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Clinical Investigation

Intratumoral Hydrogen Peroxide With Radiation Therapy in Locally Advanced Breast Cancer: Results From a Phase 1 Clinical Trial

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Purpose: Hydrogen peroxide (H_2O_2) plays a vital role in normal cellular processes but at supraphysiological concentrations causes oxidative stress and cytotoxicity, a property that is potentially exploitable for the treatment of cancer in combination with radiation therapy (RT). We report the first phase 1 trial testing the safety and tolerability of intratumoral H_2O_2 + external beam RT as a novel combination in patients with breast cancer and exploratory plasma marker analyses investigating possible mechanisms of action.

Methods and Materials: Twelve patients with breast tumors ≥3 cm (surgically or medically inoperable) received intratumoral H_2O_2 with either 36 Gy in 6 twice-weekly fractions (n = 6) or 49.5 Gy in 18 daily fractions (n = 6) to the whole breast ±

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locoregional lymph nodes in a single-center, nonrandomized study. H_2O_2 was mixed in 1% sodium hyaluronate gel (final H_2O_2 concentration 0.5%) before administration to slow drug release and minimize local discomfort. The mixture was injected intratumorally under ultrasound guidance twice weekly 1 hour before RT. The primary endpoint was patient-reported maximum intratumoral pain intensity before and 24 hours postinjection. Secondary endpoints included grade $\geq$3 skin toxicity and tumor response by ultrasound. Blood samples were collected before, during, and at the end of treatment for cell-death and immune marker analysis.

Results: Compliance with H_2O_2 and RT was 100%. Five of 12 patients reported moderate pain after injection (grade 2 Common Terminology Criteria for Adverse Events v4.02) with median duration 60 minutes (interquartile range, 20-120 minutes). Skin toxicity was comparable to RT alone, with maintained partial/complete tumor response relative to baseline in 11 of 12 patients at last follow-up (median 12 months). Blood marker analysis highlighted significant associations of TRAIL, IL-1β, IL-4, and MIP-1α with tumor response.

Conclusions: Intratumoral H_2O_2 with RT is well tolerated with no additional toxicity compared with RT alone. If efficacy is confirmed in a randomized phase 2 trial, the approach has potential as a cost-effective radiation response enhancer in multiple cancer types in which locoregional control after RT alone remains poor.

Introduction

Breast cancer presents a global challenge, with an estimated incidence of 2 million patients worldwide, 80% of whom present with locally advanced disease.[1,2] In the United Kingdom, where women with locally advanced disease represent a minority (7%-13%) of the 55,000 new patient presentations, the lifetime morbidity of progressive local disease is significant.[3-5] Treatment is challenging in frail or elderly individuals who are unfit for or refuse surgery and for whom radiation therapy (RT) $\pm$ hormone therapy is often the most appropriate option for relief of breast ulceration, bleeding, and pain. Locally advanced inoperable primary or recurrent cancers infiltrating the breast/chest wall and/or axilla, with or without metastases, are typically associated with life expectancies measured in years rather than months and present significant challenges to patients and medical professionals. This represents an area of unmet clinical need, in which innovative approaches to enhance response to radiation would be highly beneficial.

An interaction at a cellular level between H_2O_2 and ionizing radiation (IR) was first reported in osteosarcoma (HS-Os-1) and prostate cancer (PC-3) cell lines, which demonstrated extreme resistance to either H_2O_2 or 30 Gy alone.[6,7] The addition of 0.1 mM H_2O_2 before IR resulted in enhanced cytotoxicity without causing DNA double strand breaks that classically mediate cell killing.[8,9] A novel mechanism was postulated to involve lysosomal membrane rupture with release of powerful oxidants, including heavy metal ions that permeabilize mitochondria and activate apoptosis.[10] In vivo use involved a mixture of 0.5% H_2O_2 in 0.83% sodium hyaluronate gel, the Kochi Oxydol-Radiation Therapy for Unresectable Carcinomas (KORTUC) strategy designed to minimize local pain at the injection site. Intratumoral injection of this H_2O_2 gel mixture into murine tumors before 30 Gy IR demonstrated clear evidence of growth delay greater than that achieved by either modality alone. No toxicity was noted.[11]

In this study we report the first systematically conducted phase 1 trial testing intratumoral H_2O_2 in combination with RT in locally advanced breast cancer (NCT02757651). The primary objective was assessment of safety and tolerability of H_2O_2 injections with moderately hypofractionated RT. Secondary endpoints included the proportion of patients requiring additional pain medication, incidence of grade $\geq$3 skin toxicity, and tumor response assessment. Exploratory analysis of plasma markers was also performed.

Methods and Materials

Study design

This nonrandomized study involved patients with locally advanced or locally recurrent breast cancer (with or without metastases) for whom RT was indicated for locoregional disease control. Patients were inoperable due to comorbidities or local disease extent, or surgery to the breast primary was not appropriate due to presence of metastatic disease.

The single-center study was conducted at The Royal Marsden NHS Foundation Trust (CCR4502). Approval by the Research Ethics Committee (REC) and the Medicines and Healthcare products Regulatory Agency (MHRA) was obtained before trial commencement (IRAS 203161, REC 16/LO/1566, EudraCT 2016-000833-40). Monitoring was undertaken by the Clinical Trials Unit at The Royal Marsden NHS Foundation Trust. The trial schema is shown in Figure 1.

