Influence of MRI Examinations on Animal Welfare and Study Results

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Objectives: Magnetic resonance imaging (MRI) is considered to be well tolerated by laboratory animals. However, no systematic study has been performed yet, proving this assumption. Therefore, the aim of this study was to investigate the possible effects of longitudinal native and contrast-enhanced (CE) 1-T and 7-T MRI examinations on mouse welfare as well as 4T1 breast cancers progression and therapy response.

Material and Methods: Forty-seven healthy and 72 breast cancer-bearing mice (4T1) were investigated. One-Tesla (ICON) and 7-T (Biospec) MRI measurements were performed thrice per week under isoflurane anesthesia in healthy BALB/c mice for 4 weeks and 3 times within 2 weeks in tumor-bearing animals. Animal welfare was examined by an observational score sheet, rotarod performance, heart rate measurements, and assessment of fecal corticosterone metabolites. Furthermore, we investigated whether CE-MRI influences the study outcome. Therefore, hemograms and organ weights were obtained, and 4T1 tumor growth, perfusion, immune cell infiltration, as well as response to the multikinase inhibitor regorafenib were investigated. Statistical comparisons between groups were performed using analysis of variance and Tukey or Bonferroni post hoc tests.

Results: Mice showed no alterations in the observational score sheet rating, rotarod performance, heart rate, and fecal corticosterone metabolites ($P > 0.05$) after repeated MRI at both field strengths. However, spleen weights were reduced in all healthy mouse groups that received isoflurane anesthesia ($P < 0.001$) including the groups investigated by 1-T and 7-T MRI ($P = 0.02$). Neither tumor progression nor response to the regorafenib treatment was affected by isoflurane anesthesia or CE-MRI monitoring. Furthermore, immunohistological tumor analysis did not indicate an effect of isoflurane and MRI on macrophage infiltration of tumors, perfusion of tumor vessels, and apoptotic cell rate ($P > 0.05$).

Conclusions: Repeated MRI did not influence the welfare of mice and did not affect tumor growth and therapy response of 4T1 tumors. However, systemic immunological effects of isoflurane anesthesia need to be considered to prevent potential bias.

Key Words: MRI, animal welfare, severity assessment, long-term effects, regorafenib, isoflurane, anesthesia, behavior, rotarod, breast cancer

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Noninvasive imaging is broadly used in (pre)clinical studies to depict morphological and (patho)physiological processes,[1,2] and is often argued to contribute to the 3Rs.[3] These were initially described in 1959 by Russel and Burch, and define the 3 main principles for the ethically correct handling of laboratory animals. In detail, they stand for replacement, reduction, and refinement. This can be achieved by reducing the number of animals needed, by refining the studies in favor of animal welfare and data quality, and by replacing animal experiments, for example, by in vitro techniques. Noninvasive imaging contributes to the 3Rs by enabling longitudinal studies of the same animal, thus reducing the number of animals and allowing intraindividual analyses.[4] However, longitudinal examinations are also discussed to induce higher burden due to suffering of the single animal for a longer period. Although imaging techniques are well tolerated in humans, in laboratory animals, few important differences need to be highlighted. First, animal handling itself can cause anxiety[5] and alter physiological parameters.[6] Second, animals need to be anesthetized throughout the whole imaging procedure. In this context, isoflurane is one of the most commonly used anesthetic agents. It acts on the γ-aminobutyric acid and glutamatergic receptors in the brain, but the mechanism of action is not yet fully known.[7] With respect to animal behavior, it was shown to decrease burrowing and open field exploratory behavior in C57BL/6JRj mice,[8] and significantly modulated immune responses.[9] Furthermore, isoflurane was shown to alter plasma corticosterone levels in the hippocampus[10] as well as activate TrkB and can therefore have an antidepressant effect.[11] These risks increase with anesthesia length and repetitions,[8] and are consequently especially relevant for MRI.[12]

Next to effects related to the anesthesia, preclinical MRI is typically performed at higher field strengths to achieve sufficient spatial resolution[13] as well as more often and in shorter time intervals[12] compared with clinical investigations. High noise levels[14] and the magnetic field itself can influence animals' behavior and even cause depressive symptoms.[15] Especially, ultrafast echo planar imaging (EPI) sequences require high gradient amplitudes and fast switching rates, which can evoke peripheral nerve stimulation[16,17] or tissue heating,[18] resulting in muscle twitching and even pain.[19] Thus, thresholds were defined to prevent sequence-related injuries for humans.[20] However, in small animal imaging, gradient amplitudes above those thresholds are often needed for sufficient spatial and temporal resolution. Depending on the scientific question, MRI can also require the injection of a contrast agent,[21,22] causing additional stress. Taken together, all aforementioned issues might influence animal welfare and maybe even study results, leading to an interpretation bias.

So far, no systematic study has been published examining the long-term effects of standard MRI protocols on animal welfare and scientific outcome in cancer research. Thus, we investigated the influence of native and contrast-enhanced (CE) MRI under isoflurane anesthesia with different field strengths on welfare and physiological parameters of healthy mice. Furthermore, effects of CE-MRI on the progression of highly vascularized 4T1 breast cancers and its response to the multikinase receptor inhibitor regorafenib were examined. This study should demonstrate possible effects of MRI on animal welfare and scientific outcome and moreover reevaluate the suitability of MRI in preclinical research.

