

Monitoring tumor reoxygenation in 3D spheroids using live-cell imaging

Tumor hypoxia is a state of diminished oxygen availability within solid tumors that arises when oxygen demand exceeds supply. Because hypoxia is a well-established cause of radiotherapy resistance, strategies to increase oxygen availability within the tumor microenvironment have long been investigated to improve the response of hypoxic tumors to radiation. Previous preclinical and Phase I clinical studies have reported that intratumoral administration of hydrogen peroxide (H_2O_2) can enhance the response to radiotherapy.¹

Building on this evidence, Nimalasena et al. investigated whether intratumoral administration of KORTUC (0.5% H_2O_2 in 1% sodium hyaluronate) could induce reoxygenation in 3D spheroid models of tumor hypoxia.¹ The researchers used the Celigo™ image cytometer to monitor dynamic changes in a fluorescent hypoxia marker in spheroids following treatment with different H_2O_2 concentrations across multiple timepoints. By combining live-cell hypoxia imaging with quantitative fluorescence analysis, the researchers could monitor how the hypoxic response changed after H_2O_2 treatment, rather than assessing hypoxia at a single fixed timepoint. Here, we explore the study's key findings and the role of the Celigo image cytometer in monitoring these changes in hypoxia over time.

Spheroid reoxygenation

To investigate whether H_2O_2 could reduce hypoxia, HCT116 (colorectal) and LICR-LON-HN5 (head and neck) cancer spheroids were formed in ultra-low attachment 96-well plates. On day 4, when spheroids reached 400–700 μm in diameter, they were treated with H_2O_2 at concentrations ranging from 0–9.6 mM. Changes in hypoxia were then monitored over time using the fluorescent hypoxia marker Image-iT™ Red Hypoxia Reagent. Brightfield and fluorescent images were acquired at multiple timepoints up to 24 hours post-treatment using the Celigo image cytometer and its 'Tumorsphere 1' application, with fluorescence intensity used as a proxy measure of hypoxia.

As shown in Figure 1, treatment with H_2O_2 resulted in a dose-dependent reduction in fluorescence in both HCT116 and LICR-LON-HN5 spheroids 1 hour after treatment, with a significant reduction observed at concentrations ≥ 1.2 mM. In HCT116 spheroids, this reduction in fluorescence was sustained for up to 6 hours following treatment with H_2O_2 concentrations ≥ 2.4 mM (data shown in original article). However, red fluorescence reappeared in both spheroid models at 24 hours, suggesting that the reoxygenation effect was transient and that hypoxia was reestablished over time. These findings were further supported by pimonidazole staining of fixed spheroids, which showed reduced hypoxia following treatment with H_2O_2 concentrations > 1.2 mM. Collectively, the results indicate that H_2O_2 -induced reoxygenation is both dose- and time-dependent in this preclinical model system.

