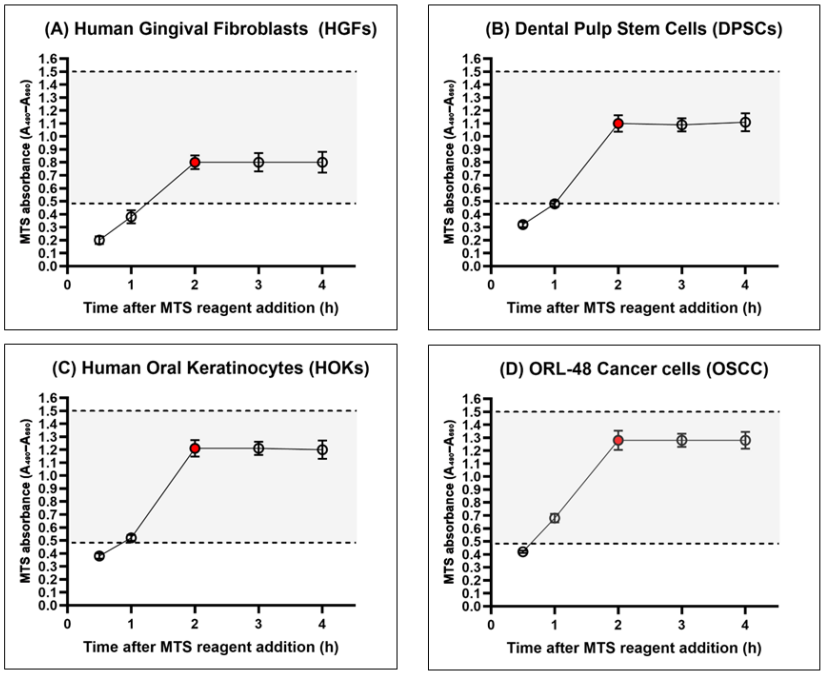


Fig. 2. Time-dependent development of MTS absorbance at the selected cell-seeding densities. Cells were cultured for 72 h before MTS reagent addition, and absorbance was measured sequentially after 0.5, 1, 2, 3 and 4 h for (A) human gingival fibroblasts at 2×10^4 cells/well, (B) dental pulp stem cells at 2×10^4 cells/well, (C) human oral keratinocytes at 8×10^3 cells/well and (D) ORL-48 oral squamous cell carcinoma cells at 8×10^3 cells/well. The grey-shaded region represents the investigator-defined absorbance range of 0.5–1.5, while red-filled symbols indicate the selected 2-h incubation time. Data are presented as mean ± SD from three independent experiments; technical triplicates were averaged within each experiment

Source: Authors' own data

3.3 Selected MTS assay conditions at the 72-h endpoint

The selected conditions combined a model-specific seeding density with a common MTS reagent incubation time (Table 1). HGF and DPSC were assigned 2×10^4 cells/well, whereas HOK and ORL-48 were assigned 8×10^3 cells/well. A 2-h incubation after MTS addition was used for all four models. These conditions were selected and validated for the 72-h endpoint in the present study.

Table 1. Summary of the selected MTS assay conditions for the four oral cell models

Cell model	Passage range	Selected seeding density (cells/well)	Selected MTS incubation time (h)	R^2
HGF	3–8	2×10^4	2	0.9598
DPSC	2–10	2×10^4	2	0.9773
HOK	3–9	8×10^3	2	0.8889
ORL-48	15–35	8×10^3	2	0.8754

Note. R^2 values describe the fit of the simple linear regression between numerical seeding density and the 2-h blank-corrected MTS absorbance at the 72-h culture endpoint.

Source: Authors' own data

4. DISCUSSION

This study optimised cell-seeding density and MTS reagent incubation time for four oral cell models after 72 h of culture. The required seeding density differed among the models, whereas a 2-h incubation after MTS addition was suitable for all four under the tested conditions. HGF and DPSC required 2×10^4 cells/well, while HOK and ORL-48 required 8×10^3 cells/well to produce absorbance values within the investigator-defined range of 0.5-1.5.