MATERIALS AND METHODS

Animal Experiments

All animal experiments were approved by the German State Office for Nature, Environment, and Consumer Protection (LANUV) North Rhine-Westphalia. Overall, 119 female BALB/cAnNRj mice (age 10–12 weeks; Janvier Labs, Saint Berthevin, France) were housed in groups of 5 animals under specific pathogen-free conditions with a 12-hour light/dark cycle. The environment was temperature-controlled (20°C–24°C) and humidity-controlled (45%–65%) according to the guidelines of the "Federation for Laboratory Animal Science Associations" (FELASA, www.felasa.eu). Acidified water and standard pellets for laboratory mice (Sniff GmbH, Soest, Germany) were offered ad libitum.

Orthotopic Tumor Inoculation and Antitumor Therapy

Murine triple-negative breast cancer cells (4T1,[23] ATCC CRL-2539, Manassas, VA; 4×10^4 cells in 50 μL RPMI 1640 cell culture medium) were injected orthotopically into the right mammary fat pad of n = 72 mice. Tumor sizes were assessed daily using caliper measurements, and the volume was calculated using the formula length × width × width × (π/6). Tumors with sizes of less than 5 mm^3 on day 6 after tumor cell injection were excluded from analyses.

Six days after tumor cell injection, animals were randomly allocated to receive a daily oral dose of either 10 mg/kg body weight regorafenib (multikinase inhibitor, Merck, Darmstadt, Germany) dissolved in polyethyleneglycol 400, 1,2-propandiol, and pluronic F68 (all from Merck, Darmstadt, Germany), as described[24] or the corresponding amount of vehicle solution.

MRI Protocol

Imaging was performed using a 1-T (ICON; Bruker, Ettlingen, Germany) or a 7-T MRI scanner (BioSpec; Bruker, Ettlingen, Germany) with a transceiver mouse volume coil (sequence details in Supplemental Digital Content 1, Tables S1, http://links.lww.com/RLI/A531 and S2, http://links.lww.com/RLI/A530).

Healthy and tumor-bearing mice of the 7-T MRI and 7-T CE-MRI groups were imaged according to a representative MRI protocol performed in our institute: (1) localization of liver and mammary fat pad by transversal T1-RARE sequences, (2) imaging of both using a T2-RARE sequence, (3) T1-weighted saturation recovery gradient echo sequence for dynamic CE-MRI with 80 measurements and a temporal resolution of 8.4 images per second resulting in a total scan time of 11.2 minutes. After a baseline measurement of 1 minute, all healthy and tumor-bearing animals of the 7-T CE-MRI group received an intravenous injection of 0.1 mmol/kg body weight Gadovist (Bayer, Germany) between the 7th and 10th repetition of the T1-FLASH sequence for dynamic contrast-enhanced imaging.

An additional group of 3 healthy mice underwent 7-T MRI of the brain and lung with different EPI sequences varying in TR (667, 990, and 3000 milliseconds; see Table S2, http://links.lww.com/RLI/A530) to investigate acute effects of these sequences on the respiration rate.

Influence of Repeated MRI With Standard Sequences on Animal Welfare and Health

Influence of repeated MRI on animal welfare and health was assessed in 44 healthy female BALB/cAnNRj mice. At least 5 animals were randomized to either experimental group: (1) no imaging (control), (2) isoflurane anesthesia, (3) 1-T MRI, (4) 7-T MRI, and (5) 7-T CE-MRI. Isoflurane anesthesia alone (30 minutes, 2% isoflurane in O_2) or in combination with MRI was carried out 3 times per week for 4 weeks, starting at day 0.

The health state of all animals was monitored daily using a previously described modified score sheet[25] (see Supplemental Digital Content 1, Table S3, http://links.lww.com/RLI/A530). Briefly, alterations in body weight, general state, spontaneous behavior, and treatment-specific parameters (tumor growth, antitumor therapy) were documented and allocated to a point grading system, where 0 points describe no alteration of the physiological state and 20 points or higher describe the highest severity and humane end point. Before the first imaging session, all animals were trained for behavioral examinations. Baseline values for rotarod performance, heart rate, and fecal corticosterone metabolite (FCM) concentrations were assessed. Changes of these parameters were then monitored twice per week (Fig. 1). On the last day of the experiment, a final assessment of all behavioral tests was performed, and blood was collected before euthanasia.

Rotarod Test

The rotarod (Panlab Harvard Apparatus, Barcelona, Spain) test was applied to discover changes in motor coordination and balance. Mice were put on a spinning cylinder, with a starting speed of 4 rotations per minute, accelerating steadily to a maximum speed of 40 rotations per minute after 5 minutes. The speed at which the mouse fell of the cylinder was recorded. Each measurement was repeated twice. Baseline values were set to 100%, and percent change of the subsequent time points were calculated on an individual basis.

Assessment of Heart Rate

Heart rate was measured in conscious mice as an indicator of stress and discomfort (CODA System; Kent Scientific Corporation, Torrington, CT). Therefore, mice were restrained in a plexiglas tube on a tempered panel. The tail was placed inside an occlusion and volume pressure cuff. Heart rate was assessed by volume pressure recording and analyzed using the suppliers' software. Each measurement consisted of 15 repetitions to compensate for movement artifacts. Baseline values were set to 100%, and percent changes at the subsequent time points were calculated on an individual basis.

