A precision 3D conformal treatment technique in rats: Application to whole-brain radiotherapy with hippocampal avoidance

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Purpose: To develop and validate three-dimensional (3D) conformal hippocampal sparing whole-brain radiation therapy (HA-WBRT) for Wistar rats utilizing precision 3D-printed immobilization and micro-blocks. This technique paves the way for future preclinical studies investigating brain treatments that reduce neurotoxicity.

Methods and materials: A novel preclinical treatment planning and delivery process was developed to enable precision 3D conformal treatment and hippocampal avoidance capability for the Xrad 225cx small animal irradiator. A range of conformal avoidance plans were evaluated consisting of equiangularly spaced coplanar axial beams, with plans containing 2, 4, 7, and 8 fields. The hippocampal sparing and coverage of these plans were investigated through Monte Carlo dose calculation (SmART-Plan Xrad 225cx planning system). Treatment delivery was implemented through a novel process where hippocampal block shapes were computer generated from an MRI rat atlas which was registered to on-board cone beam CT of the rat in treatment position. The blocks were 3D printed with a tungsten-doped filament at lateral resolution of 80 μm . Precision immobilization was achieved utilizing a 3D-printed support system which enabled angled positioning of the rat head in supine position and bite block to improve coverage of the central diencephalon. Treatment delivery was verified on rodent-morphic Presage[®] 3D dosimeters optically scanned at 0.2-mm isotropic resolution. Biological verification of hippocampal avoidance was performed with immunohistologic staining.

Results: All simulated plans spared the hippocampus while delivering high dose to the brain (22.5–26.2 Gy mean dose to brain at mean hippocampal dose of 7 Gy). No significant improvement in hippocampal sparing was observed by adding beams beyond four fields. Dosimetric sparing of hippocampal region of the four-field plan was verified with the Presage[®] dosimeter (mean dose = 9.6 Gy, D100% = 7.1 Gy). Simulation and dosimeter match at distance-to-agreement of 2 mm and dose difference of $\pm 3\%$ at 91.7% gamma passing rate (passing criteria of $\gamma < 1$). Agreement is less at 1 mm and $\pm 5\%$ at 69.0% gamma passing rate. The four-field plan was further validated with immunohistochemistry and showed a significant reduction in DNA double-strand breaks within the spared region compared with whole-brain irradiated groups ($P = 0.021$). However, coverage of the whole brain was low at 48.5–57.8% of the volume receiving 30Gy at 7Gy mean hippocampal dose in simulation and 46.7–52.5% in dosimetric measurements. This can be attributed to the shape of the rat hippocampus and the inability of treatment platform to employ non-coplanar beams.

Conclusion: A novel approach for conformal microradiation therapy using 3D-printing technology was developed, implemented, and validated. A workflow was developed to generate accurate 3D-printed blocks from registered high-resolution rat MRI atlas structures. Although hippocampus was spared with this technique, whole-brain target coverage was suboptimal, indicating that non-coplanar beams and IMRT capability may be required to meet stringent dose criteria associated with current

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Key words: 3D printer, hippocampal avoidance, preclinical study, RTOG 0933, small animal irradiator

1. INTRODUCTION

Therapeutic whole-brain radiotherapy (WBRT) for brain metastases has been used since the 1950s.¹ Despite its therapeutic benefits in patients with a high burden of intracranial disease, many patients experience some degree of neurocognitive decline after treatment.^{2,3} Metastases to the limbic circuit (including the hippocampus) are rare, and because the hippocampus is known to play an integral role in memory, the concept of WBRT with hippocampal avoidance (HA-WBRT) evolved as a treatment to try to reduce neurocognitive decline after radiotherapy.^{1,4} The potential value of hippocampal avoidance is supported by preclinical studies investigating the role of the hippocampus in memory and the recent RTOG 0933 trial which provides preliminary data that the rate of neurocognitive declines less for patients receiving WBRT with hippocampal avoidance than the decline seen after WBRT seen in historical controls.^{1,3} There are now several ongoing trials in the United States and abroad to investigate the value of hippocampal avoidance during radiotherapy.

