Offset of apparent hyperpolarized $^{13}$C lactate flux by the use of adjuvant metformin in ionizing radiation therapy in vivo

Young-Suk Choi | Joonsung Lee | Han-Sol Lee | Jae Eun Song | Dong-Hyun Kim | Ho-Taek Song

Department of Radiology and Research Institute of Radiological Science, Yonsei University College of Medicine, Seoul, South Korea
Biomedical Science Institute, Yonsei University College of Medicine, Seoul, South Korea
GE Healthcare, Seoul, South Korea
Department of Electrical and Electronic Engineering, Yonsei University, Seoul, South Korea

Correspondence
Professor Ho-Taek Song, Department of Radiology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, South Korea.
Email: hotsong@yuhs.ac

Funding information
Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea, Grant/Award Number: H111C0032; National Research Foundation of Korea (NRF), Grant/Award Number: 2020R1F1A1074342; Yonsei University College of Medicine, Grant/Award Number: 6-2019-0076

An increase in hyperpolarized (HP) $[1-^{13}$C$]$lactate production has been suggested as a biomarker for cancer occurrence as well as for response monitoring of cancer treatment. Recently, the use of metformin has been suggested as an anticancer or adjuvant treatment. By regulating the cytosolic NAD$^+$/NADH redox state, metformin stimulates lactate production and increases the HP $[1-^{13}$C$]$lactate conversion rate in the kidney, liver, and heart. In general, increased HP $[1-^{13}$C$]$lactate is regarded as a sign of cancer occurrence or tumor growth. Thus, the relationship between the tumor suppression effect of metformin and the change in metabolism monitored by HP $[1-^{13}$C$]$pyruvate MRS in cancer treatment needs to be investigated. The present study was performed using a brain metastasis animal model with MDA-MB-231(BR)-Luc breast cancer cells. HP $[1-^{13}$C$]$pyruvate MRS, $T_2$-weighted MRI, and bioluminescence imaging were performed in groups treated with metformin or adjuvant metformin and radiation therapy. Metformin treatment alone did not display a tumor suppression effect, and the HP $[1-^{13}$C$]$lactate conversion rate increased. In radiation therapy, the HP $[1-^{13}$C$]$lactate conversion rate decreased with tumor suppression, with a $p$-value of 0.028. In the adjuvant metformin and radiation treatment, the tumor suppression effect increased, with a $p$-value of 0.001. However, the apparent HP $[1-^{13}$C$]$lactate conversion rate ($K_{pl}$) was observed to be offset by two opposite effects: a decrease on radiation therapy and an increase caused by metformin treatment. Although HP $[1-^{13}$C$]$pyruvate MRS could not evaluate the tumor suppression effect of adjuvant metformin and radiation therapy due to the offset phenomenon, metabolic changes following only metformin pre-treatment could be monitored. Therefore, our results indicate that the interpretation of HP $[1-^{13}$C$]$pyruvate MRS for response monitoring of cancer treatment should be carried out with caution when metformin is used as an adjuvant cancer therapy.

KEYWORDS
$^{13}$C pyruvate, cancer metabolism, dynamic nuclear polarization, hyperpolarization, MRS, metformin, NAD$^+$/NADH redox, therapeutic response

Abbreviations: FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HP, hyperpolarized; IR, ionizing radiation therapy; $K_{pl}$ apparent conversion rate from HP$[1-^{13}$C$]$pyruvate to HP$[1-^{13}$C$]$lactate; LDHA, lactate dehydrogenase A; MCT, monocarboxylate transporter; NAD, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; PI, propidium iodide; SEM, standard error of the mean; TBST, Tris-buffered saline containing 0.05% Tween 20.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2021 The Authors. NMR in Biomedicine published by John Wiley & Sons Ltd.
Aerobic glycolysis is a crucial feature of aberrant metabolism in cancer, represented by increased glucose uptake and increased lactate production.\textsuperscript{1,2} Hyperpolarized (HP) \(^{13}\)C MRS is an efficient diagnosis technique for imaging metabolism in vivo.\textsuperscript{3} This technology provides a real-time in vivo quantification of metabolites by amplifying the signal to noise ratio over 10 000-fold using a dynamic nuclear polarization technique.\textsuperscript{4–6} Since increased lactate production is a characteristic feature of aerobic glycolysis, \([1-^{13}\text{C}]\text{pyruvate MRS is used to investigate cancer metabolism for increased HP [1-^{13}\text{C}]lactate production in patients with prostate cancer and brain tumors.}\textsuperscript{7,8} The cancer therapeutic efficacy evaluated using HP [1-^{13}\text{C}]\text{pyruvate MRS in animal models reported decreased HP [1-^{13}\text{C}]lactate production after chemotherapy with etoposide, imatinib, and FX11 and radiation therapy.}\textsuperscript{9–16}