FCM Measurements

Feces was collected during every animal handling and stored at −80°C. Samples of 3 consecutive days were pooled and dried for 24 hours at 50°C. Then, an aliquot of 50 mg was dissolved in 80% methanol (1 mL, Merck, Darmstadt, Germany) over night at 4°C. Samples were homogenized and centrifuged for 10 minutes at 3000*g* (relative centrifugal acceleration) (Fresco 21 and Pico 21 Heraeus, Hanau, Germany). Subsequently, the samples were analyzed by 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay[26] at the Institute of Physiology, Pathophysiology, and Experimental Endocrinology of the University of Veterinary Medicine in Vienna, Austria. Baseline values were set to 100%, and percent changes at the subsequent time points were calculated on an individual basis.

Influence of Echo Planar Imaging on Animal Welfare and Respiratory Rate

For the evaluation of acute irritating effects on the central nervous system deriving from EPI sequences, 3 animals were investigated 3 times within 1 week, and respiratory rates were documented every 5 seconds during the scans.

Influence of Repeated MRI on Tumor Pathophysiology

To test whether repeated MRI influences 4T1 tumor pathophysiology, 72 regorafenib-treated or vehicle-treated animals were randomized to the following experimental groups: (1) no imaging, (2) isoflurane anesthesia, and (3) 7-T CE-MRI. Isoflurane anesthesia alone (30 minutes, 2% isoflurane in O_2) or in combination with CE-MRI was performed on days 7, 10, and 14 after tumor inoculation. Before the first imaging session, all animals were trained, and baseline values assessed as described for healthy animals. Changes were monitored on days 11,

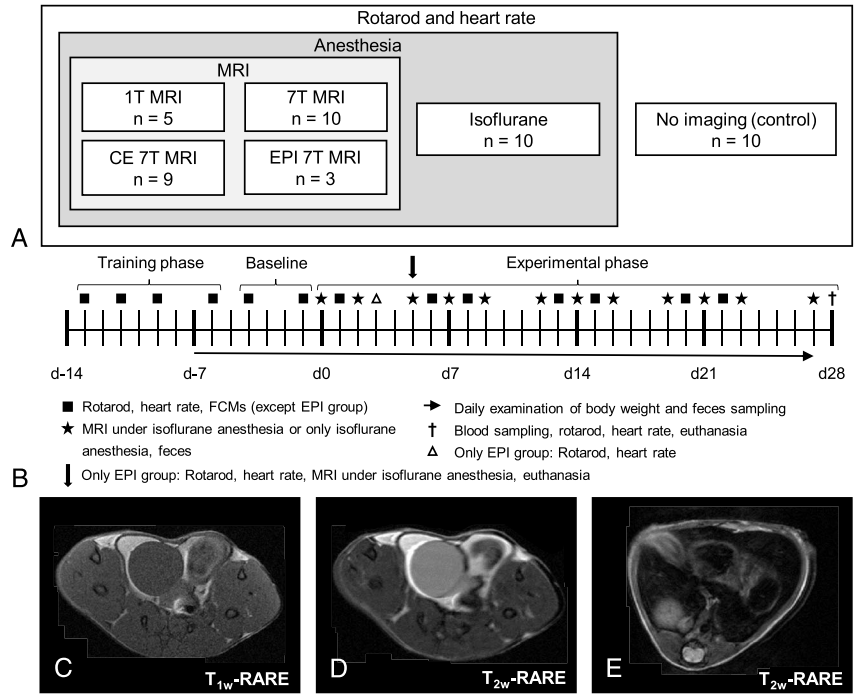

FIGURE 1. Overview of (A) experimental groups and (B) timeline for healthy BALB/c mice to evaluate the influence of MRI on animal behavior and health state. FCMs, fecal corticosterone metabolites. Animals were imaged thrice per week over a 4 weeks period with MRI sequences commonly used in drug response studies. Representative transversal images of the mammary fat pad acquired with (C) a T_{1w} RARE and (D) a T_{2w} RARE sequence. E, Representative MRI of a healthy mouse liver assessed with a T_{2w} RARE sequence.

13, and 15 after tumor cell injection (Fig. 2). On the last day of the experiment, a final assessment of all behavioral tests was performed, and blood was collected before euthanasia.

Hemograms

Blood was collected by retrobulbar sinus puncture and analyzed for numbers of leukocytes, erythrocytes, thrombocytes, hemoglobin amount, and hematocrit concentration with Celltac alpha MEK-6550 (Nihon Kohden, Shinjuku, Japan).

Organ and Tumor Tissue Preparation

Before euthanasia, mice were anesthetized with ketamine/xylazine in 0.9% NaCl (120 mg/kg body weight ketamine/16 mg/kg body weight xylazine; 30 µL/10 g body weight intraperitoneal injection).

All tumor-bearing mice were injected intravenously with rhodamine-labeled *Ricinus communis* agglutinin I (15 mg/kg body weight; Vector Laboratories, Burlingame, CA) to stain perfused tumor vessels. After 15 minutes mice hearts were perfused with 10-mL phosphate buffer saline (Merck, Darmstadt, Germany) through the left ventricle of the heart. Organs of interest (brain, heart, lungs, liver, spleen, kidneys) were macroscopically examined for abnormalities and weighted. Tumors were excised, weighted, embedded in Tissue Tek O. C.T. Compound (Sakura, Alphen aan den Rijn, the Netherlands), and stored at −80°C.