Despite intense clinical interest in hippocampal avoidance, a reliable and accurate animal model of the technique to allow for better preclinical data collection is lacking. Largely, this can be attributed to the technical hurdles associated with delivering precise, conformal treatment to the brain of small animals. In an effort to develop a reliable model that allows for reverse translation, a rodent model of hippocampal avoidance with MRI-based treatment planning was previously developed.⁵ In this initial work, a lateral-opposed two-field HA-WBRT plan was developed for 8-week-old Wistar rats with two conformal lead hand-cut blocks held at a distance in front of the x-ray source to shield the hippocampus. The coverage of the target volume (brain minus hippocampus) was not ideal due to under-dosing of the central diencephalon. To address these shortcomings, four improvements and innovations on this previous approach have been made including (a) a broad investigation of three-dimensional (3D) conformal treatments (not 2D parallel opposed, but up to eight multiple conformal beams); (b) a novel accurate method of conformal block creation from auto-segmented MRI atlas utilizing precision 3D printing; (c) an improved immobilization method incorporating a novel 3D-printed device to angle the rat's head in a supine position to expose the central diencephalon to radiation [Fig. 1(a)]; and (d) a precision mounting tool for accurate and consistent conformal block placement [Figs. 1(c) and 1(d)]. The main goal of current work is to develop, evaluate, and verify this novel 3D conformal treatment technique in rats for delivering whole-brain radiotherapy with hippocampal avoidance. Accuracy of the treatment set-up was validated with Monte Carlo simulation software,

high-resolution 3D optical dosimetry, and immunohistology. In the process, a platform for precise radiation delivery with 3D-printed conformal blocks was developed and verified.

2. MATERIALS AND METHODS

2.A. Animals and overview of study design

All animal experiments were reviewed and approved by the Duke Institutional Animal Care and Use Committee (IACUC) under protocol number A125-14-05. Wistar rats were housed in pairs (purchased from the Charles River Laboratory). A custom 3D-printed bite block was developed to immobilize and position rats in the supine position with their heads angled off the table. This head angle positioned the hippocampus relative to the gantry for conformal treatment with better access to diencephalon (Fig. 1). A low-dose, bone-contrasted cone beam CT (CBCT) was acquired in an 8-week-old Wistar rat immobilized with the 3D-printed bite block while sedated with 3% isoflurane anesthesia on a Xrad 225Cx MicroCT irradiator (CBCT at 40 kVp, 2.5 mA, 1×1 binning, 0.8 mm voxels).⁶⁻⁸ This CBCT was registered to an MRI Wistar rat atlas provided by the Center for In Vivo Microscopy (CIVM) at Duke.⁹ The registration process via bony landmarks has been described in detail previously.⁵ The CBCT and the segmented hippocampus were processed on an in-house block generation graphical user interface (GUI) based on MATLAB to generate digital block models for conformal hippocampal sparing and to simulate a CBCT with radiation blocks for SmART-Plan, a small animal Monte Carlo simulation software.¹⁰ The blocks were then 3D-printed from acrylonitrile butadiene styrene (ABS) metal-composite 3D-printing material GMASS™ (Turner MedTech, Inc., Orem, UT, USA) intended for x-ray shielding. Treatment dosimetry was verified with one cylindrical and two rodent-morphic Presage® dosimeters and simulated with SmART-Plan Monte Carlo software. Subsequently, rats were irradiated with a single 4-Gy treatment and killed immediately after treatment to allow for *in vivo* verification of hippocampal sparing with anti-phospho-Histone H2A.X immunohistochemical (IHC) microscopy, which visualizes double-stranded breaks in DNA due to radiation damage.

2.B. Treatment localization: immobilization and block generation software

The procedure for generating precision 3D-printed blocks for hippocampal sparing is illustrated in Fig. 2. To ensure reliable positioning and localization of the hippocampus on every rat, a custom 3D-printed bite block immobilization tool

FIG. 1. Materials for immobilization of Wistar rats and high precision positioning of 3D-printed micro-blocks. (a) Computer rendering of the bite block used for rat immobilization for supine irradiation. (b) CBCTs of two different rats restrained with the bite block demonstrate consistency of position between the two subject rats. Bony landmarks close to the hippocampus demonstrate 1.81-mm shift in position at maximum, which is corrected during image-guided treatment process. (c) Collimator mount and block tray for radiation block positioning with respect to beam center. (d) Picture of treatment set-up. Collimator mount and the block tray position the radiation block with respect to the center of the beam. Fluoroscope taken with this set-up (inset) confirms that the radiation block effectively stops the radiation. [Color figure can be viewed at wileyonlinelibrary.com]

was developed (Fig. 1) based on a design by the Duke Center for In Vivo Microscopy (CIVM).¹¹ The immobilization features a locking nose cone and bite bar, with channel for isoflurane flow. To optimize dose conformality around the hippocampus, the block holds a supine animal with its head angled upward at 15 degrees.