MTS is a metabolic readout rather than a direct enumeration assay. Tetrazolium reduction can be influenced by cell number, cellular metabolic activity, culture conditions and reagent exposure time (Berridge et al., 2005; Riss et al., 2016; Stockert et al., 2018). Absorbance should therefore not be interpreted automatically as a proportional change in viable cell number or proliferation. The present study used absorbance as a measure of MTS-reducing activity and selected assay conditions empirically for each model.

The 0.5-1.5 absorbance range was an investigator-defined selection criterion. Although the Infinite 200 PRO has a manufacturer-specified measurement capacity of 0-4 OD (Tecan Austria GmbH, 2024), that capacity does not demonstrate a linear relationship between cell number and MTS absorbance throughout the interval. The lower study boundary was used to avoid weak baseline signals, and the upper boundary was used to avoid high baseline signals that might limit detection of a further increase. The selected range is therefore specific to this optimisation strategy and should not be presented as a universal linear range.

The density-response results show why each model required separate optimisation. HGF and DPSC reached the study-defined absorbance range at 2×10^4 cells/well, whereas HOK and ORL-48 reached it at 8×10^3 cells/well. These differences may reflect variation in attachment, growth or MTS-reducing activity, but the present experiments did not determine which factor was responsible. The lower R^2 values for HOK and ORL-48, together with the graphical flattening at higher densities, indicate that a single straight line did not describe the complete series equally well for all models. Linear regression was therefore used only to summarise the overall density-absorbance association; density selection was based on the observed absorbance values and the pre-specified selection criteria.

Published studies support the need for model- and protocol-specific verification rather than direct transfer of numerical settings. Gingival fibroblast and DPSC studies have used different cell sources, tetrazolium reagents and exposure conditions (Nizam et al., 2014; Tabatabaei & Torshabi, 2016; Huynh et al., 2016). These reports provide biological and methodological context, but they do not validate the numerical densities selected in the present MTS system. Differences in cell source, passage, medium, reagent chemistry and culture duration can alter the conditions required for a usable signal.

The same caution applies to HOK and ORL-48. Hujiahemaiti et al. (2018) measured HOK responses using CCK-8, scratch and Transwell assays. These assays address different aspects of cellular response and their results should not be treated as interchangeable. ORL-48 studies have likewise interpreted metabolic measurements together with morphological, trypan-blue, apoptotic or cell-cycle findings (Kamaruddin et al., 2019; Ahmad et al., 2024). These examples support the use of complementary measurements when a direct biological conclusion is required; they do not establish equivalence between the assays.

Two hours was the earliest common measurement at which the mean absorbance for all four selected densities was within 0.5-1.5. The means increased from 0.5 to 2 h and showed no marked further increase through 4 h on descriptive visual assessment. Extending incubation beyond 2 h therefore did not improve

condition selection in this experiment. The conclusion is limited by the absence of a prospectively defined quantitative plateau threshold.

The 72-h endpoint was prioritised because prolonged culture carries a greater risk of cell accumulation and high baseline absorbance. This decision does not establish that the same densities are optimal at 24 or 48 h. The selected densities may be used as candidate starting conditions for shorter experiments, but their performance should be verified at each endpoint using time-matched untreated or vehicle controls.

Assay optimisation may influence later stages of a study. A weak baseline can make treatment-related decreases difficult to detect, whereas a high baseline can make further increases difficult to resolve. Either condition may affect concentration selection before migration, scratch-closure, gene-expression or protein analyses. Defining the assay conditions, controls and interpretation of the metabolic readout before screening reduces this risk (Niles et al., 2009; Adan et al., 2016; Riss et al., 2016).