Immunohistology

Tumors were cryosectioned into 8-µm-thick slices (CM3050S; Leica, Wetzlar, Germany). For immunohistological staining, tumor slices were fixed with methanol (80%, Merck, Darmstadt, Germany) and ice-cold acetone (100%, Merck, Darmstadt, Germany). Blood vessels were stained with rat-anti-mouse PECAM-1 monoclonal CD31 (50 µg/mL, BD Bioscience, San Jose, CA, #553370), followed by donkey-anti-rat IgG (H + L) (0.001 µg/µL, #712–546-153 Dianova, Hamburg, Germany). Macrophages were stained with F4/80 rat-anti-mouse antibody (20 µg/mL, #MCA497GA Bio-Rad, Hercules, CA), followed by donkey-anti-rat IgG (H + L) (0.001 µg/µL, #712–546-153 Dianova, Hamburg, Germany). For detection of apoptotic cells, tumor slices were fixed with 4% paraformaldehyde and stained using the in situ cell death detection kit labeled with fluorescein (TUNEL, Roche, Basel, Switzerland). All slices were counterstained with 4′,6-diamidino-2-phenylindole (0.5 µg/mL DAPI, Merck, Darmstadt, Germany) to visualize nuclei. Five fluorescent micrographs per section were captured using the Axio Imager.M2 microscope (Zeiss, Göttingen, Germany) with a high-resolution camera (AxioCam MRm Rev.3, Zeiss, Göttingen, Germany) and quantified using ImageJ2[27] (National Institute of Health, Bethesda, MD).

Vessel perfusion was quantified by determining the percentage of colocalized CD31 and *R. communis* agglutinin I–positive area fraction. The positive area fraction of $F4/80^+$ cells and $TUNEL^+$ cells was determined, and the relation to $DAPI^+$ cells was calculated to quantify the percentage of macrophages and apoptotic cells, respectively.

Statistical Analysis

Statistical analysis was performed with SPSS (IBM Corp, v25, Sanborn, NY, academic license) and GraphPad Prism5 (Graphpad Software, v5.01, San Diego, CA, academic license). Data were tested for normality and analyzed with one-way analysis of variance and Tukey post hoc test or repeated measures analysis of variance with Bonferroni post hoc test on a 95% confidence interval. *P* values less than 0.05

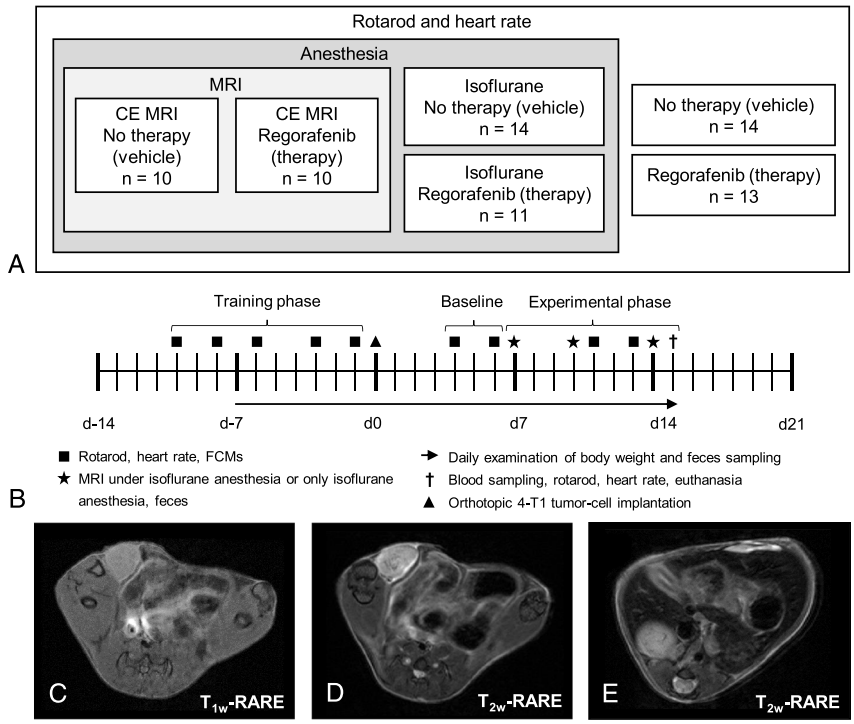

FIGURE 2. Overview of (A) experimental groups and (B) timeline for 4T1 tumor-bearing BALB/c mice. FCMs, fecal corticosterone metabolites. The influence of isoflurane anesthesia and MRI with contrast agent (Gadovist, Bayer) on study results was investigated 3 times over 2 weeks in treated (regorafenib) and untreated (vehicle) 4T1 tumor-bearing mice with MRI sequences commonly used in drug response studies. Representative transversal images of a 4T1 tumor located in the mammary fat pad acquired using (C) a T_{1w} RARE and (D) a T_{2w} RARE sequence. Liver metastases were excluded by T_{2w} MRI using a RARE sequence. E, A representative MRI at the level of the liver.

were considered statistically significant. All data are presented as mean ± standard deviation.

RESULTS

Influence of Repeated MRI on Animal Welfare and Health

First, the influence of repeated MRI at different field strengths with and without contrast agent was studied in healthy BALB/c mice. The general burden of each animal was rated daily by evaluation of several welfare parameters defined in a score sheet (Supplemental Digital Content 1, Table S3, http://links.lww.com/RLI/A530). In all groups, scores did not exceed a mild burden throughout the experiments (Fig. 3A).

In line with this, no alteration of the rotarod performance could be detected after repeated anesthesia or MRI when compared with baseline values ($P = 0.086$; Table 1; Fig. 3B). Furthermore, stress-associated parameters such as heart rate ($P = 0.272$; Table 1; Fig. 3C) and FCMs ($P = 0.117$; Table 1; Fig. 3D) did not show any alteration after repeated anesthesia or MRI.