An in-house general-purpose radiation block generation software [Fig. 2(b)] was created to calculate precise conformal 3D-block shapes for arbitrary gantry angles based on segmented volumes to spare. The software requires as input a CBCT of a Wistar rat brain with the hippocampus already segmented. Hippocampal segmentation was performed via registration to an MRI atlas in 3D slicer,¹² following the bony landmark registration method outlined in Cramer *et al.* (2015). Users specify SAD, source-to-block distance (SBD), gantry angles, margin, and treatment isocenter position. The software then computes the block dimensions using projection ray tracing to determine block dimensions at the SBD for conformal sparing of the structure located at SAD. Users can also specify the margins from the segmented structure to block edge during block generation. After processing, the software outputs models of each block as a 3D-mesh stereolithography file, which can be directly 3D printed on any nominal fused-deposition modeling 3D printer in any desired material. GMASSTM was chosen for this study due to its high concentration of tungsten (30% by volume) and utility as x-ray shielding material. A custom tray system was developed to rigidly and consistently mount each block at the center of an Xrad treatment field using $40 \times 40 \text{ mm}^2$ collimator and a

3D-printed collimator mount [Figs. 1(c) and 2(b)]. The software also outputs 2D block printed templates with crosshairs denoting beam center and which fit into the tray. The tray locks into the collimator mount so that the crosshair converges at the center of the collimator. This allows precise positioning and alignment of blocks onto individual trays for multi-field treatment plans.

The accuracy and repeatability of block mounting on the Xrad 225Cx was evaluated by analysis of multiple portal images (radiographic) each taken after repetitive unmounting and re-mounting of the block tray and block (Fig. 2). The analysis consisted of determining the center-of-mass deviations between seven repeat images using ImageJ software tools (<https://imagej.nih.gov/ij/>). The accuracy of measured center-of-mass was determined by comparison of the radiographic portal image with the printed block template.

2.C. Treatment planning

The block generation software described above locates the treatment isocenter and exports a planning CBCT data set of the Wistar rat with simulated radiation blocks “burned in” as cerrobend which can then be used as input for dose calculation in the Xrad 225Cx treatment planning software SmART-Plan. The density of the segmented radiation blocks was artificially set as cerrobend with very high density $\sim 100 \text{ g/cm}^3$ such that $< 0.01\%$ transmission is modeled through the block. This was a work-around to circumvent limitations of the SmART-Plan software to faithfully

FIG. 2. Flowchart of process for precision micro-block fabrication and subsequent Monte Carlo simulation via in-house “Block Generation” software. (a) MRI-ATLAS and CBCT of a Wistar rat are registered, then a volume of interest (hippocampus) is segmented out for input into block generation software. (b) The Block Generation software takes as inputs the segmented volume and treatment beam angles to generate precision 3D-printable micro-blocks. It also “burns” virtual blocks into the CBCT data to enable dose calculation in SmART-Plan. (c) Visual representation of the algorithm, which traces the segmented volume toward the X-ray source to generate the geometry required to shield it at source-to-block distance (SBD), given the source-to-axis distance (SAD) and beam angle. [Color figure can be viewed at wileyonlinelibrary.com]

simulate tungsten ABS. With this new capability, a range of 3D conformal plans with 2, 4, 7, and 8 fields (2F, 4F, 7F, and 8F) were developed utilizing coplanar beams (non-coplanar beams are not possible with the hardware limitations of the Xrad 225Cx couch). On SmART-Plan, 4-Gy prescription dose to the isocenter placed within the cerebral cortex for the 3D conformal treatment cases were simulated.

Dose volume histograms (DVH) based on segmented whole brain and hippocampal volumes extracted from the atlas were generated during planning for all conformal HA-WBRT plans with 1-mm margin for hippocampus. All SmART-Plan simulated dose distributions were scaled by a constant to maximize the percentage of planning treatment volume (PTV) receiving 30 Gy or higher (V30), where the PTV is the brain minus the hippocampus, without violating the other RTOG 0933 limits (hippocampal dose $D_{100\%} < 9$ Gy and $D_{\max} < 16$ Gy; PTV volume receiving greater than 40 Gy $V_{40} < 2\%$) and keeping the mean hippocampal dose among the plans equal. Most RTOG limits were met when hippocampal dose was 7.0 Gy. Different dose scales

were attempted such as normalization to PTV $V_{30} = 90\%$, but that resulted in excessive brain dose (~ 100 Gy) and resulted in no clear differentiation between the conformal plans in terms of hippocampal sparing. After normalization, RTOG 0933 statistics were tabulated, including V30 and V40 for the PTV and $D_{100\%}$ and $D_{\max}$ of hippocampus, to compare hippocampal sparing and PTV dose homogeneity between the conformal plans. All DVH calculations were performed in SlicerRT, a radiotherapy platform on 3D Slicer designed for preclinical radiological studies.¹³