For decades, metformin has been one of the most widely used medicines for patients with diabetes.\textsuperscript{17} Retrospective studies have reported reduced cancer incidence and improved cancer outcomes with metformin use.\textsuperscript{18,19} Metformin has been suggested as an anticancer drug or as an adjuvant drug, with around 55 clinical trials ongoing worldwide.\textsuperscript{20} Although metformin has been used clinically for over half a century, its mechanisms of action are not fully understood. Madiraju et al recently suggested that metformin inhibits endogenous glucose production by decreasing the cytosolic oxidized/reduced nicotinamide adenine dinucleotide (NAD\(^+\)/NADH) redox state, which, in turn, inhibits the conversion of lactate and glycerol-3-phosphate—the substrates of gluconeogenesis—to pyruvate and dihydroyxacetone phosphate.\textsuperscript{21,22} Recent studies on HP [1-^{13}\text{C}] pyruvate MRS reported an increased HP [1-^{13}\text{C}]lactate conversion rate, thereby showing an altered cytosolic NAD\(^+\)/NADH redox state due to metformin treatment.\textsuperscript{23,24} An increased HP [1-^{13}\text{C}] lactate conversion rate in cancers is generally indicative of cancer occurrence or tumor growth. Therefore, there is a need to be cautious to interpret HP [1-^{13}\text{C}]\text{pyruvate MRS when metformin is used as an anticancer therapy during cancer response monitoring. In this study, we demonstrate the interpretation of HP [1-^{13}\text{C}]\text{pyruvate MRS in a brain metastasis animal model when adjuvant metformin and ionizing radiation therapy (IR) are used as an anticancer treatment.}

2 MATERIALS AND METHODS

2.1 Animal model

The study was approved by the Animal Care and Use Committee of Yonsei University College of Medicine, and all procedures were in compliance with the guidelines of the committee. Luciferase-tagged brain-seeking MDA-MB-231 breast cancer cells (MDA-MB-231(BR)-Luc) were used.\textsuperscript{25} Cells were harvested, suspended using phosphate buffered saline (PBS), and kept on ice at a concentration of \(1 \times 10^6\) cells/10 \(\mu\)L. Six-week-old female BALB/c nude mice were anesthetized using 1-3\% isoflurane gas in oxygen. Then, \(2 \times 10^5\) MDA-MB-231(BR)-Luc cells (2 \(\mu\)L volume) were injected into the brain 2 mm posterior, 1.5 mm right lateral, and 3.5 mm deep from the bregma. Cells were injected at a speed of 0.5 \(\mu\)L/min using a 33-gauge needle with a Hamilton syringe.

2.1.1 Effect of metformin treatment on HP [1-^{13}\text{C}]\text{pyruvate MRS}

To evaluate metformin's effect on HP [1-^{13}\text{C}]\text{pyruvate MRS (Experiment 1), mice were divided into control and metformin groups (}}n = 5\text{ per group. One week after tumor implantation, metformin was administered at 300 mg/kg, five times a week, via a gastric tube during follow-up. A T}_2\text{-weighted MR scan was performed at 2 weeks, and it was observed that one mouse in the metformin group failed to form a tumor mass. At 3 weeks, HP [1-^{13}\text{C}]\text{pyruvate MRS was performed in the control group (}}n = 5\text{) and the metformin group (}}n = 4\text{). Immediately after finishing HP MRS, two mice in the control group died. Therefore, survival was traced in the control group (}}n = 3\text{) and metformin group (}}n = 4\text{).}

2.1.2 Effect of adjuvant metformin and IR treatment on HP [1-^{13}\text{C}]\text{pyruvate MRS}

Next, five mice each were randomly grouped (Experiment 2) into a vehicle group (Veh), an IR group, and an adjuvant metformin and radiation therapy group (Met+IR). One week after tumor implantation, 300 mg/kg metformin was administered five times a week via a gastric tube during follow-up. A T\textsubscript{2} -weighted MR scan was performed at two weeks, and one mouse in the Met+IR group failed to form a tumor mass. Radiation therapy was performed 14 d after tumor cell injection. Anesthetized mice were exposed to 15 Gy by applying 3 Gy of X-rays for five consecutive days using an X-Rad 320 (Precision X-Ray, North Branford, CT). HP [1-^{13}\text{C}]\text{pyruvate MRS was performed on the brain of the mice in the Veh (}}n = 5\text{), IR (}}n = 5\text{), and Met+IR (}}n = 4\text{) groups at both 2 weeks and 3 weeks after tumor cell injection. Bioluminescence signal and survival were traced in these three groups (methods mentioned in the ensuing sub-sections).
2.2 | [1-13C]pyruvate preparation

[1-13C]pyruvate was mixed with 15 mM trityl radical OX-063 (Oxford Instruments, Oxford, UK), and 0.75 mM gadoterate meglumine (Dotarem, Guerbet, Villepinte, France) was added before polarization. Then, 21.2 μL of the [1-13C]pyruvate mixture was polarized using a HyperSense (Oxford Instruments, Oxford, UK), and the sample was dissolved in 3.8 mL of buffer (40 mM Tris, pH 7.4, 50 mM NaCl, 1 g/L EDTA, 80 mM NaOH). Pyruvate was nearly 20% polarized, and 350 μL of the dissolved sample was injected into the mice via their tail veins.

2.3 | In vivo HP 13C MRS

All experiments were performed on a 9.4 T MRI scanner system (Bruker BioSpin MRI, Ettlingen, Germany) with a 20 mm 1H,13C dual-tuned surface transmit/receive coil. The surface coil was placed directly above the mouse brain. For HP 13C MRS, slice-selective dynamic 13C free induction decay data with a flip angle of 10° and time resolution of 1 s were acquired using a pulse and acquire sequence.

A 10 mm thick axial slice was selected to include the whole brain. Dynamic acquisitions were started just before injecting HP [1-13C]pyruvate into the mouse and were acquired for 2 min. The spectral bandwidth was 6510 Hz collected into 2048 points. The conversion rate from pyruvate to lactate (Kpl) was calculated using the model-free approach previously presented by Hill et al.26 The HP 13C dynamic curve is shown in Supplementary Figure S1.