Eligible patients were older than 18 years of age, had histologically confirmed breast cancer, and required breast RT for local control and/or palliation of locoregional symptoms. They had at least 1 breast tumor measuring $\geq$3 cm in diameter in a superficial location accessible for injection. Any combination of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression was allowed. Exclusion criteria included prior RT to the breast and concomitant biological therapies other than trastuzumab, pertuzumab, and denosumab. Pregnancy was excluded in female patients of child-

bearing age. Patients were excluded if the anatomic location of the breast tumor, such as proximity to blood vessels or the brachial plexus, precluded safe access for intratumoral injection. This precaution minimized the risk of injection into a blood vessel causing embolism, an adverse effect that has not been described in the literature in relation to intratumoral H_2O_2.[12,13]

Drug formulation

A slow-release 0.5% H_2O_2 solution was created by mixing 0.4 mL of 3% H_2O_2 (2.0 mL sterile ampoules supplied by Stockport Pharmaceuticals, UK) with 2.0 mL OSTENIL (20 mg sodium hyaluronate in a 2.0-mL preloaded syringe provided by AAH Pharmaceuticals, UK), the latter licensed for intra-articular injection of arthritic joints.[14] The low molecular weight of H_2O_2 (34 g/mol) ensures rapid equilibration of drug within the gel. The mixture is a colorless, viscous solution (pH 6.8-7.8) stored at room temperature and stable for 2 hours after preparation, as determined by viscosity measurements (performed by Stockport Pharmaceuticals, UK). The gel allows slow release of H_2O_2 for at least 24 hours, as evidenced by generation of oxygen microbubbles during injection, a feature that provides a strong rationale for twice-weekly administration during RT.[15] In the trial, the drug and gel were mixed under aseptic conditions using 2 syringes connected via a 2-way tap. Once made, each syringe contained 2.4 mL of 0.5% H_2O_2, the contents of both syringes typically needed for tumors measuring 30 to 60 mm in diameter.

Radiation therapy

Six patients received 49.5 Gy in 18 daily fractions of 2.75 Gy, and 6 were treated with 36 Gy in 6 twice-weekly

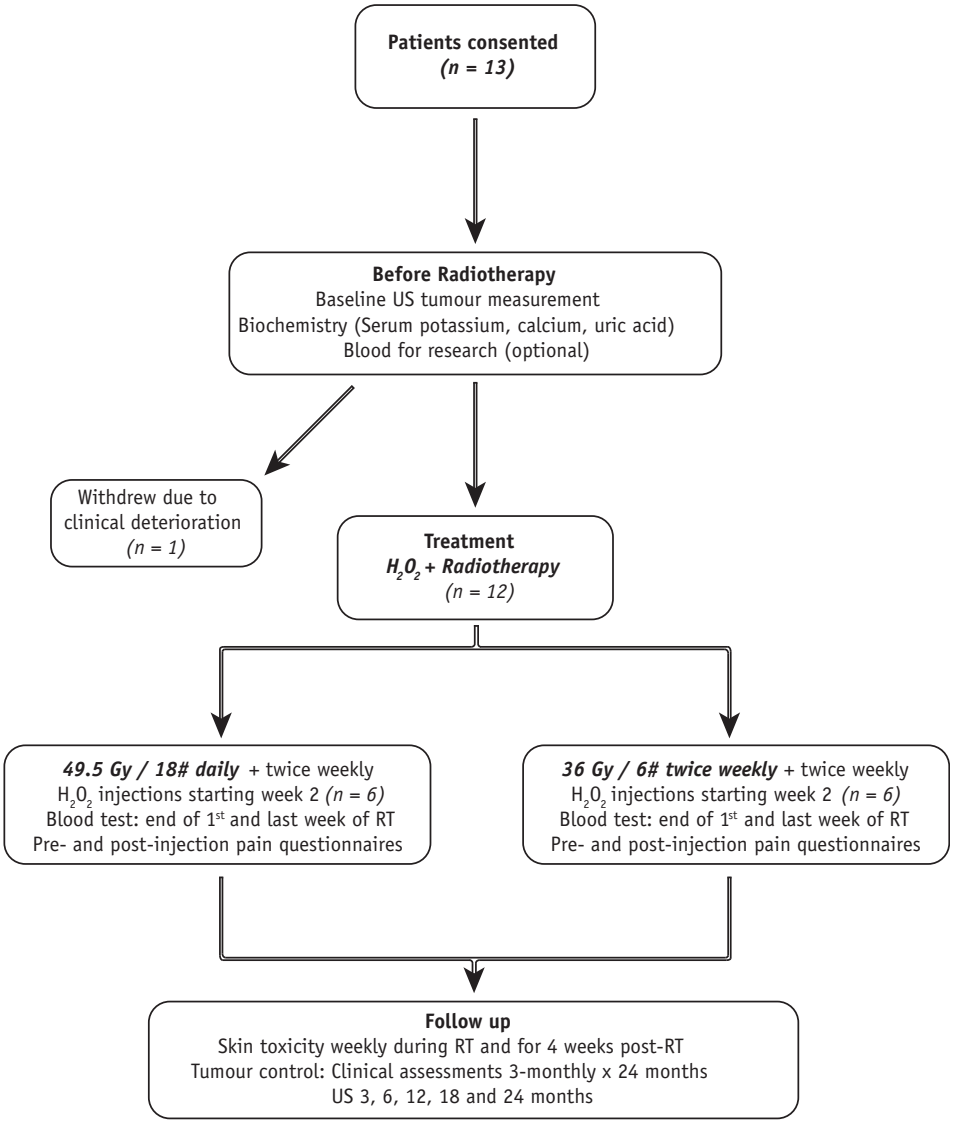

Fig. 1. Phase 1 trial schema. A nonrandomized study design testing intratumoral H_2O_2 in sodium hyaluronate gel in combination with 2 radiation therapy fractionation schedules in patients with locally advanced breast cancer and corresponding follow-up schedule. *Abbreviations:* RT = radiation therapy; # = radiation therapy fraction; US = ultrasound.

fractions of 6 Gy to the whole breast ± locoregional lymph nodes. The equivalent RT dose expressed in conventional 2-Gy fractions (EQD_2) was 57 Gy and 65 Gy for these 2 schedules, respectively (Fig. E1). Patients on the 6-Gy twice-weekly schedule, requiring lymph node irradiation in addition to the breast, were treated to a total dose of 30 Gy in 5 twice-weekly fractions of 6 Gy to the nodal regions, to not exceed brachial plexus tolerance dose (as per standard institutional guidelines).