2.D. Treatment verification

2.D.1. Dosimetric verification

For physical verification of the dose deliveries, a custom rodent-morphic Presage[®] dosimeter with the same dimensions of the Wistar rat⁷ was irradiated with the four-field conformal treatment, which was determined from simulation to be the optimal plan. The exact beam-on time required to

deliver 4 Gy to the isocenter was obtained from SmART-Plan simulations. A margin of 1 mm was applied to each radiation block, to account for any positional uncertainties. The dosimeters were fabricated from the CBCT of Wistar rats via snake segmentation in itk-snap¹⁴ to generate a surface rendering of the rat head. All irradiations were performed at 225 kVp with 0.35 mm Cu filter, with SAD of 30.76 cm and SBD of 23.07 cm.

Subsequently, a complex eight-field HA-WBRT conformal treatment was delivered to a 2.5-cm-diameter cylindrical Presage[®] dosimeter to single fraction of 30 Gy (beams at gantry angles 0°, 45°, 90°, 135°, 180°, 225°, 270°, and 315°). Hippocampal margins were set at 0 mm for this experiment to test the positional accuracy of the blocks. A particular care was taken in placement and printing of each blocks to ensure the blocks were correctly positioned and to attempt to meet RTOG limits by minimizing errors in block positioning. After irradiations, dosimeters were scanned with duke large field-of-view optical scanner (DLOS)¹⁵ to reconstruct the dose delivered. Dosimeters were reconstructed at a resolution of $1 \times 1 \times 1 \text{ mm}^3$ using 360 projections.

Following irradiation, the 3D dose distribution for the four-field case was compared with its corresponding Monte Carlo simulated dose distribution. The Presage[®] dose distribution was scaled to maximize gamma passing rate with distance-to-agreement (DTA) criteria of 2 mm, $\pm 3\%$ of dose difference, and $\gamma < 1$. Gamma calculation was restricted to the whole brain to exclude the skull, where heightened bone dose in SmART-Plan cannot be reproduced in Presage[®].¹⁶

2.D.2. In vivo assessment of DNA damage with immunofluorescence

To assess *in vivo* the ability of the conformal treatment plans to spare the hippocampus, radiation-induced DNA double-strand breaks with anti-phospho-Histone H2A.X antibody was evaluated after treatment with four-field HA-WBRT. Six Wistar rats (8 weeks old) were treated with either WBRT (“positive control,” $n = 2$), HA-WBRT ($n = 2$), or no RT (“negative control,” $n = 2$). Before treatment of every rat, a treatment CBCT was taken. This CBCT was registered to planning CBCT used in SmART-Plan simulation studies before irradiation, obviating the need for manual fluoroscopic positioning performed during earlier work in Cramer et al. (2015). Rats in the WBRT and HA-WBRT groups received a single fraction of 4 Gy using a four-field treatment. Rats in the control group were irradiated at 225 kVp with 0.35 mm Cu filter, with SAD of 30.76 cm and SBD of 23.07 cm. Within 1 hour of irradiation, the rats were terminally sedated with CO₂, decapitated, and their brains removed. The investigator performing the immunofluorescence (KMJ) was blind to the treatment groups. The brains were embedded in clear frozen sectioning compound (VWR, West Chester, PA, USA), frozen on dry ice, and then sliced into 10- μm coronal sections with a cryostat at -20°C . Frozen sections were fixed with cold 4% paraformaldehyde for 10 min and then permeabilized with PBS containing 0.25% Triton X-100 (PBST) for

10 min. The sections were blocked by 10% goat serum in PBST for 30 min at room temperature. Mouse monoclonal anti-phospho-Histone H2A.X (Ser139) Antibody (1:250 in 10% goat serum PBST, #05-636, Millipore, Billerica, MA, USA) were applied to the sections for 1 h at room temperature. Nuclei were then stained with Hoechst 33342 (1:100 in PBS) for 2 min at room temperature. Fluorescence images were acquired with a Leica microscope. Relative signal intensities for phospho-Histone H2A.X were determined using Image J by averaging pixel values by total pixel numbers. The intensities were calculated in the cerebral cortex and hippocampal region for each group, and then were compared by unpaired Student’s *t*-test with significance set at $P = 0.05$.