Brain metastasis was mapped and localized by a single time point of HP 13C MR imaging obtained using a centric-ordered chemical shift image sequence. A slice-selective excitation pulse with a 10° flip angle was used, and the slice thickness was 2 mm, including the tumor region with a 16 × 16 mm² field of view and a matrix size of 8 × 8. The spectral bandwidth was 6510 Hz, collected into 512 samples. Chemical shift image acquisition was started 20 s after the administration of the pyruvate, and the total scan time was about 10 s, with a repetition time of 81 ms and two averages.

T2-weighted images were acquired and used for chemical shift image overlay and tumor volume measurement with matrix size 192 × 192, slice thickness 0.5 mm, number of slices four, and RARE factor 4.

Analysis of the 13C MRS data was performed on software developed using MATLAB 2017a (MathWorks, Natick, MA). The stacks of spectral peaks were used to calculate the exchange ratio of [1-13C]lactate from [1-13C]pyruvate.

2.4 | Bioluminescence imaging

Tumor growth was assessed by bioluminescence imaging once a week from 12 to 74 d after tumor inoculation using a Xenogen IVIS imaging system (Caliper Life Science, Hopkinton, MA). Mice were subcutaneously injected with 150 mg/kg of D-luciferin (100 μL). After 10 min, the luminescence was measured from each mouse. Luminescence was reported as photons per second per square centimeter per square radian (p/s/cm²/sr).

2.5 | Cell culture

MDA-MB-231(BR)-Luc cells were cultured with 10% fetal bovine serum (FBS, Corning, Corning, NY) in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Logan, UT) in T75 cell culture flasks (SPL Life Science, Pocheon, Korea). Cells were incubated at 37 °C with 5% CO₂. Mycoplasma elimination was performed using a BioMycoid elimination kit (CellSafe, Yongin, Korea), and Mycoplasma-negative cells were confirmed using HiSense mycoplasma detection kits (CellSafe). The cells were validated as MDA-MB-231 by short tandem repeat DNA fingerprinting certified by the Korea Cell Line Bank.

2.6 | Cell growth assay

Two methods measured cell growth: the CellTiter 96 AQueous one solution cell proliferation assay (MTS) (Promega, Madison, WI) and the bioluminescent assay (Caliper Life Science, Hopkinton, MA). MDA-MB-231(BR)-Luc cells were seeded in 96-well plates at 5000 cells per well. After 24 h, cells were treated with different metformin concentrations in DMEM supplemented with 10% FBS for 4 h and then exposed to 10 Gy X-rays. After incubation for 72 h, we measured cell growth. For luciferase assay, D-luciferin (Caliper Life Science, Hopkinton, MA) was added to 200 μL of culture medium at 150 μg/mL concentration. After 5 min, a luciferase signal was measured using a luminometer (Berthold Centro LB 960, Bad Wildbad, Germany).
2.7 | Cell cycle analysis

A total of $4 \times 10^5$ cells were seeded in a 6 cm dish. The effect of metformin on radiation was evaluated with 5 mM metformin treatment for 4 h and exposure to 10 Gy of X-rays. After 48 h, cell cycle analysis was performed. Trypsinized cells were washed and re-suspended in 0.3 mL of PBS supplemented with 10% FBS. Cells were fixed by adding 0.7 mL of 100% ethanol and stored at $-20^\circ$C for 2 h. Cells were washed with PBS and stained with propidium iodide (PI, Sigma, Burlington, MA) staining solution (50 mg/L PI, 0.1% Triton X-100, 0.1 mmol/L EDTA, and 50 mg/L RNase A) for 30 min in the dark. Flow cytometry was performed on a BD Biosciences flow cytometer (Franklin Lakes, NJ) and analyzed with FlowJo.

2.8 | Annexin V apoptosis assay

MDA-MB-231(BR)-Luc cells were seeded in a 6 cm dish at a density of $4 \times 10^5$ cells. After 24 h, cells treated with 5 mM metformin in 10% FBS containing DMEM for 4 h were exposed to 10 Gy X-rays, and annexin V staining was performed at 48 h. All procedures were performed using an annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit (Sigma). Briefly, trypsinized cells were washed and re-suspended in 1 mL binding buffer. Then, 10 μL PI and 5 μL annexin V (Promega) were added and incubated for 10 min in the dark. Flow cytometry was performed on a BD Biosciences flow cytometer and analyzed with FlowJo.

2.9 | Immunofluorescence staining

Tumor-bearing brain tissue sections of 3 μm thickness were deparaffinized in xylene for 10 min. A tissue sample was hydrated by placing the slide gradually in 95%, 90%, 85%, 80%, and 70% ethanol for 5 min each. Antigens were retrieved in 10 mM sodium citrate buffer at pH 6.0. Tissue samples were blocked in 5% normal donkey serum for 60 min and incubated with goat antibodies against monocarboxylate transporter 1 (MCT1) (1:500, SC-14917, Santa Cruz Biotechnology, Dallas, TX) and rabbit antibodies against MCT4 (1:500, SC-14917, Santa Cruz Biotechnology) at 4°C overnight. Slides were washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBST), FITC-labeled donkey anti-rabbit antibodies and Texas Red-labeled donkey anti-goat antibodies (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) were added. Slides were incubated for 2 h at 20°C and washed three times using TBST. Then, 0.3% Sudan Black B in 70% ethanol was added, incubated for 1 h, and washed three times to decrease autofluorescence. Counterstain was performed using 4',6-diamidino-2-phenylindole VECTASHIELD medium (Vector Laboratories, Burlingame, CA). A Carl Zeiss Axioskop microscope (Oberkochen, Germany) was used for visualization.