HGF, DPSC and HOK were included because they represent cellular contexts relevant to oral tissue repair (Gronthos et al., 2000; Huynh et al., 2016; Hujiahemaiti et al., 2018). ORL-48 was included separately as a malignant oral comparator, not as a wound-healing model. Its inclusion allows treatment-associated MTS responses in a malignant cell line to be observed alongside those in the normal repair-associated models. Such a response would require further investigation and should not be interpreted by MTS alone.

An increase in ORL-48 MTS absorbance does not by itself prove increased cancer-cell proliferation, and the absence of an increase does not demonstrate anticancer selectivity. A relevant direct or complementary measurement, such as cell counting, DNA synthesis, clonogenic survival, apoptosis or cell-cycle analysis, is required according to the biological question. Previous ORL-48 studies illustrate this cautious approach by interpreting metabolic measurements together with other cellular findings (Kamaruddin et al., 2019; Ahmad et al., 2024).

This study has several limitations. The selected conditions apply to the cell sources, passage ranges, media, plate format, MTS reagent and reader used in this study. The 0.5-1.5 range was investigator-defined and was not validated as a universal linear range. MTS absorbance was not confirmed by direct cell counting or a DNA-based assay. The main analysis was performed after 72 h of culture; therefore, applicability at 24 and 48 h requires separate verification. Only passage ranges were available for reporting, and passage-related variability cannot be excluded, particularly for ORL-48. The full density series for HOK and ORL-48 also showed deviation from a simple linear pattern at higher densities. These limitations restrict generalisation of the numerical settings to other laboratories.

5. CONCLUSION

Cell-seeding density required model-specific optimisation for the MTS assay. After 72 h of culture, 2×10^4 cells/well for HGF and DPSC and 8×10^3 cells/well for HOK and ORL-48 produced 2-h MTS absorbance values within the investigator-defined range of 0.5-1.5. At these densities, 2 h was the earliest common post-reagent measurement that met this criterion, and the mean values showed no marked further increase through 4 h on descriptive assessment. These findings apply to the tested 72-h conditions. They provide candidate starting settings for subsequent experiments, but shorter endpoints and altered culture systems require separate verification. MTS findings should also be interpreted as metabolic activity and confirmed

with a direct or complementary method when conclusions about cell number, proliferation, migration or wound healing are required.

6. ACKNOWLEDGEMENTS/FUNDING

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7. CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

8. AUTHORS' CONTRIBUTIONS

Vivi Noryati Ahmad: Conceptualisation, methodology, formal analysis, investigation, data curation, visualisation, validation, supervision, writing–original draft, and writing–review and editing. The original optimisation experiments involving all four cell models and the follow-up experiments involving HOK cells were conducted by Vivi Noryati Ahmad; **Afiqah Salhudin**: Investigation and validation involving HGF cells; **Alea Nur Athirah Shaiful Ezan**: Investigation and validation involving DPSC cells; **Puteri Nur Balqis Rabbiatul Addawiyah Mohd Riduwan**: Investigation and validation involving ORL-48 cells. All authors reviewed and approved the final manuscript.

9. DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this manuscript, ChatGPT (OpenAI) was used as a language and editorial support tool to improve grammar, clarity, readability and the organisation of selected passages. It was not used to generate research data, perform data analysis, or determine the interpretation and conclusions of the study. The research concept, experimental work, scientific content and critical decisions were developed and verified by the authors. All AI-assisted text was carefully reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the final manuscript.

10. DATA AVAILABILITY/SUPPLEMENTARY MATERIALS

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

11. ETHICS STATEMENT

The study protocol, which included the optimisation experiments involving the four cell models examined in this study, was reviewed and granted ethics review exemption by the UiTM Faculty of Dentistry Ethics Review Committee (Reference No. ERC/04/2026 [UG/5]). The study used established commercially

available cell models and did not involve the recruitment of human participants, collection of new human tissues or specimens, or animal experimentation. All experimental procedures were conducted in accordance with the institutional Safety, Health and Environmental protocols of Universiti Teknologi MARA.

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