3. RESULTS

3.A. Comparison between various HA-WBRT methods

SmART-Plan simulated dose distributions and associated DVHs for all HA-WBRT plans (2F, 4F, 7F, and 8F) are shown in Fig. 3. All dose distributions and DVHs were normalized to give a mean hippocampal dose of 7.0 Gy. Simulated blocks can be observed throughout the axial slices in Fig. 3(a) at the periphery of the rat. The treatment couch and bite block have been accounted for in simulation, as can be observed by noting the dose in these structures. Axial slices clearly show a central very low-dose region in the brain corresponding to the shielded hippocampal volume. In Fig. 3(b), DVHs of the hippocampal volume (blue) and the brain (PTV, pink) are presented for each simulated plan. The brain DVH features a step-like appearance for parallel-opposed 2F plan at around 70% due to missed central diencephalon. With addition of more fields, this curve “smooths out,” indicating the volume of the brain missed on the 2F plan is now receiving at least a partial dose. Measured RTOG metrics from these curves are compiled in Fig. 3(c) and compared with the RTOG criteria.

Figure 4 compares the measured rodent-morphic Presage[®] dose distribution with simulated SmART-Plan Monte Carlo dose distribution for the four-field HA-WBRT treatment. In Fig. 4(a), the two distributions are shown on the rat CBCT. The maximum gamma passing rate obtained by scaling the Presage[®] dose to match the simulation was 91.7% with criteria of 2-mm distance-to-agreement and 3% dose difference within the whole brain. The high dose measured at edge of the Presage[®] dosimeter (0.5–1 mm edge ring) results from a well-known edge artifact in optical CT. Data in this outer rim are, therefore, ignored. Dose profiles 1–4 (shown as dotted lines) through the hippocampal region are shown in Fig. 4(b) for the registered measurement (green) and simulation (red) dose distributions. Profile 1 includes bone dose to demonstrate effects of dose heterogeneity to simulated dose. Calculated DVHs of hippocampus with 1-mm margin (hippo+margin) and brain without hippocampus (brain wo hippo, PTV) from these two distributions are compared in Fig. 4(c).

FIG. 3. SmART-Plan dose distribution comparison between 2-, 4-, 7-, and 8-field 3D conformal HA-WBRT simulations. (a) Simulated dose distributions after scaling dose to best match RTOG criteria while keeping mean hippocampal dose the same among the four plans. (b) Corresponding DVH for hippocampus with 1-mm margin (hippo + margin, blue) and for brain without hippocampus (brain wo hippo, pink). (c) Comparison of RTOG-relevant dose statistics among the conformal plans. [Color figure can be viewed at wileyonlinelibrary.com]

3.B. Dosimetric and geometric verification of 3D-printed blocks

The accuracy and repeatability of block mounting on the Xrad 225Cx, evaluated by analysis of multiple portal images, is shown in Table I. A placement error of 0.12 mm in the left–right axis and 0.03 mm in superior–inferior axis from planned block position was observed. The repeatability of block placement in left–right axis was ± 0.10 mm and in the superior–inferior axis was ± 0.05 mm.

The optical-CT reconstruction of the 2.5-cm-diameter cylindrical Presage[®] irradiated to 30 Gy with eight-field HA-WBRT is shown in Fig. 5. The axial and coronal views of the reconstructed dose distribution reveal a region of low dose in the center of the dosimeter corresponding to spared hippocampal volume [Figs. 5(b) and 5(c)]. A star-shaped pattern is observed on the axial slice reminiscent of the eight-field simulation in Fig. 3. The reconstructed dose volume was scaled by a constant value to produce DVH that maximizes V30 while satisfying all RTOG trial limits. Scaled DVHs obtained from segmentations of the hippocampus (without margin) and the PTV (brain wo hippocampus) are shown in Fig. 5(d). Most RTOG limits could be satisfied, but fractional volume of the

PTV receiving 30 Gy, V30, was unmet (ideally $> 90\%$ but measurement was 52.5%). The geometric conformity of presented sparing technique is best visualized with a rendering of the 9 Gy and Dmax (16 Gy for hippocampus) isodose surface of the scaled Presage[®] data surrounding the hippocampal avoidance structure [Fig. 5(e)]. Most notably, the 9-Gy isodose almost entirely envelops the hippocampal volume.