2.10 | Western blotting

Twenty micrograms of protein was separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blots were probed with primary antibodies against MCT1 (NBP1-59656; Novusbio, MO), MCT4 (SC-14917, Santa Cruz Biotechnology, Dallas, TX), and lactate dehydrogenase A (LDHA; NBP1-48336; Novusbio, Saint Charles, MO), using β-actin (sc-47,778; Santa Cruz Biotechnology, Dallas, TX) or α-tubulin (05-829; Millipore, Burlington, MA) as an internal control. Protein bands were visualized on AGFA blue medical X-ray film using a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence kit (Thermo, AB Frontier, Seoul, Korea). Western blot bands were quantified using the NIH ImageJ software (Version 1.53C), and protein levels were normalized to the internal control.

2.11 | Lactate measurement

MDA-MB-231(BR)-Luc cells were seeded in a 6 cm dish at a density of $4 \times 10^5$ cells. After 24 h, the cells were treated with 5 mM of metformin or various radiation doses in 10% FBS containing DMEM for the indicated time. The effect of metformin on radiation was evaluated by treatment with metformin for 4 h and exposure to 10 Gy X-rays. After 24 h, the cells were harvested using trypsin-EDTA and lysed using NP-40 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% NP-40). The lysate concentration was measured using a BCA protein kit (Thermo Scientific, Waltham, MA), and the lactate concentration was measured using a lactate colorimetric assay kit (K607, BioVision, Milpitas, CA) according to the manufacturer’s instructions and calculated in 40 μg of lysate.
2.12 2-NDBG glucose uptake

Glucose uptake was measured using a 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-d-glucose (2-NBDG) glucose uptake assay kit (K682, BioVision) following a previous report. MDA-MB-231(BR)-Luc cells were seeded in a 6 cm dish at a density of 2 \times 10^5 cells. Cells were treated with 5 mM metformin for 4 h, exposed to 10 Gy X-rays, and harvested after 24 h using trypsin-EDTA. Cells were incubated with 2-NBDG for 1 h in DMEM containing 0.5% FBS in a CO2 incubator, washed, and suspended using an analysis buffer. Flow cytometry was performed on a BD Biosciences flow cytometer and analyzed with FlowJo.

2.13 NAD measurement

NAD levels were measured using an NADH colorimetric kit (K337, BioVision). MDA-MB-231(BR)-Luc cells were treated with 5 mM metformin for 24 h and exposed to 10 Gy X-rays. Since poly ADP-ribose accumulation is a very early response to DNA damage, and this process uses NAD\(^+\), this reaction can influence the total amount of NAD. Therefore, we harvested cells 30 min after irradiation and followed the manufacturer’s instructions. The lysed cell concentration was measured using a BCA-based assay, and 40 \(\mu\)g of lysate was used to detect NAD\(^+\) and NADH concentrations. According to the manufacturer's protocol, the absorbance was measured at 450 nm using a spectrometer (VersaMax, Molecular Devices, San Jose, CA).

2.14 ATP measurement

MDA-MB-231(BR)-Luc cells were treated with 5 mM of metformin for 4 h, exposed to 10 Gy of X-rays, and then maintained for 24 h. Trypsin-treated cells were counted, and 1 \times 10^5 cells were used for the ATP measurement according to the manufacturer’s protocol (K354, BioVision).

2.15 Statistical analysis

In vivo data are presented as mean ± standard error (SEM) and in vitro data are presented as mean ± standard deviation. The sample size indicates experimental replicates from a single representative experiment validated by independent repetitions. \(p < 0.05\) was considered statistically significant.

![Figure 1](image-url)  
**Figure 1** HP [1-13C]lactate localized in the brain metastasis. (A), The spectrum (above left) and the interpolated HP [1-13C]lactate map (above right) were overlaid on 1H imaging. The lactate map was estimated as a peak signal to noise ratio with an arbitrary unit (AU). HP [1-13C] pyruvate and HP [1-13C]lactate spectra were detected at 173 ppm and 185 ppm, respectively. Higher HP [1-13C]lactate peaks were detected in the voxels containing metastasis. (B), MCT1 and MCT4 were stained with Texas Red (red) and FITC (green), respectively, and nuclei were stained with DAPI. Scale bar, 100 \(\mu\)m.
significant. The p-value was determined by Student’s two-tailed t-test or ANOVA followed by Tukey’s test for post hoc comparison using statistical software (Prism Version 6.0, GraphPad, San Diego, CA).

3 | RESULTS

3.1 | HP [1-13C]lactate localized in brain metastasis

Most HP [1-13C]lactate signals localized in the metastasized brain tumors (Figure 1A). Note that 3 x 3 voxels were selected in 8 x 8 chemical shift imaging and overlaid on 1H imaging. We examined the MCT1 and MCT4 expression patterns in a control mouse exhibiting metastasis. A high expression of MCT1 and MCT4 was observed in the tumor area, correlating with positively detected HP [1-13C] lactate signal (Figure 1B).

3.2 | Metformin alone showed no tumor suppression but increased HP [1-13C]lactate conversion rate

The effect of metformin on HP [1-13C]pyruvate MRS in the mouse model was investigated according to the schedule shown in Figure 2A. T2-weighted MRI was used to monitor tumor growth. Metformin alone showed no difference in tumor growth (Figure 2B and 2C) or survival (Figure 2D). The apparent conversion rate (Kpl) from HP [1-13C]pyruvate to HP [1-13C]lactate was estimated using the HP 13C MRS dynamic curve (Figure 2E). Metformin-treated mice showed increased HP [1-13C]lactate conversion compared with vehicle-treated mice (Figure 2F and Supplementary Figure S2). However, metformin-treated mice without brain metastasis showed no increase in HP [1-13C]
lactate (Figure 2G), showing the difference in response to metformin treatment between the normal brain tissue and tumor tissue in BALB/c nude mice.