Hemograms, assessed on the last day of the experiment, also revealed no deterioration of the animals' health reflected by a comparable number of leukocytes (Fig. 3E), erythrocytes, thrombocytes, and hemoglobin as well as hematocrit values in the different experimental groups (Supplemental Digital Content 1, Table S4, http://links.lww.com/RLI/A530). However, it needs to be mentioned that 5 animals per group of the control, isoflurane, and 7-T MRI group had to be excluded from analysis due to an internal error of the measurement device. Statistical analysis was performed with the remaining 5 mice for the control, isoflurane, and 7-T group.

Finally, gross necropsy of brain, lungs, heart, liver, spleen, and kidneys did not show any macroscopic abnormalities. However, the spleen weights of control mice were significantly higher ($P < 0.001$) compared with all other experimental groups of healthy mice that received isoflurane anesthesia alone or in combination with MRI (control: 0.109 ± 0.007 g, isoflurane: 0.096 ± 0.007 g, 1-T MRI: 0.096 ± 0.009 g, 7-T MRI: 0.091 ± 0.009 g, 7-T CE-MRI: 0.095 ± 0.01 g; Fig. 3F). Importantly, no significant difference was observed in case of the net isoflurane group compared with the imaging groups (that are anesthetized during the measurements with isoflurane), indicating that the decrease in spleen weights is solely an effect of inhalation anesthesia.

Influence of EPI Sequences on Animal Welfare and Respiratory Rate

Within a pilot study, the influence of EPI MRI on the respiratory rate and animal welfare was investigated in 3 animals. In line with our results of repeated MRI, EPI sequences had no impact on rotarod performance and heart rate measurements in healthy mice. Furthermore, no changes of respiratory rates were assessed during the measurements that would indicate an irritation of the brainstem (Supplemental Digital Content 2, Fig. S1, http://links.lww.com/RLI/A531). Because no alterations were observed, no additional animals were examined for ethical reasons.

Influence of Repeated MRI on Tumor Progression and Therapy Response

Next, the influence of repeated CE-MRI on progression and therapy response of 4T1 murine breast cancers was investigated. In addition, the animals' wellbeing was assessed as described for healthy mice.

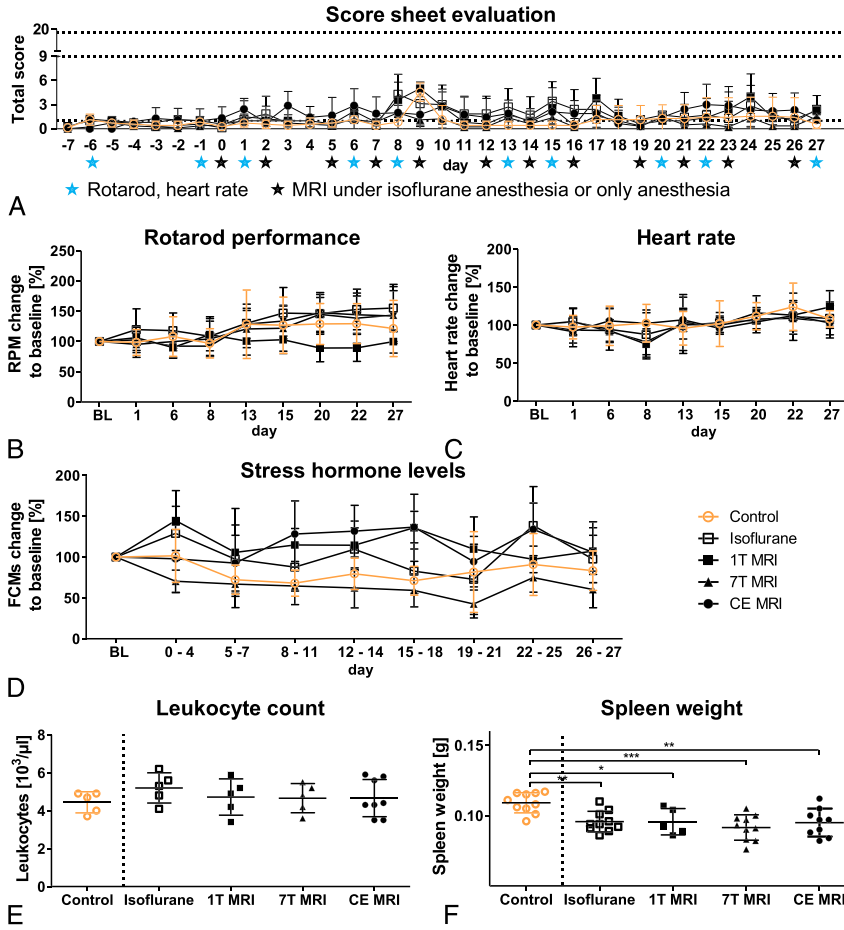

FIGURE 3. Influence of MRI on animal welfare, leukocyte counts, and spleen weights (healthy BALB/c mice). BL, baseline. A, The score sheet evaluation (based on body weight, fur appearance, body openings, behavior, body temperature, and heart rate) shows a mild burden for all animals independent of MRI. B, Longitudinal rotarod evaluation (rotations per minute) suggests low stress levels in mice of all groups independent of the performance of MRI scans. C, Longitudinal heart rate measurements indicate no change over time. D, Fecal corticosterone metabolites (FCMs) (stress hormone levels) are stable in all groups over time. E, Repeated MRI examinations have no influence on leukocyte counts. F, Spleen weights are reduced in all anesthetized groups ($P < 0.001$), but not further affected by MRI examinations.