The RT schedule was selected according to the patient's performance status and comorbidities, with fitter patients selected for the daily treatment schedule. RT was delivered using a linear accelerator with 6 to 10 MV photons, 3-dimensionally planned using data from a computed tomography planning scan and using standard tangentially opposed fields. Patients were simulated and treated in the supine position on a breast board with both arms abducted. The clinical target volume comprised the entire ipsilateral breast, including the deep fascia, but excluding underlying muscle or overlying skin (when not involved with disease). The RT dose was prescribed to the 100% isodose, ensuring the target volume was within the 95% to 107% isodose lines. Organs at risk including the heart, lung, and contralateral breast were outlined and standard guidelines for dose tolerances were followed. A standard treatment verification protocol was used, consisting of daily imaging for the first 3 days and subsequent weekly imaging. In cases in which there was skin involvement by tumor, treatment included 5 mm wax bolus throughout RT to maximize dose to skin, in keeping with standard practice. In patients treated with 49.5 Gy in 18 fractions, a sequential boost dose to the tumor bed (13.35 Gy in 5 daily fractions using minitangential opposed beams or a directly applied electron beam) was allowed, but this needed to be declared at time of trial entry. A tumor bed boost dose increased the EQD_2 to that comparable with 36 Gy in 6 fractions and with dose intensities previously reported in earlier patient cohorts treated with the same drug preparation.[16,17]

Intratumoral injections of H_2O_2 in sodium hyaluronate gel

Transdermal intratumoral KORTUC injections were administered twice weekly commencing in the second calendar week of RT. Each patient received 4 to 6 doses in total (median = 5 injections), the smaller number given to patients prescribed 6 fractions. The rationale for starting KORTUC in the second week was to allow for reduction in tumor interstitial pressure during the first week of RT, enabling technically easier and more tolerable injections for the patient. Injections were performed (23-gauge needle) under ultrasound (US) guidance by a trained radiologist or radiographer after 0.5% lignocaine injection to anesthetize the skin. For tumors measuring 30 to 60 mm in size, 2 syringes (4.8 mL) of 0.5% H_2O_2 in sodium hyaluronate gel were injected at each time point. Three syringes (7.2 mL) were required for tumors >60 mm in size.

Uniform and accurate delivery under US guidance via 2 to 3 differently angled needle tracks was aided by the immediate appearance of oxygen microbubbles as H_2O_2 degraded to oxygen and water within the tumor (Fig. 2A). The needle tip was positioned at the deepest aspect of the tumor and the gel released slowly while withdrawing the needle toward the surface. For smaller tumors, it was possible to achieve even distribution of the H_2O_2 gel mixture within the tumor via a single skin puncture site and by altering the angle of the needle (working from left to right or top to bottom within the tumor). For some larger tumors (eg, >60 mm) it was necessary to inject the tumor via more than 1 skin entry point from different directions to ensure even distribution of oxygen microbubbles throughout the tumor volume. The number of needle tracks within the tumor and skin entry points were decided by the radiologist during the US scan and guided by the extent and distribution of oxygen microbubbles during the injection procedure. If any gel tracked back to the skin surface during withdrawal of the needle, it was promptly wiped away with sterile gauze. If patients had >1 distinct tumor in the breast/axilla, the clinician/radiologist was required to clearly document the injected lesion (usually the largest) to aid response assessment. RT was delivered within 1 to 2 hours after H_2O_2 injection.

Treatment monitoring

Within each RT group (daily or twice-weekly fractions), a minimum gap of 1 week was stipulated between the first and second patient, during which acute toxicity data associated with intratumoral injections (pain, skin toxicity, and tumor lysis) were reviewed by an independent data monitoring committee. Based on predetermined criteria, the second and third patients in each group and subsequently the fourth, fifth, and sixth patients in each group were allowed to be treated concomitantly.

Primary endpoint

This related to the timing, severity, and duration of pain postinjection recorded via a self-reported questionnaire completed by patients at home. An 11-point numerical scale ranging from 0 ("no pain") to 10 ("worst possible pain") recorded severity and duration before and more than 24 hours after each H_2O_2 injection (Fig. E1). Patient-reported scores were used to calculate (1) the proportion of patients with pain scores ≥5 points greater than baseline after any of the intratumoral injections and (2) the requirement for additional pain medication.