3.3 | Adjuvant metformin offset the $K_{pl}$ of HP [1-13C]pyruvate MRS

We investigated the tumor response to the vehicle, radiation therapy, and adjuvant metformin along with radiation therapy using MRI, bioluminescence imaging, and HP [1-13C]pyruvate MRS results according to the treatment schedule (Figure 3A). $T_2$-weighted imaging showed a threefold increase in tumor volume (from $1.35 \pm 0.37$ mm$^3$ to $4.43 \pm 0.82$ mm$^3$) in the vehicle group for a week. Meanwhile, the tumor volume did not increase in the radiation therapy or adjuvant metformin and radiation therapy group (Figure 3B and 3C).

In the HP [1-13C]pyruvate MRS assessment, the vehicle group showed no change in the $K_{pl}$. The radiation therapy group showed decreased $K_{pl}$ after treatment, with a $p$-value of 0.028. However, the adjuvant metformin and radiation therapy group did not show any difference in $K_{pl}$ after treatment (Figure 3D).

We investigated tumor growth using serial bioluminescence imaging (Figure 3E and 3F) and traced the survival date (Figure 3G). Compared with the other therapy groups, the adjuvant metformin and radiation therapy group showed a more potent tumor suppression effect as per the bioluminescence imaging results and survival date. Furthermore, histologic analysis obtained directly after radiation therapy confirmed a decrease

![Image](image-url)

**FIGURE 3** Offset of $K_{pl}$ in adjuvant metformin for radiation therapy. (A), Experimental schedule of $T_2$-weighted MRI and HP [1-13C]pyruvate MRS for adjuvant metformin use with radiation therapy in a brain metastasis animal model. (B), The tumor response monitoring using $T_2$-weighted MRI at 2 weeks (before radiation) and 3 weeks (after radiation) for the vehicle group (Veh), IR group, and adjuvant metformin and radiation therapy group (Met+IR). (C), Tumor volume at 2 weeks and 3 weeks after injecting tumor cells. (D), $K_{pl}$ decreased in the radiation therapy group only. (E), Representative bioluminescence images during the follow-up. (F), Bioluminescence signals acquired from each group at 12 to 74 d after tumor cell implantation, displayed on a log scale. (G), Each group's survival (Veh, $n = 5$; IR, $n = 5$; Met+IR, $n = 4$). Data displayed as box plot or as mean ± SEM. $p$-values in tumor volume and $K_{pl}$ were obtained from a Student t-test between 2 weeks and 3 weeks. ANOVA test was used for bioluminescence and survival assays, and a Tukey test for post hoc comparison. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
in the cancer cell population in the adjuvant metformin and radiation treatment group (Supplementary Figure S3), even though we could not determine the size difference using MRI.

### 3.4 | Adjuvant metformin enhanced tumor suppression by increasing apoptosis

MDA-MB-231(Br)-Luc cells were exposed to various doses of X-rays and metformin for 4 h. Tumor suppression was observed in a dose-dependent manner (Figure 4A-C) and 10 Gy was selected for subsequent experiments. The effect of the adjuvant metformin and 10 Gy X-ray treatment on the cell proliferation was measured using the MTS (Figure 4B) and luciferase assays (Figure 4C). The MTS assay results showed an increase in the tumor suppression effect after therapy with 20 mM metformin and 10 Gy X-ray irradiation. The luciferase assay results did not indicate enhanced inhibition. However, microscopic examination indicated that treatment with 5 mM metformin and 10 Gy of X-rays resulted in a synergistic anticancer effect (Figure 4D and Supplementary Figure S4) after 48 h.

In cell cycle analysis, 48 h after adjuvant 5 mM metformin and 10 Gy X-ray irradiation, no additional effect was observed on radiation-induced G2M arrest (Figure 5A). However, annexin V cytometric analysis showed that adjuvant metformin increased radiation-induced apoptosis (Figure 5B).

### 3.5 | Effect of metformin and radiation treatment on cellular metabolism

The endogenous lactate pool is a dominant factor for HP [1-13C]lactate conversion. Therefore, we measured the lactate concentration according to metformin, radiation, and adjuvant metformin combined with radiation treatment using MDA-MB-231(BR)-Luc cells. The amount of lactate increased in a dose-dependent manner after metformin treatment in 4 h (Figure 6A). The lactate concentration increased in 30 min after 10 Gy X-ray irradiation (Figure 6B). Furthermore, the lactate concentration increased with 5 mM adjuvant metformin and 10 Gy X-ray treatment compared with only 5 mM metformin, with a p-value of 0.0023, or 10 Gy X-ray treatment, with a p-value less than 0.0001 (Figure 6C).
To elucidate the cause of increased lactate concentration, we investigated the related factors, including the degree of glucose uptake, MCT, LDHA activity, and cytosolic NAD\(^+\)/NADH redox state. Glucose uptake was measured using the fluorescent glucose analogue 2-NBDG (Figure 6D). Treatment with metformin alone did not affect glucose uptake, but it was increased 1.61-fold in the 10 Gy X-ray exposure group and 1.28-fold in the 10 Gy X-ray combined with adjuvant metformin treatment group. The MCT4 protein level was decreased only after adjuvant metformin and X-ray treatment, with a \(p\)-value of 0.0637 (Figure 6E). However, there were no differences in the levels of MCT1 (Figure 6F), LDHA (Figure 6G), or total NADs (Figure 6H). Metformin decreased the NAD\(^+\)/NADH ratio (Figure 6I; with a \(p\)-value 0.0003) and ATP levels (Figure 6J; with a \(p\)-value less than 0.0001). The 10 Gy X-ray exposure did not affect the NAD\(^+\)/NADH ratio but decreased ATP levels, with a \(p\)-value less than 0.0001.