The tumors of regorafenib-treated animals were significantly smaller compared with vehicle-treated tumors from day 11 after tumor cell injection on ($P = 0.015$; Figs. 4A–B). The therapeutic effect of regorafenib on 4T1 tumors could be confirmed by histological analysis showing a slightly ($P > 0.05$) lower macrophage infiltration (Fig. 4D) and a considerably higher percentage of apoptotic cells in regorafenib-treated compared with vehicle-treated tumors (Fig. 4E). However, no alteration in vessel perfusion (Fig. 4C) was found. Furthermore, no influence of isoflurane anesthesia or MRI on tumor growth could be detected in neither vehicle-treated ($P = 0.291$) nor regorafenib-treated ($P = 0.930$) animals. In this line, isoflurane and MRI did not change perfusion, macrophage infiltration, and apoptosis in tumors treated with vehicle or regorafenib (Figs. 4C–E).

TABLE 1. Results of the Rotarod Test, Heart Rate, and FCM Measurements in Healthy BALB/c Mice

Parameter	Control	Isoflurane	1-T MRI	7-T MRI	7-T CE-MRI
Rotarod, %	126 ± 46	155 ± 39	142 ± 42	143 ± 47	97 ± 20
Heart rate, %	108 ± 11	109 ± 22	124 ± 22	104 ± 21	103 ± 7
FCMs, %	83 ± 24	98 ± 29	107 ± 29	60 ± 22	106 ± 37

Values at day 27 of the experiment are given as % change to the baseline.

Score sheet evaluation of tumor-bearing animals revealed higher scores as compared with healthy animals. Nevertheless, the burden exceeded the mild level only for a few individual animals on the last day of the experiment, which could be attributed to cancer progression (Supplemental Digital Content 3, Fig. S2A, http://links.lww.com/RLI/A532). Treatment of tumor-bearing mice with regorafenib had no influence on animal welfare assessed by score sheet evaluation when compared with vehicle-treated ones. Furthermore, neither isoflurane anesthesia nor MRI altered welfare of tumor-bearing animals according to the point grading system from our score sheet evaluation (Table 2). In this line, rotarod performance ($P = 0.093$; Table 2; Fig. S2B, http://links.lww.com/RLI/A532), heart rate ($P = 0.924$; Table 2; Fig. S2C, http://links.lww.com/RLI/A532), and FCMs ($P = 0.089$, Table 2, Fig. S2D, http://links.lww.com/RLI/A532) did not differ in tumor-bearing mice, indicating that tumor growth, regorafenib therapy, and isoflurane anesthesia as well as MRI did not affect motor coordination and stress levels, and therefore, animal welfare (Figs. S2B–D, http://links.lww.com/RLI/A532).

In tumor-bearing mice, the hemograms showed no differences in the number of erythrocytes, hemoglobin, or hematocrit content compared with healthy mice. However, leukocyte counts were significantly