Secondary endpoints

Secondary endpoints included acute RT-induced skin toxicity, serum biochemistry, and tumor response. Skin

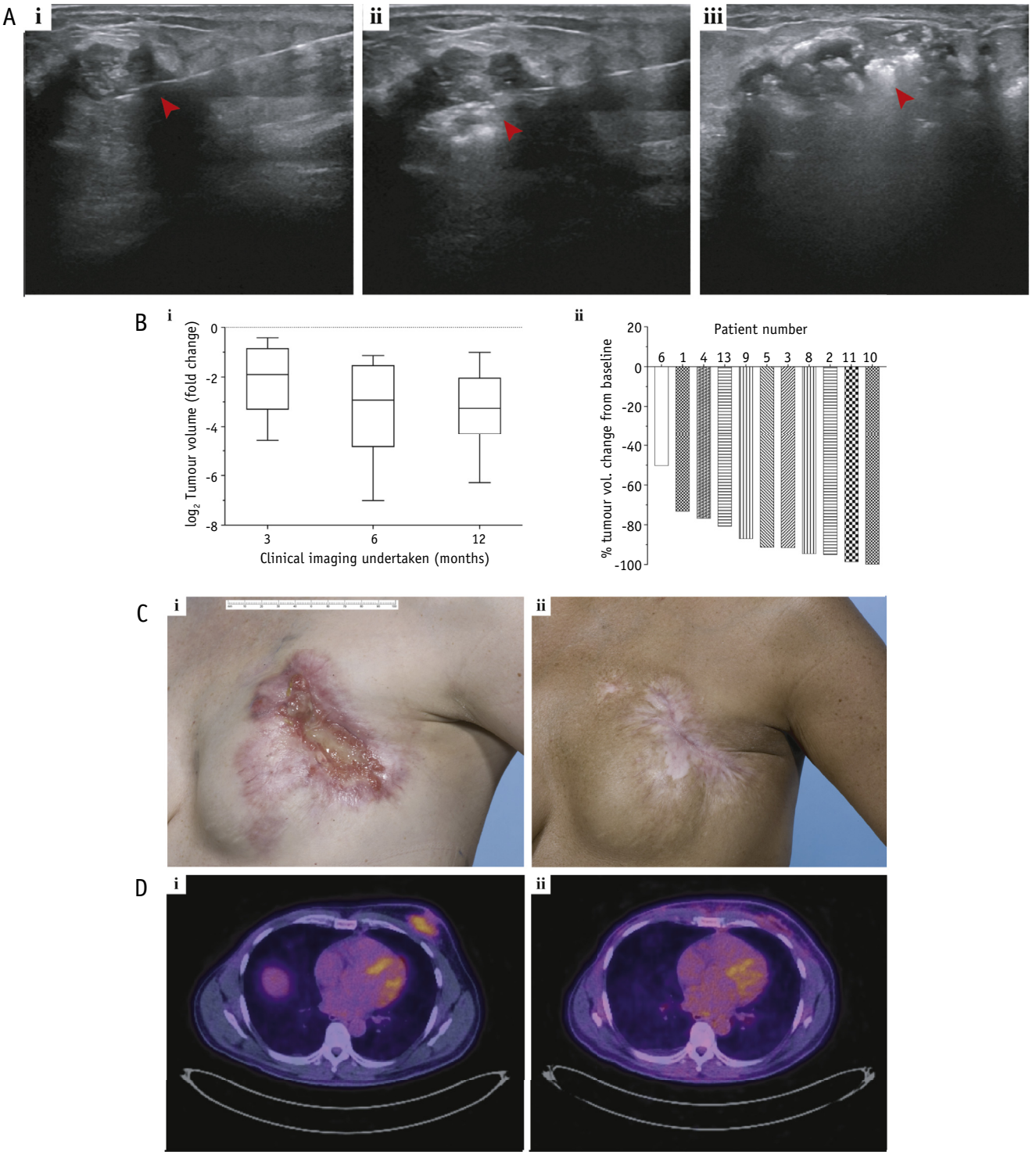

Fig. 2. Intratumoral H_2O_2 administration and tumor response. (A) Sequence of ultrasound images of a breast tumor showing H_2O_2 administration, red arrow indicating (i) needle entering under ultrasound guidance, (ii) H_2O_2 + sodium hyaluronate gel mixture being injected intratumorally, and (iii) breakdown of H_2O_2 with formation of echogenic oxygen microbubbles (white) within the tumor. (B) Tumor volume changes: (i) box plot showing the cumulative fold decrease (log2 transformed) for all 12 patients, at the indicated time points post-RT and (ii) waterfall plot showing % tumor volume change up to 12 months post-RT normalized to baseline tumor measurement (data represent tumor measurements at 9 and 12 months post-RT for 3 and 8 patients, respectively). (C) Clinical photographs of patient 10: (i) left breast with fungating tumor (baseline) and (ii) 12 months posttreatment with H_2O_2 + RT. (D) ^{18}F-FDG PET scans of patient 8: (i) high tracer uptake in left breast tumor at baseline and (ii) complete metabolic response at 12 months posttreatment. *Abbreviations:* FDG = fluorodeoxyglucose; PET = positron emission tomography; RT = radiation therapy.

toxicity was assessed weekly in all patients during and for 4 weeks after RT by a member of the clinical team. Standardized proformas recorded the degree of erythema and desquamation of the skin of the breast. In each of the RT groups, if no more than 1 of the first 3 patients had a persistent Common Terminology Criteria for Adverse Events (CTCAE) (v4.02) skin toxicity grade $\geq$3 at 6 weeks after RT, the independent data monitoring committee allowed recruitment to continue for a further 3 patients within that RT schedule. If moist desquamation was seen beyond skin folds, weekly assessments were continued until severity was reduced to grade $\leq$1. The proportion of patients with grade $\geq$3 skin toxicity at any time from the start of RT to 4 weeks post-RT and the worst grade of skin toxicity reported from the start of RT to 4 weeks post-RT were recorded in these cases. However, it was recognized that if cancer infiltrated skin, patients would typically experience grade $\geq$3 skin toxicity after RT alone.