4 | DISCUSSION

4.1 | HP \([1-^{13}C]\)puvurate MRS for tumor response monitoring

This study showed that the results of the tumor response monitored using the HP \([1-^{13}C]\)lactate conversion rate may not be consistent with the anticancer effect. A decreased HP \([1-^{13}C]\)lactate conversion rate has been presented as an early metabolic response to anticancer therapy in animal models and patients.\(^3\) In the breast cancer patients, the HP \([1-^{13}C]\)lactate/pyruvate ratio showed a strong correlation with tumor volume and suggested that the hypoxia-mediated lactate production in a large tumor may account for this positive correlation.\(^3\) However, a condition that may affect the NAD\(^+\)/NADH redox state or lactate concentration can likely affect the apparent HP \([1-^{13}C]\)lactate conversion ratio. Recently, the anticancer effect of pazopanib, a tyrosine kinase inhibitor, was reported to increase the HP \([1-^{13}C]\)lactate conversion after two days of treatment, suggesting that anti-angiogenic effects may lead to elevated lactate production.\(^3\) The amount of lactate has been regarded as a dominant factor for HP \([1-^{13}C]\)lactate conversion. However, inconsistencies have been reported between the lactate concentration and the HP \([1-^{13}C]\)lactate/pyruvate conversion rate.\(^3\) In xenografts using MDA-MB-231, the HP \([1-^{13}C]\)lactate/pyruvate ratio was negatively correlated with the wet tumor weight, and it is possible that the necrotic center in larger tumors contributed to this negative correlation.\(^3\)
Therefore, preliminary information about various factors, such as tumor size, pyruvate delivery, membrane transport, enzyme activity, drugs, or any condition affecting the lactate pool and NAD+/NADH redox state, is required to interpret the HP [1-13C]lactate conversion rate correctly.

4.2 Metformin's tumor suppression effect

Metformin has been reported to decrease the polyp growth in colon cancer and suppress the proliferation of endometrial cancer. However, other types of cancer failed to show the tumor suppression ability of metformin. The plasma concentration of metformin is approximately 10-30 μM, and the dosage must not exceed 35 mg/kg/d. Most in vitro studies reported that an anticancer effect was observed in a dose range of 1-50 mM of effective metformin concentration for cancer therapy, so it seems debatable whether the dose used in the clinical trial had a therapeutic effect. In this in vivo study, 300 mg/kg/d, a dose 10 times higher than the human dose of oral metformin, was used. Its metabolic effects on the cytosolic redox state were shown by the increased [1-13C]lactate conversion rate. Metformin alone did not have a tumor suppression effect in vivo, even at high dosage. However, when it was used as an adjuvant, metformin sensitized the anticancer effect of radiation therapy.

Therefore, our results support the anticancer effect of metformin as an adjuvant therapy rather than monotherapy, similarly to the findings of other clinical studies that reported an improved treatment with adjuvant metformin in radiation therapy and chemotherapy.

Although the MTS and bioluminescence assays did not appropriately estimate the radiation-sensitizing effect of adjuvant metformin and irradiation, our in vitro cell proliferation study showed a decrease in the tumor cell population. In MTS cell proliferation assays, the MTS tetrazolium compound generates a colored formazan dye, and this conversion is carried out by NAD(P)H-dependent dehydrogenase enzymes. Therefore, the metformin treatment, which leads to changes in the NAD+/NADH ratio, may affect the assay results. Moreover, the intensity of the bioluminescence signal reflects the cell numbers, and this assay uses ATP to emit the bioluminescence. Thus, an alteration in the ATP level caused by metformin treatment may affect the assay results.
4.3 | $K_{pl}$ and metformin

There can be several explanations for the radio-sensitizing effect of metformin. According to an animal model study using MDA-MB-231 and 4T1 cells, a treatment with 225 mg/kg of metformin improved vascular maturity, reduced leakage, and alleviated the hypoxic conditions in the tumor region in breast-lung metastasis. Because hypoxic cancer cells are resistant to radiation therapy, alleviated hypoxia by metformin via vascular normalization may enhance the anticancer effect of radiation. Moreover, alteration in metabolism by metformin may have increased the radiation-mediated anticancer effects. A recently published paper supports our results that the triple-negative breast cancer cells, MDA-MB-231 and SUM159PT, on treatment with 8 Gy radiation, showed increased glucose uptake and lactate production, suggesting that the re-wiring of glucose metabolism may be necessary for cell survival and resistance to radiation. Interestingly, we found that the treatment with adjuvant metformin and 10 Gy radiation increased the glucose uptake, but the degree of glucose uptake was less than that observed after treatment with radiation alone. Furthermore, the MCT4 protein level was markedly reduced after adjuvant metformin and radiation treatment.

MCT proteins transport pyruvate, lactate, and ketone bodies. The expression of MCT proteins is elevated in many tumors, wherein some cancer cells use them as metabolic fuel or to prevent acidification by excreting lactate and $\text{H}^+$. Recently, the combined treatment of an MCT inhibitor and metformin showed a remarkably potent anticancer effect. MCT inhibitors inhibit LDH via end-product inhibition, whereas metformin inhibits the mitochondrial respiratory chain complex I. NAD$^+$ is required for glycolysis and is generated from NADH via LDH in the cytoplasm. Thus, the decreased lactate export via MCT4 and a decrease of the NAD$^+$/NADH redox state by mitochondrial respiratory chain complex I inhibition leads to glycolytic blockage, ATP depletion, and cell death.