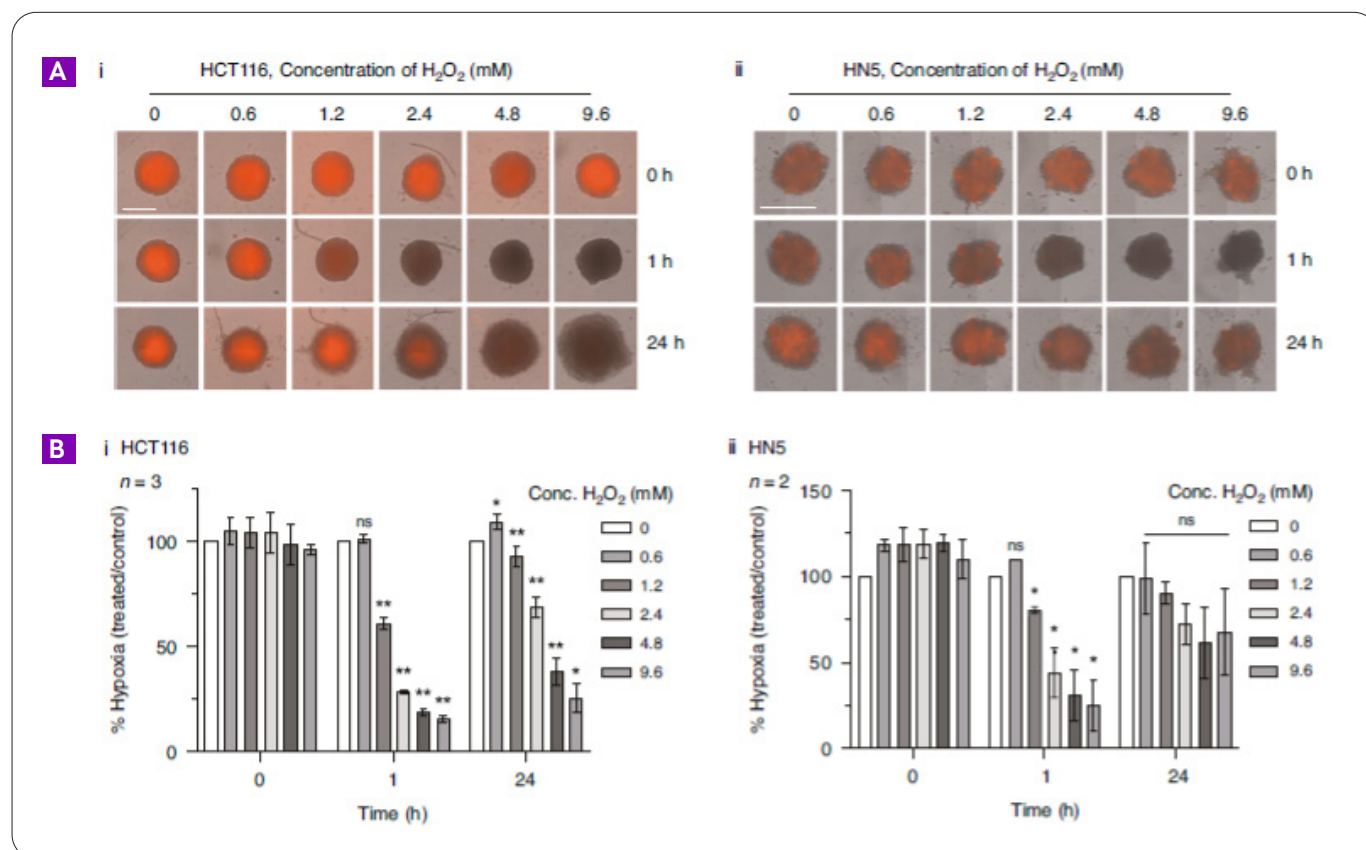


Figure 1: Dose-dependent reoxygenation following hydrogen peroxide treatment of spheroids. (a) Merged brightfield and fluorescence images of (i) HCT116 and (ii) HN5 spheroids pre-treated with Image-iT™ Red for 24 hours and subsequently treated with H_2O_2 (0–9.6 mM). Representative spheroids are shown. The apparent increase in HCT116 spheroid size at higher H_2O_2 concentrations at 24 hours is attributed to spheroid disaggregation resulting from H_2O_2 -induced cytotoxicity. Scale bar = 500 μ m. (b) Changes in Image-iT Red fluorescence intensity in (i) HCT116 (n = 3 independent experiments) and (ii) HN5 (n = 2 independent experiments) spheroids relative to untreated controls. Data are mean $\pm$ SD from 14–16 spheroids per condition for each experiment. *p < 0.05, **p < 0.01, ns, not significant; paired Student's t-test. Image credit: Panels a and b reproduced without modification from Nimalasena et al., licensed under CC BY 4.0 (<http://creativecommons.org/licenses/by/4.0/>).

Ultrasound confirms oxygen release *in vivo*

Having demonstrated H_2O_2 -induced reoxygenation in spheroid models, the researchers next set out to establish whether KORTUC could generate oxygen within tumors *in vivo*. To investigate this, the team used ultrasound imaging during and after intratumoral injections in xenograft models. Oxygen microbubbles formed immediately following KORTUC injection and remained visible for up to 1 hour.

Interestingly, no microbubble formation was observed following intratumoral injection of sodium hyaluronate alone, supporting H_2O_2 as the source of oxygen generation. The authors note that, in the clinical studies described,¹ radiotherapy is delivered approximately 1 hour following intratumoral KORTUC injection, making this finding contextually relevant to the study's translational rationale.

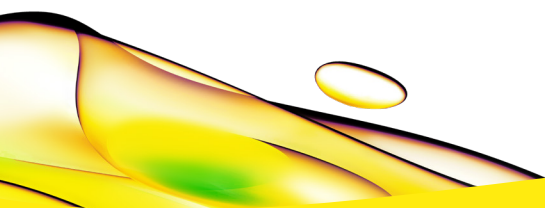
Conclusion

In the present study, Nimalasena et al. demonstrated that intratumoral injection of H_2O_2 was associated with a reduction in tumor hypoxia in spheroid models arising from two cancer cell lines. They showed that this reduction in fluorescence was maintained for several hours in HCT116 spheroids at higher H_2O_2 concentrations, but that fluorescence re-emerged by 24 hours in both models. By using a fluorescent hypoxia marker with the Celigo image cytometer, the researchers were able to quantify these changes across multiple timepoints, revealing a dynamic and transient response that would not have been captured by a single endpoint measurement.

The findings were further supported by pimonidazole staining and then ultrasound imaging in xenograft models, which demonstrated oxygen microbubble formation persisting for up to 1 hour following KORTUC injection. Together, these results suggest that H_2O_2 treatment can produce a temporary reduction in hypoxia in these preclinical models and that KORTUC generates oxygen within tumors for at least 1 hour, which may be relevant given that this is the interval between KORTUC injection and radiotherapy delivery reported in prior clinical studies.¹ The results also highlight the importance of considering both dose and timing when investigating H_2O_2 -induced reoxygenation.

References

1. Nimalasena, S., Anbalagan, S., Box, C., Yu, S., Boulton, J. K. R., Bush, N., Howell, L., Sinnett, V., Murphy, W., Yarnold, J., Robinson, S. P., & Somaiah, N. (2024). Tumour reoxygenation after intratumoural hydrogen peroxide (KORTUC) injection: a novel approach to enhance radiosensitivity. *BJC Reports*, 2(1), 78. <https://doi.org/10.1038/s44276-024-00098-y>



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