In every patient, routine biochemistry including serum potassium, calcium, and uric acid were measured 2 days after the first H_2O_2 dose to rule out tumor lysis before proceeding with subsequent doses.[18]

Tumor response were assessed at 3, 6, and 12 months posttreatment. At each timepoint, 3-dimensional US measurements were obtained and the tumor volume was calculated on the assumption that breast tumors assume a hemiellipsoid shape, as previously demonstrated.[19] Maximum tumor dimension alone was not considered an accurate representation of tumor response, especially when tumors "flatten" after RT. Tumor volumes were compared against pretreatment measurements applying RECIST-like principles, with complete response (CR) defined as disappearance of the target lesion, partial response (PR) as at least a 30% reduction in tumor volume, and stable disease (SD) as less than a 30% reduction or 20% increase in tumor volume.

Statistical considerations

Based on the previously published data, 30% to 100% of patients experienced pain described as no worse than "mild" (or CTCAE grade 1) for several hours after injection.[16,20-22] A single case of tumor lysis syndrome (mild) was reported in a total of 139 breast cancer patients in the Japanese literature. Given this knowledge of the safety of H_2O_2 plus RT, the phase 1 trial required 12 patients to be recruited. Patients treated with once-daily and twice-weekly fractions of RT were analyzed as a single stratum, and the study population was defined as all patients who registered for the trial and received at least 1 dose of intratumoral H_2O_2. Tumor volumes were calculated using 3-dimensional measurements obtained from US scans.

Plasma markers

Blood samples were obtained pre-RT and at the end of the first and last weeks of RT. Blood (two 9-mL K3-EDTA tubes) was obtained by venipuncture and processed within 30 minutes. Plasma and buffy coat were isolated by centrifugation at 1600 $\times$ g for 10 minutes at room temperature and frozen in aliquots at -80°C. All the plasma samples subsequently underwent 1 freeze-thaw cycle before assay.

ELISA and Luminex assay

Frozen plasma samples were thawed on ice and brought to room temperature. They were spun at 10,000 $\times$ g for 10 minutes and the plasma supernatant assayed either by ELISA or Luminex assay as per the manufacturers' protocols. Results were measured against a normal plasma control obtained from a healthy donor (Cambridge BioScience Ltd). The absorbance for ELISA was obtained using a POLAR star Omega plate reader spectrophotometer (BMG Labtech). The Luminex Human XL Cytokine Discovery Panel (15-plex, #FCSTM18-15, R&D System) was carried out with a minimum of 100 events per bead using a Luminex 200 system with xPONENT v3.1 software (Millipore). The plasma samples were assayed in duplicate wells for individual targets at their corresponding time points (pre-RT, end of first week of RT, and post-H_2O_2 + RT), and the log2 fold change was calculated. Further information regarding the ELISA and Luminex analytes, their dilutions, and kit details used in this study are provided in Table E1.

Exploratory translational endpoints

Exploratory analyses of plasma biomarkers of cell death, inflammation, and immune response were conducted to test the feasibility of investigating novel mechanisms of action and biomarkers of response in trial patients. A linear mixed effect model was used to model the random effect of longitudinal data that the markers generated.[23] The model was built to study the effect of each marker and to quantify its significance in terms of association with tumor shrinkage over time. The difference between individual and temporal variability was treated as a random effect. The impact of each marker was regarded as a fixed effect. The model used was

Tumor_volume = marker + time + marker: time + random_effect (time)

The ":" sign denotes the variation of each plasma marker with time. Each marker was used to fit a mixed model individually with all patient samples, and 20 models were fitted in total. A P value for the coefficient of each marker was calculated to indicate whether the fixed effect had significance (at the 5% level). All graphs were generated using Graphpad Prism v8.1 (Mac OS), Graphpad Software (La Jolla, CA).

Results

Patient characteristics

Patient demographics, tumor characteristics, prior treatment, and RT target volumes are summarized in Table 1.

Thirteen patients (11 female, 2 male) were recruited to the study between February 2017 and August 2018. All patients had locally advanced or recurrent breast cancer and were inoperable due to comorbidities, local extent of disease, or metastatic disease. One patient withdrew due to clinical deterioration unrelated to the trial before starting RT and received no H_2O_2, so an additional (13th) patient was recruited. Median age was 77 years (range, 45-93). Three patients were wheelchair-bound due to comorbidities and frailty. Breast tumor stage was T2 in 5 of 12 patients and T4 in 7 of 12 patients. Six of 12 patients had N0 and 6 of 12 N1 disease (axillary node involvement). Eight of 12 patients had distant metastases. Breast tumor size varied from 30 mm to 164 mm (maximum dimension). Ten patients had ER+/HER2− disease, and 2 had triple negative disease. There were no patients with inflammatory breast cancer. All patients had received 1 to 4 previous lines of treatment for their breast cancer, and the majority had progressed on prior systemic treatment. Three patients had prior surgery for breast cancer but had locally recurrent disease. During RT, 7 of 12 patients continued taking concurrent hormone therapy, and 2 of 12 continued bisphosphonate therapy for metastatic bone disease.

Compliance with treatment protocol and follow-up

Compliance with H_2O_2 injections was 100% in all patients, including 1 with needle phobia. All patients received RT within the prescribed 1 to 2 hours of receiving the H_2O_2 injection, with a single exception of a patient given 1 RT fraction before H_2O_2 injection in error. Results are reported at a minimum follow-up of 12 months for all patients alive at the time of reporting (range, 2-24 months). Eleven patients completed 12 months of follow-up, and the 12th died of rapidly progressive metastatic disease slightly less than 2 months after RT.