Metformin treatment increased lactate production in vitro and increased $K_{pl}$ in vivo, showing consistency between the lactate pool size and the HP $[1-^{13}C]$lactate conversion rate. X-ray exposure increased lactate production in vitro but decreased $K_{pl}$ after treatment in vivo, showing inconsistency between the in vitro and in vivo results. Decreased MCT4 and NAD$^+$/NADH leads to an increased intracellular lactate pool in vitro. Two opposite effects offset the $K_{pl}$ levels in vivo: a decrease by radiation therapy and an increase caused by metformin treatment.

A limitation of our study is that we could not obtain the ex vivo measurements, including lactate level, NAD$^+$/NADH redox state, MCT1, MCT4, and LDHA, from the tumor-bearing animal model used in the study due to survival follow-up.

5 | CONCLUSION

This study evaluated the metabolic change of metastatic brain tumors after adjuvant metformin and radiation treatment using HP $[1-^{13}C]$pyruvate MRS in an animal model of breast cancer brain metastasis. Metformin alone did not show an anticancer effect, and the HP $[1-^{13}C]$lactate conversion rate increased compared with that in the control mice. Radiation therapy resulted in tumor suppression with a significantly decreased HP $[1-^{13}C]$lactate conversion rate. When metformin was used as an adjuvant for radiation therapy, radiation-mediated anticancer effects were elevated, but the HP $[1-^{13}C]$lactate conversion rate was offset. Therefore, our results indicate that the interpretation of HP $[1-^{13}C]$pyruvate MRS for response monitoring of cancer treatment should be done with caution when metformin is used as an adjuvant cancer therapy.

ACKNOWLEDGEMENT

The authors acknowledge the grant provided by the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (grant number HI111C0032), the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2020R1F1A1074342), and the faculty research grant of Yonsei University College of Medicine (6-2019-0076).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ho-Taek Song https://orcid.org/0000-0002-6655-2575

REFERENCES

1. San-Millán I, Brooks GA. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. Carcinogenesis. 2017;38(2):119-133. https://doi.org/10.1093/carcin/bgw127
2. Liberti MV, Locasale JW. Correction to: ‘The Warburg effect: how does it benefit cancer cells?’ [Trends in Biochemical Sciences, 41 (2016) 211]. Trends Biochem Sci. 2016;41(3):211-218. https://doi.org/10.1016/j.tibs.2016.01.004
3. Zaccagna F, Grist JT, Deen SS, et al. Hyperpolarized carbon-13 magnetic resonance spectroscopic imaging: a clinical tool for studying tumour metabolism. Br J Radiol. 2018;91(1085):20170688. https://doi.org/10.1259/bjr/20170688
4. Ardenkjaer-Larsen JH, Fridlund B, Gram A, et al. Increase in signal-to-noise ratio of > 10,000 times in liquid-state NMR. Proc Natl Acad Sci U S A. 2003; 100(18):10158-10163. https://doi.org/10.1073/pnas.1733835100
5. Golman K, Isn't Zandt R, Thaning M. Real-time metabolic imaging. Proc Natl Acad Sci U S A. 2006;103(30):11270-11275. https://doi.org/10.1073/pnas.0601319103

6. Golman K, Olsson LE, Axelsson O, Månsson S, Karlsson M, Petersson JS. Molecular imaging using hyperpolarized 13C. Br J Radiol. 2003;76(Spec No 2):S118-S127. https://doi.org/10.1259/bjr/26631666

7. Milloushev VZ, Granlund KL, Boltynskiy R, et al. Metabolic imaging of the human brain with hyperpolarized 13C pyruvate demonstrates 13C lactate production in brain tumor patients. Cancer Res. 2018;78(14):3755-3760. https://doi.org/10.1158/0008-5472.Can-18-0221

8. Nelson SJ, Kjurhanewicz J, Vigneron DB, et al. Metabolic imaging of patients with prostate cancer using hyperpolarized [1-13C]pyruvate. Sci Transl Med. 2013;5(198):198ra108. https://doi.org/10.1126/scitranslmed.3006070

9. Chen AP, Wu C, Gu YP, Cunningham CH. Probing early tumor response to radiation therapy using hyperpolarized [1-13C]pyruvate in MDA-MB-231 xenografts. PLoS ONE. 2013;8(2):e56551. https://doi.org/10.1371/journal.pone.0056551

10. Lodi A, Woods SM, Ronen SM. Treatment with the MEK inhibitor U0126 induces decreased hyperpolarized pyruvate to lactate conversion in breast, but not prostate, cancer cells. NMR Biomed. 2013;26(3):299-306. https://doi.org/10.1002/nbm.2848

11. Saito K, Matsumoto S, Takakusagi Y, et al. 13C-MR spectroscopic imaging with hyperpolarized [1-13C]pyruvate detects early response to radiotherapy in SCC tumors and HT-29 tumors. Clin Cancer Res. 2015;21(22):5073-5081. https://doi.org/10.1158/1078-0432.Ccr-14-1717

12. Day SE, Kettunen MI, Cherukuri MK, et al. Detecting response of rat C6 glioma tumors to radiotherapy using hyperpolarized [1-13C]pyruvate and 13C magnetic resonance spectroscopic imaging. Magn Reson Med. 2011;65(2):557-563. https://doi.org/10.1002/mrm.22698