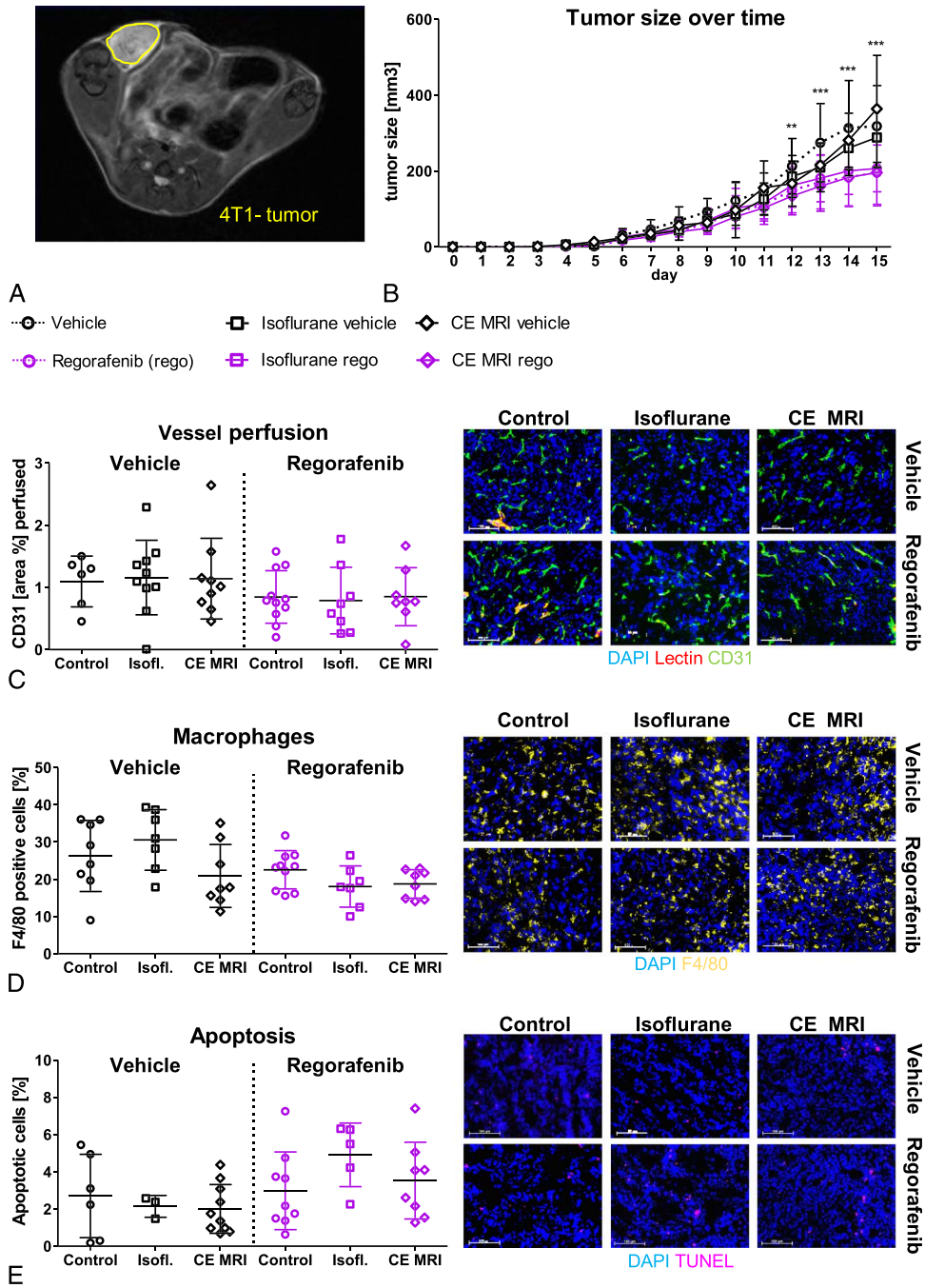

FIGURE 4. Influence of MRI on therapy response of 4T1 tumors in BALB/c mice. Isofl, isoflurane. A, T_{2w} MRI scan (7 T) of a 4T1 tumor located in the mammary fat pat. B, CE-MRI and anesthesia have no influence on tumor growth in untreated and treated animals. Dotted lines indicate vehicle and regorafenib control groups. C, Staining of perfused vessels with CD31 and lectin does not indicate differences between the groups. D, Macrophage (F4/80 staining) infiltration in tumors does not change if isoflurane or CE-MRI scans are performed. E, TUNEL staining indicates no differences in apoptotic cell rates in tumors of vehicle-treated and regorafenib-treated mice after isoflurane anesthesia or CE-MRI. Scale bar: 100 µm (images taken at 20× magnification).

higher ($P = 0.002$), and the number of thrombocytes significantly reduced ($P < 0.001$; Table S5, http://links.lww.com/RLI/A530). Within tumor-bearing groups, regorafenib therapy significantly reduced the number of leukocytes ($P = 0.019$; Fig. 5A). In vehicle-treated animals, isoflurane anesthesia as well as MRI resulted in a considerable reduction ($P = 0.093$) of leukocyte numbers, whereas leukocyte counts in regorafenib-treated animals remained unchanged ($P = 0.239$; vehicle [control: 69.45 ± 38.58 $\times 10^3$/µL, isoflurane: 53.07 ± 27.38 $\times 10^3$/µL, CE-MRI: 38.57 ± 25.09 $\times 10^3$/µL]; regorafenib [control: 43.29 ± 26.5 $\times 10^3$/µL, isoflurane: 40.88 ± 12.59 $\times 10^3$/µL, CE-MRI: 32.32 ± 16.40 $\times 10^3$/µL]) (Fig. 5A).

Furthermore, spleen weights were significantly higher in tumor bearing mice in comparison to healthy animals ($P < 0.001$) and regorafenib resulted in a significant reduction in spleen weights compared with

TABLE 2. Results of the Rotarod Test, Heart Rate, and FCM Measurements in 4T1 Tumor-Bearing BALB/c Mice

	Vehicle			Regorafenib		
Parameter	**Control**	**Isoflurane**	**CE-MRI**	**Control**	**Isoflurane**	**CE-MRI**
Score, points	5.7 ± 1.6	9.0 ± 5.5	8.2 ± 2.4	5.0 ± 1.6	9.9 ± 4.2	8.0 ± 2.5
Rotarod, %	103 ± 30	114 ± 35	95 ± 35	118 ± 35	125 ± 30	98 ± 29
Heart rate, %	106 ± 22	96 ± 34	100 ± 20	96 ± 26	98 ± 21	100 ± 15
FCMs, %	99 ± 40	111 ± 44	110 ± 36	132 ± 88	132 ± 87	160 ± 26

Values at day 15 of the experiment are given as % change to the baseline.

vehicle controls (Fig. 5B) ($P = 0.007$). In addition, the spleen weights of the vehicle-treated cohort were reduced in the isoflurane group and significantly reduced in the CE-MRI group ($P = 0.022$) (vehicle [control: 0.298 ± 0.071 g, isoflurane: 0.252 ± 0.05 g, CE-MRI: 0.219 ± 0.077 g]; regorafenib [control: 0.220 ± 0.063 g, isoflurane: 0.236 ± 0.053 g, CE-MRI: 0.186 ± 0.031 g]) (Fig. 5B).