3.C. In vivo verification with immunohistology

Figure 6 shows immunofluorescence results of histological slices through the cortex and the hippocampus for all three rat groups: WBRT (or “positive control,” PC), four-field HA-WBRT (“hippocampal sparing,” HS), and no irradiation negative control (NC). Anti-phospho-Histone H2A.X immunofluorescence intensity is shown as green staining. Hoechst staining of cell nuclei are shown as blue. Relative signal intensity (RSI) of gamma-H2A.X fluorescence is increased in the cortex following WBRT and HS, but RSI in the hippocampus (hippo) is significantly less after HS compared with WBRT [Fig. 6(c)]. As expected, the relative signal intensity (RSI) of H2A.X fluorescence of the HS group is elevated compared with those of NC in the cortices

FIG. 4. Comparison of rodent-morphic Presage® measurement and SmART-Plan dose calculation of the 4F conformal HA-WBRT plan. The relative measured dose was normalized to maximum gamma pass rate with the calculated distribution. (a) Coronal and axial dose distributions of measured (Presage) and calculated (SmART-Plan) doses. Contours of the hippocampus with 1-mm margin (hippo + margin) and the PTV (whole brain without hippocampus) used for DVH calculations are shown. (b) Dose profiles in the coronal (lines 1 and 2) and axial (lines 3 and 4) indicate good agreement of the measured and calculated distributions. (c) Corresponding DVHs of hippocampus (including 1mm margin) and PTV (brain w/o hippo). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE I. Positional variation of seven mounted blocks expected at isocenter, measured via fluoroscopy.

Block CM position	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Planned position	Bias	SD
Fluoro										
X (mm)	11.85	11.85	12.11	12.09	11.90	11.90	11.87	12.06	L0.12	±0.10 mm
Y (mm)	13.62	13.62	13.49	13.59	13.62	13.65	13.64	13.35	I 0.03	±0.05 mm

CM, center of mass; SD, standard deviation; L/R, patient left/right; S/I, superior/inferior.

($RSI_{cortex,NC} = 2.77$, $RSI_{cortex,HS} = 4.08$, $P = 0.032$). In addition, the RSI of H2A.X fluorescence in hippocampus for PC ($RSI_{hippo,PC} = 6.09$) is significantly higher than NC ($P = 0.011$) or HS ($P = 0.021$). A slight increase in H2A.X fluorescence is noted in the hippocampus for HS compared with that for NC ($RSI_{hippo,HS} = 3.40$, $RSI_{hippo,NC} = 2.91$, $P = 0.067$), which is consistent with dosimetric and

simulated results that the hippocampus does receive limited dose with $D100\% > 5$ Gy.

4. DISCUSSION

The capability of the novel conformal coplanar treatment approach for HA-WBRT is explored in Fig. 3. Prior work⁵

FIG. 6. *In vivo* validation of hippocampal avoidance with radiation in rats treated with brain irradiation with hippocampal avoidance. (a) Representative immunofluorescence images of cerebral cortices in rats treated with no irradiation (NC for negative control), whole-brain irradiation (PC for positive control), and hippocampal sparing brain irradiation (HS). In the cortex, there is no difference in Hoechst staining or anti-phospho-Histone H2A.X between the PC and HS groups. Scale bar = 50 microns. (b) Representative immunofluorescence images using hippocampal slices (granule cell layer of the dentate gyrus) from each treatment group. In the hippocampus, anti-phospho-Histone gamma-H2A.X immunofluorescence is significantly reduced in the HS group compared with the PC. Scale bar = 50 microns. (c) Anti-phospho-Histone gamma-H2A.X antibody immunofluorescence signal intensity analysis. The radiation-induced phosphorylation of Histone H2A.X in the hippocampus is significantly decreased when the brain is irradiated with hippocampal avoidance, $*P < 0.05$ (by Student's *t*-test). [Color figure can be viewed at wileyonlinelibrary.com]