Primary endpoint

The pain scores are summarized in Table 2, with respective grades detailed in Figure E1 (iii). Three of 12 patients experienced grade 1 (mild) tumor pain postinjection, and 5 of 12 experienced grade 2 pain (moderate severity, limiting activities of daily living) as per CTCAE v4.02.[24] The remainder did not report any additional pain after intratumoral injection. Median pain duration was 60 minutes with an interquartile range of 20 to 120 minutes.

Four of 12 patients reported pain $\geq$5 points above baseline during treatment. One patient was taking opiate analgesia (oral morphine) before starting RT to control pain resulting from a fungating breast tumor. Six of 12 patients required additional analgesia to manage their symptoms (paracetamol and codeine-based). In these cases, management included ensuring compliance with pre-existing painkillers and optimizing analgesia $\pm$ anxiolytics for the remainder of their treatment.

Table 1 Summary of patient demographics, tumor characteristics, previous lines of treatment, and RT treatment volumes

Patient	Age	Sex	PS* (ECOG)	Baseline TNM stage	Tumor phenotype	Prior treatment (no. of lines of therapy)	RT target volume
1	77	F	1	T2N1M1	$ER^+/HER2^-$	Endocrine (3)	Breast + axillary LN levels I-IV
2	69	F	0	T4N0M1	$ER^+/HER2^-$	Endocrine (2)	Breast
3	79	F	3	T4N0M0	$ER^+/HER2^-$	Endocrine (3)	Breast
4	80	M	2	T4N0M0	$ER^+/HER2^-$	Endocrine (2)	Breast
5	89	F	3	T2N0M0	$ER^+/HER2^-$	Endocrine (1)	Breast
6	78	F	2	T4N1M1	$ER^+/HER2^-$	Endocrine (2) Chemotherapy (1)	Breast + axillary LN levels I-IV
8	53	M	0	T2N0M1	$ER^-/HER2^-$	Surgery − Chemotherapy (4) RT (contralateral)	Breast
9	53	F	2	T2N1M1	$ER^+/HER2^-$	Surgery Endocrine (3) Chemotherapy (2) RT (contralateral)	Breast + axillary LN levels I-IV
10	45	F	0	T4N1M1	$ER^+/HER2^-$	Chemotherapy (1)	Breast + axillary LN levels I-IV
11	75	F	3	T4N1M1	$ER^+/HER2^-$	Surgery Endocrine (3)	Breast
12	45	F	1	T4N1M1	$ER^-/HER2^-$	Chemotherapy (2)	Breast + axillary LN levels I-IV
13	93	F	3	T2N0M0	$ER^+/HER2^-$	None	Breast

Abbreviations: ECOG = Eastern Cooperative Oncology Group; RT = radiation therapy; TNM = tumor, node, metastasis.
Patients 1, 3, 4, 5, 11, and 13 received 36 Gy/6 fractions, and 2, 6, 8, 9, 10, and 12 received 49.5 Gy/18 fractions.
* Performance status (Eastern Cooperative Oncology Group).

Table 2 Summary of pain scores and RT acute skin toxicity scores

Patient number	Maximum pain intensity		Extra analgesia required	Median difference in pain score (pre- and post-RT)	Effect of pain on ADLs	Maximum RT acute skin toxicity score	Bolus during RT
	Score	Period					
1	4	2 h	N	3	Y—housework, shopping	3	Y
2	0	0 min	N	0	N	3	Y
3	3	30 min	N	2.5	N	2	N
4	4	30 min	Y	0.5	N	2	N
5	0	0 min	N	0	N	0	N
6*	10	6 h	Y	5	N	1	N
8†	10	6 h	Y	6	Y—driving	2	N
9	6	5 h	N	4	N	2	N
10	8	2 h	Y	7	Y—housework	3	Y
11	0	0 min	N	0	N	3	Y
12‡	0	0 min	Y	0	N	3	Y
13	6	1 h	Y	5	N	0	N

Abbreviations: ADL = activities of daily living; CTCAE = Common Terminology Criteria for Adverse Events; RT = radiation therapy.

Pain intensity scored from 0 to 10 via patient self-assessment questionnaires (median calculated from difference in pain score pre- and post-H_2O_2 injection for each patient throughout treatment course (4-6 injections in total for each patient)); RT acute skin toxicity scored from 0 to 5 using CTCAE v4.02 by clinicians.

* Patient with needle phobia.

† Patient had significant breast pain before H_2O_2 injection and poor compliance with analgesia.

‡ Patient with significant breast pain and was taking opiate analgesia before RT, explaining the pain score of 0.

Secondary endpoints

Skin toxicity and tumor lysis

The highest grade of skin toxicity reported was grade 3 in 5 of 12 patients, grade 2 in 4 of 12 patients, grade 1 in 1 of 12 patients, and grade 0 in 2 of 12 patients (Table 2 and Fig. E1 [iv]). All 5 patients who experienced grade 3 skin toxicity had been treated with bolus during RT (due to skin involvement by tumor). There was no suggestion of enhancement of erythema due to local leakage of H_2O_2. The acute radiation skin toxicity observed in the trial was comparable to that expected with standard RT alone, including in patients with cancer infiltrating overlying skin.[25] There were no cases of tumor lysis syndrome.

Tumor response

Figure 2B (i) and Table E2 detail the tumor response based on US measurements at successive time points posttreatment. At the last imaging assessment percentage tumor volume reduction was between 50% and 100%, as shown in Figure 2B (ii). All evaluable patients in this study maintained locoregional control in the irradiated target lesion at last clinical follow-up (median, 12 months; range, 2-24 months). Patient 12 died of metastatic disease 6 weeks after RT and was not evaluable at the 3-month endpoint for tumor response.