13. Dafni H, Larson PE, Hu S, et al. Hyperpolarized 13C spectroscopic imaging informs on hypoxia-inducible factor-1 and Myc activity downstream of platelet-derived growth factor receptor. Cancer Res. 2010;70(19):7400-7410. https://doi.org/10.1158/0008-5472.Can-10-0883

14. Rajeshkumar NV, Dutta P, Yabuuchi S, et al. Therapeutic targeting of the Warburg effect in pancreatic cancer relies on an absence of p53 function. Cancer Res. 2015;75(16):3355-3364. https://doi.org/10.1158/0008-5472.Can-15-0108

15. Zacharias NM, Baran N, Shannugavelandy SS, et al. Assessing metabolic intervention with a glutaminase inhibitor in real-time by hyperpolarized magnetic resonance in acute myeloid leukemia. Mol Cancer Ther. 2019;18(11):1937-1946. https://doi.org/10.1158/1535-7163.Mct-18-0985

16. Sandulache VC, Chen Y, Lee J, et al. Evaluation of hyperpolarized 13C-[1-13C]pyruvate by magnetic resonance to detect ionizing radiation effects in real time. PLoS ONE. 2014;9(1):e78731. https://doi.org/10.1371/journal.pone.0078731

17. Bailey CJ. Metformin: historical overview. Diabetologia. 2017;60(9):1566-1576. https://doi.org/10.1007/s00125-017-4318-z

18. Decensi A, Puntoni M, Goodwin P, et al. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. Cancer Prev Res. 2010;3(11):1451-1461. https://doi.org/10.1158/1940-6207.Capr-10-0157

19. Schuler KM, Rambally BS, DiFurio MJ, et al. Antiproliferative and metabolic effects of metformin in a preoperative window clinical trial for endometrial cancer. Cancer Med. 2015;4(2):161-173. https://doi.org/10.1002/cam4.353

20. Coyle C, Cafferty FH, Vale C, Langley RE. Metformin as an adjuvant treatment for cancer: a systematic review and meta-analysis. Ann Oncol. 2016;27(12):2184-2195. https://doi.org/10.1093/annonc/mdw410

21. Madiraju AK, Qiu Y, Perry RJ, et al. Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo. Nat Med. 2018;24(9):1384-1394. https://doi.org/10.1038/s41591-018-0125-4

22. Madiraju AK, Matsumoto S, Takakusagi Y, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature. 2014;510(7506):542-546. https://doi.org/10.1038/nature13270

23. Decensi A, Puntoni M, Goodwin P, et al. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. Cancer Prev Res. 2010;3(11):1451-1461. https://doi.org/10.1158/1940-6207.Capr-10-0157

24. Lewis AJ, Miller JJ, McCallum C, et al. Assessment of metformin-induced changes in cardiac and hepatic redox state using hyperpolarized[1-13C] pyruvate. Diabetes. 2016;65(12):3544-3551. https://doi.org/10.2337/db16-0804

25. Qi H, Nielsen PM, Schroeder M, Bertelsen LB, Palm F, Laustsen C. Acute renal metabolic effect of metformin assessed with hyperpolarised MRI in rats. Diabetologia. 2018;61(2):445-454. https://doi.org/10.1007/s00125-017-4540-8

26. Yoneda T, Williams PJ, Hira T, Niewolna M, Nishimura R. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res. 2001;16(8):1486-1495. https://doi.org/10.1097/00004703-200106.000000-00000

27. Hill DK, Orton MR, Mariotti E, et al. Model free approach to kinetic analysis of real-time hyperpolarized 13C magnetic resonance spectroscopy data. PLoS ONE. 2013;8(9):e71996. https://doi.org/10.1371/journal.pone.0071996

28. Zhang L, Bailleul J, Yazal T, et al. PK-M2-mediated metabolic changes in breast cancer cells induced by ionizing radiation. Biol Pharm Bull. 2019;42(1):612-632. https://doi.org/10.1177/1747493018778713

29. Ravoori MK, Singh SP, Lee J, Bankson JA, Kundra V. In vivo assessment of ovarian tumor response to tyrosine kinase inhibitor pazopanib by using hyperpolarized 13C-pyruvate MR spectroscopy and 18F-FDG PET/CT imaging in a mouse model. Radiology. 2017;285(3):830-838. https://doi.org/10.1148/radiol.2017161772

30. Lee CY, Lau JYC, Geraghty BJ, Chen AP, Gu YP, Cunningham CH. Correlation of hyperpolarized 13C-MRI data with tissue extract measurements. NMR Biomed. 2020;33(5):e4269. https://doi.org/10.1002/nbm.4269

31. Feuerecker B, Michalk M, Hundshammer C, et al. Assessment of 213Bi-anti-EGFR MAb treatment efficacy in malignant cancer cells with [1-13C] pyruvate and [18F]FDG. Sci Rep. 2019;9(1):8294. https://doi.org/10.1038/s41598-019-44484-w

32. Higurashi T, Hosono K, Takahashi H, et al. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. Lancet Oncol. 2016;17(4):475-483. https://doi.org/10.1016/s1470-2045(15)00565-3
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Choi Y-S, Lee J, Lee H-S, Song JE, Kim D-H, Song H-T. Offset of apparent hyperpolarized $^{13}$C lactate flux by the use of adjuvant metformin in ionizing radiation therapy in vivo. NMR in Biomedicine. 2021;34:e4561. https://doi.org/10.1002/nbm.4561