calculated 3D doses is quite close as shown in Fig. 4(c). Both the measurement and the simulation indicate that the hippocampus is significantly spared at $D_{max} < 16$ Gy and $D_{100\%} \leq 7.1$ Gy. However, the Presage[®] dose distribution is marked by broader dose penumbra at the block edges, as can be seen on profile 2 at around 11 mm and 15–20 mm. Possible causes include the incorrect assumption of the Monte Carlo simulation of an infinitesimal x-ray focal spot size, the error of which is amplified in this set-up due to proximity of the radiation block to the x-ray source, and the effect of heterogeneities (e.g., skull bones) that effect the Monte Carlo calculation but are not present in the Presage measurement. Dose heterogeneity effects are observed as dose peaks for the skull in Fig. 4(b), profile 1. These effects could also be the cause for the minor deviations between simulation and measured DVHs despite the well-matched dose profiles. In addition, SmART-Plan simulation was calculated on rat CBCT, which contains high-Z structures which the tissue-equivalent rodent-morphic dosimeters lack.

The optical-CT reconstruction of the 2.5-cm-diameter cylindrical Presage[®] dosimeter irradiated to 30 Gy with an eight-field HA-WBRT plan demonstrate excellent hippocampal sparing (Fig. 5). The geometric accuracy and extent of sparing is reflected in the dose volume histogram (DVH) calculation generated from the Presage[®] cylinder data [Fig. 5(d)] and 9-Gy and 15-Gy isodose surfaces [Fig. 5(e)]. Geometrical congruence of hippocampus and the 9-Gy isodose strongly suggest that the presented workflow enables precise shielding of a complex 3D shape.

Immunohistology studies with four-field HA-WBRT confirm that the set-up is valid for *in vivo* treatment of rats with hippocampal avoidance. Negative control (NC) groups exhibit minimal signal in both cortex and hippocampus, which is expected since the rat received no dose

other than from the CBCT. The WBRT (PC) group delivered uniform dose to all parts of the brain. This is reflected in the distinct increase in green fluorescent signal for both cortex and hippocampus. For rats with hippocampal avoidance protocol (HS), radiation therapy induced significant phosphorylation of Histone H2A.X in the cortex; however, gamma-H2A.X staining is limited in the hippocampus for the same rat, indicating that the treatment protocol delivered significant dose to the cortex while limiting dose to the hippocampus.

Despite the hippocampal avoidance demonstrated, even the complex eight-field HA-WBRT 3D conformal plan was unable to deliver uniform dose to the PTV on par with the criteria specified in current human clinical trials, specifically the V30 (fractional volume of the brain receiving 30 Gy or larger was less than 90% of that in the current human hippocampal avoidance VMAT trials). The geometrical shape of the hippocampal volume provides explanation as to why [Fig. 5(e)]. Unlike human, a prone or supine rat hippocampal volume is nearly coplanar with the gantry. This means that sparing the hippocampus conformally also spares the central part of the hippocampus. We hypothesize that a non-coplanar beam is likely needed to achieve sufficient dose coverage and hippocampal sparing consistent with RTOG criteria in humans, which is $V_{30} > 90\%$, $V_{40} < 2\%$ for brain coverage and $D_{100\%} < 9$ Gy, $D_{max} < 16$ Gy for hippocampus. These results, therefore, support the conclusion that substantial innovation is required to meet the RTOG criteria, including non-coplanar beams and possibly intensity modulation.

The results in this article point to successful hippocampal sparing and establishes a platform for small animal conformal and conformal avoidance radiation therapy. With an improved ability to deliver whole-brain radiation therapy to rats with

hippocampal avoidance, future studies can employ this treatment plan to investigate the extent that this treatment spares neurotoxicity relative to whole-brain radiotherapy with opposed lateral fields. In addition to histological endpoints of neuronal cell death, hippocampus-dependent neurocognitive tests, such as the Morris water maze,¹⁷ can be performed. However, the conformal treatments used here still do not provide optimal target coverage although they achieve excellent hippocampal sparing. In addition, only two rats were tested per group on the in vivo immunohistological studies. More subjects will be tested in the future to further test the treatment accuracy in vivo.

5. CONCLUSION

This work introduces a new technique and workflow for precision conformal 3D treatment of hippocampal avoidance in rats which will enable preclinical experiments to investigate the mechanisms of radiation-induced cognitive decline and the benefit of dose reduction to the hippocampus. A very high level of precision is required to achieve accurate hippocampal sparing. The technique utilizes high precision 3D-printing technologies (immobilization and block generation and mounting) and high-resolution MRI atlases to achieve HA-WBRT.

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