As an illustrative example, Figure 2C shows tumor extent in patient 10 pre-RT and 12 months posttreatment (patient maintained CR at 18 months). Only 1 of 12 patients had >1 distinct tumor lesion within the RT treatment volume. In this patient only the tumor injected with H_2O_2 showed maintained PR at 12 months, whereas the 2 lesions receiving the same RT alone showed SD (the noninjected lesions acting as internal controls). With regard to tumor response assessment, there were discrepancies in 2 patients between US and clinical response assessments (Table E2). In patient 9, US measurements between 6 and 12 months suggested an increase in tumor size despite an excellent partial response on clinical examination. Radiology review of the US images at 12 months posttreatment indicated changes consistent with fibrosis rather than active tumor. Similarly, patient 8 demonstrated a CR on clinical assessment at 12 months, despite the presence of stable measurable disease on US. A staging positron emission tomography/computed tomography scan performed concurrently confirmed complete metabolic response in the H_2O_2 + RT-treated breast tumor, as shown in Figure 2D.

Exploratory secondary endpoint (post hoc analysis)

Ten of 12 patients consented to provide blood for research at the time points shown in Figure 3A. The exploratory target panel for ELISA and Luminex assays comprised 21 markers involved in cell death, the immune checkpoint, chemo-attraction, immune regulation, and angiogenesis (Table E3). Log2-transformed fold change of targets normalized to their baseline (pre-RT) expression was plotted for all patients, comparing levels after RT alone (at the end of the first week of treatment) and after H_2O_2 + RT (end of treatment) (Figs. 3B, E3). There was no consistent trend when comparing RT alone versus H_2O_2 + RT in this small exploratory cohort. However, upregulation of markers involved in inflammation, immune modulation,

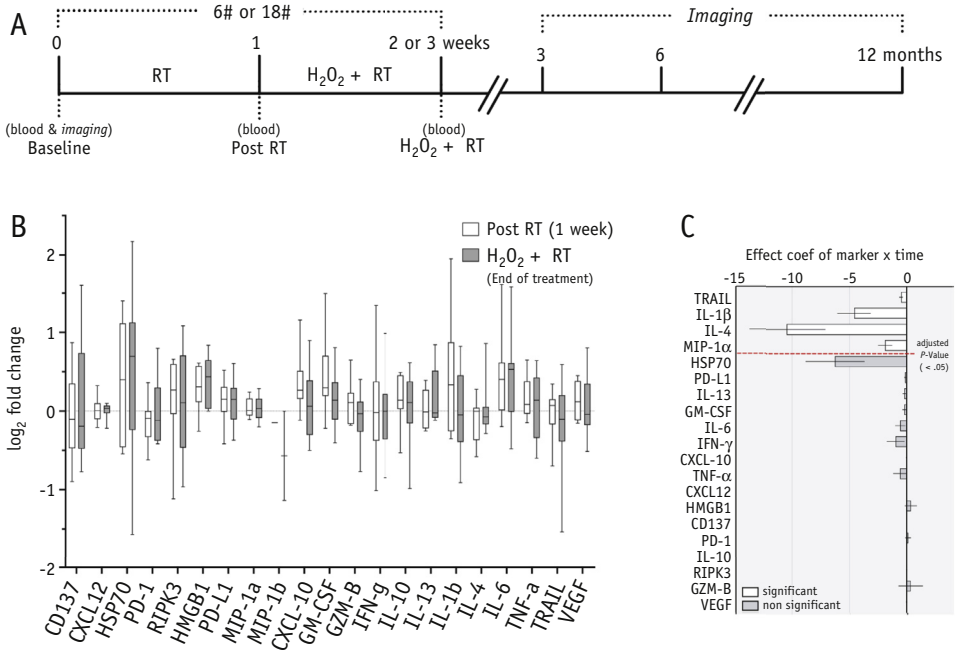

Fig. 3. Analysis of phase 1 trial plasma markers. (A) Scheme showing clinical imaging and blood sampling performed in this study. (B) Box and whisker plot depicting log2-transformed fold change of analyzed targets from individual patients. Values are normalized to their baseline expression for all 21 targets. (C) Plot shows the significant markers associated with tumor volume shrinkage, with multicomparison adjusted P value $< .05$. The bar shows the fixed effect of each marker with error bars, ranked by P values. A negative coefficient means that the marker is positively correlated with tumor shrinkage. *Abbreviation:* # = radiation therapy fraction.

and damage-associated molecular patterns (DAMPs) was noted.[26] Application of a linear mixed effect model identified 4 significant ($P < .05$) associations with tumor shrinkage, suggesting TRAIL mediated apoptosis with increased activated T cell signaling (IL-4, MIP-1α) and macrophage stimulation (IL-1β) (Fig. 3C and Table E4).

Discussion

This phase 1 study raised no concerns relating to local or systemic toxicity when intratumoral H_2O_2 is delivered with RT doses per fraction up to 6 Gy in patients with locally advanced primary or recurrent breast cancer unsuitable for primary surgery or palliative debulking. The intervention is well tolerated even by frail, older patients and those with needle phobia. In those patients with pre-existing pain symptoms, it was important and straightforward to optimize pain medication before starting treatment.