DISCUSSION

There is a general agreement of researchers and regulatory boards that the number of laboratory animals and their suffering should be minimized. Although, imaging procedures are assigned to the mild severity category,[28] hardly any data are available about imaging-related suffering or effects on study results. Our results of score sheet evaluations in healthy animals indicate that even after longitudinal MRI animal burden does not exceed a mild range. Furthermore, rotarod performance, heart rate, and FCM analysis indicated no long-term effects induced by MRI. In line with our findings, the few preliminary studies showed no effects of magnetic fields[29–31] or MRI[32] between 0.15 T and 6.3 T on exploratory behavior, locomotion, memory, or fetal development in rats and mice, when exposed 20 to 120 minutes to a magnetic field. However, also contrary results can be found describing, for example, circling behavior after 30 minutes exposure of rats to 7-T or 14-T fields.[15] Comparable findings were reported for mice along with conditioned taste aversions.[33] However, in the aforementioned studies, animals were awake and effects on their behavior were investigated directly after magnetic field exposure. In the present study, our aim was to explore the effects of a standard MRI setup with T_{1w} and T_{2w} sequences, which includes isoflurane anesthesia. Thus, these acute effects could not be captured due to the well-known anesthesia-induced behavioral alterations in the immediate postanesthetic phase.[8] However, it is noteworthy that also in the group exposed to EPI MRI sequences, no changes in the respiratory rate, pointing to irritation of the brainstem, could be seen. The latter was also assumed to be responsible for the circling behavior and taste aversion in rats found by Houpt and coworkers.[15]

Despite these encouraging results for the use of MRI, we need to mention, as a limitation of our study, that the rotarod test only measures motor coordination and balance and might not be most sensitive for measuring anxiety or stress.[34] Thus, future studies need to comprise further tests like the open field, combining visualization of motor function with a sensitive analysis of exploratory behavior of individual animals.[35]

With respect to physiological alterations, healthy mice showed a decrease in spleen weights after isoflurane anesthesia independent of MRI. Comparable findings were reported for acepromazine and propofol administration in dogs,[36] but were not yet described after repeated isoflurane anesthesia in mice. However, isoflurane has an impact on the immune system and is known to decrease the numbers of B- and T-cells (both present in spleen) in humans[9] and mice.[37]

In line with our results on healthy animals, isoflurane anesthesia and MRI had no impact on animal welfare in 4T1 tumor-bearing mice. The score sheet evaluation showed an elevated total score at the end of the experiment up to a moderate range caused by cancer progression. In this context, tumor growth induced an immune response resulting in increased leukocyte counts and spleen weights compared with healthy mice, which is in line with the literature.[38] Treatment of tumor-bearing mice with regorafenib slightly reduced leukocyte counts and spleen weights most likely due to myelosuppression.[39] As in healthy mice, lower spleen weights were measured in vehicle-treated animals after isoflurane anesthesia, whereas this effect could not be observed in regorafenib-treated mice. In the latter group, the reduction in spleen weights induced by regorafenib may have masked the isoflurane-related effect.

With respect to disease progression, regorafenib significantly inhibited 4T1 tumor growth; however, the imaging procedure itself (with contrast agent) had no detectable effect on tumor physiology nor growth nor therapy response. Although there are no reports on magnetic fields influencing tumor growth in mice, this needs to be considered

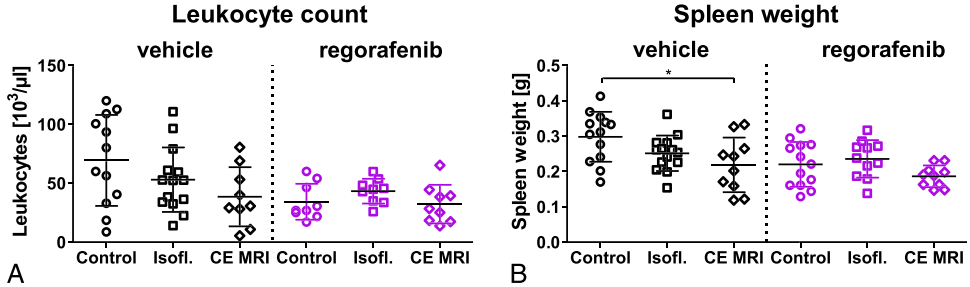

FIGURE 5. Influence of CE-MRI on leukocyte counts and spleen weights in 4T1 tumor-bearing BALB/c mice. Isofl, isoflurane anesthesia A, The highest leukocyte counts are found in untreated control animals. Isoflurane and CE-MRI exposure tend to decrease leucocyte levels in untreated animals. Regorafenib-treated animals have lower leucocyte concentrations at the end of the experiment. However, there is no difference between the regorafenib-treated control group and the regorafenib-treated isoflurane and CE-MRI exposed groups. B, In untreated animals, spleen weights are reduced after isoflurane anesthesia and significantly lower in animals after CE-MRI examinations ($P = 0.022$). In regorafenib-treated animals spleen weights are lower than in untreated control animals. However, no significant differences between the control, isoflurane, and CE-MRI groups are found.

since a reduction of tumor size in male hamsters has been described after exposure to a 586-mT static magnetic field for 3 hours.[40] In addition, several effects of intravenous (eg, ketamine) and volatile (eg, isoflurane) anesthetics on tumor development and the immune system were described caused by, for example, enhancing hypoxia-inducible factor-1α activity, apoptotic resistance of tumor cells, or attenuation of natural killer cell activity.[41] In this context, it is a limitation of our study that only female mice were included, since tumor response to isoflurane might be sex-dependent. For example, administration of isoflurane resulted in faster melanoma growth in male, but not in female mice, possibly by sex-dependent differences in their immune response.[42] However, because our study focused on orthotopic breast cancers, the choice of female mice was reasonable.

In conclusion, we systematically evaluated potential risks and limitations of repeated MRI with regard to animal welfare and cancer research. Based on our findings, we consider MRI a safe tool for longitudinal functional and morphological investigations with respect to animal welfare. By doing this, we hopefully erase concerns associated with preclinical repeated MRI of small laboratory animals. However, the influence of isoflurane on the immune system has to be considered, especially in immunotherapy-related research.

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