Commencing H_2O_2 injections in the second calendar week of RT ensured that there were no technical challenges (ie, resistance due to tissue turgor) to injecting the prescribed volume of drug in any of the patients. Given that H_2O_2 breaks down to O_2 within the tumor (2 molecules of H_2O_2 degrade to 1 molecule of O_2 and 2 molecules of water), it is hypothesized that this may contribute to reoxygenation of hypoxic areas, thereby alleviating radiation resistance. Therefore, aside from the classical DNA damage effects, another mechanism of synergy between H_2O_2 and RT may result from reoxygenation. This is currently being investigated in the laboratory setting. In tumors that do not reoxygenate spontaneously during fractionated RT, H_2O_2 is expected to be most effective after the second week of RT, when such tumors are likely to be enriched with hypoxic radioresistant subpopulations.[27-29]

Acute skin toxicity was no different to that expected after the same RT alone. As predicted, grade 3 radiation dermatitis occurred only in those patients with tumor involving skin, when a 5-mm layer of wax "blanket" ensured 100% prescribed RT dose to skin instead of approximately 70% of prescribed dose in patients without skin involvement.[30] Grade 3 skin desquamation managed with standard supportive measures including barrier creams and dressings ensured complete resolution of symptoms in every case. Overall, toxicity and tolerability were entirely consistent with extant literature, and these phase 1 data will contribute to an application for regulatory approval if the planned randomized phase 2 study confirms efficacy.

In view of the limitations of US in response assessment, magnetic resonance imaging has been selected as the imaging modality to monitor tumor response in the forthcoming phase 2 trial. In patients with locally advanced breast cancers treated with RT alone at equivalent doses to those used in this study, local control rates would be expected to be 45% to 57% at 3 years posttreatment, with lower rates associated with larger tumors.[31,32] Although it

is impossible to draw conclusions on efficacy in this phase 1 trial, anecdotal tumor responses are suggestive of enhanced antitumor effect. Because lifetime control of symptomatic locally advanced breast cancer is a major determinant of patient quality of life as well as survivorship, there is potential for an effective treatment to be globally beneficial, in which women with inoperable breast cancer often have limited access to effective treatment.[33,34] Intratumoral H_2O_2 injections are inexpensive and easy to administer, requiring minimal additional training and infrastructure.

Our study has also established that circulating plasma markers can be successfully quantified using the ELISA and Luminex platforms, providing insights into mechanisms of cell death after treatment. IR and H_2O_2 induce reactive oxygen species, inflammatory signaling, DNA damage, senescence, and cell death. In addition, IR can modulate the immunoinflammatory axis through the generation of ROS and DAMPs.[35] The wide range of potential mechanisms of interaction informed the choice of 21 markers in our exploratory panel.

The effect of H_2O_2 within the cell is concentration dependent, having a role in signaling and homeostasis at nanomolar concentrations (nM) and triggering cell death at supraphysiological (mM) concentrations.[36] The physiological outcome within the cell is modulated by antioxidant enzymes such as catalases, peroxidases, and thioredoxin-linked systems.[37] By affecting protein kinases and phosphatases, H_2O_2 influences a number of signaling cascades including ERK, JNK, MAPK, p38, TNFα, NFκB, IL-1β, IL-6, IL-8, MCP-1, and MIP.[36,38,39] Several publications have demonstrated apoptosis as the principal mode of cell death after H_2O_2 treatment.[10,40-42] The intrinsic mitochondrial pathway is thought to be the predominant mechanism of apoptosis.[43] One study reported apoptosis induction after exposure to H_2O_2 levels <0.4 mM and upregulation of RIP, a gene associated with necrosis, at higher concentrations.[44]

Exposure to H_2O_2 can result in increased expression of inflammatory cytokines.[45] IL-1 is a key mediator of T cell and dendritic cell function. Increased IL-1α levels occur in cells undergoing necrosis, whereas IL-1β signals toward apoptosis.[46,47] Another study reported that treating murine splenic T cells with H_2O_2 resulted in a significant increase in IL-4 production, a key regulator of humoral and adaptive immunity.[48] Both IL-1β and IL-4 were significantly associated with tumor shrinkage in our study.

In our plasma analysis, a significant association between TRAIL and tumor shrinkage was also found. Intracellular ROS such as H_2O_2 is thought to mediate apoptosis via death receptor ligands such as TRAIL.[43] A study in an astroglial cell line demonstrated an increase in TRAIL gene expression in cells treated with H_2O_2 in a dose-dependent manner up to a concentration of 0.8 mM.[49] TRAIL-dependent apoptosis regulates the priming of $CD8^+$ memory T cells by $CD4^+$ T_H1 cells.[50] In a study using a murine macrophage cell line (B10R), exposure to H_2O_2 increased the transcription of the chemokines MIP-1α, MIP-1β, MIP-2, and MCP-1.[51] MIP-1β was undetectable in 7 of 10 patients, but MIP-1α expression was detected to a varying degree in all cases, suggesting activation of $CD8^+$ T lymphocytes.

In summary, the analysis of blood biomarkers showed correlations with clinical tumor response and suggest an inflammatory/immune response associated with apoptotic cell death. These mechanisms of action are potentially relevant to an interaction between IR and drug. However, in this cohort, all of whom received H_2O_2 + RT, it is difficult to distinguish the contribution of H_2O_2 over and above that of RT alone. The plasma analyses have been valuable in informing the selection of markers to investigate in a subsequent trial.

Conclusions

The results from this phase 1 trial confirm intratumoral H_2O_2 in combination with RT is a safe and simple intervention with the potential for high global impact if efficacy is confirmed in the forthcoming randomized phase 2 trial. Proof of concept in breast cancer could lead to rapid evaluation in other challenging and accessible primary sites, including cancers of the head and neck, cervix uteri, and soft tissue sarcomas, where locoregional control with RT alone is poor.

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