Effect of formalin fixation on measured concentrations of deposited gadolinium in human tissue: an autopsy study

Abstract

Background:

Generally, studies of Gd deposition in humans measure concentration by analyzing formalin fixed postmortem tissue. However, the effect of formalin fixation on measured Gd concentration has not been well investigated.

Purpose

To evaluate the effect of fixation by comparing Gd concentration in fresh vs. formalin fixed postmortem human tissues.

Material and Methods:

Fresh samples of bone and skin were collected from autopsy cases with previous exposure to Gd-based contrast agents (GBCAs). The type of GBCA administered, dose, and eGFR were recorded. Each tissue sample was cut into three aliquotes. Paired samples were stored fresh frozen while the remaining two were stored in 10% neutral

buffered formalin for one and three months, respectively. Gd concentration was measured using ICP-MS.

Results:

Of 18 autopsy cases studied, twelve were exposed to only macrocyclic GBCA, 1 to only linear agents and 5 received both macrocyclic and linear agents. On average, Gd concentration for bone decreased 30.7% after one month of fixation ($p=0.043$) compared to non-fixed values. There was minimal, if any change in concentration between one and three months (average decrease 1.5% ($p=0.89$). The findings were numerically similar for skin tissue with an average decrease of 36.9% after one month ($p=0.11$) and 6.0% ($p=0.73$) between one and three months.

Conclusion:

Formalin fixation appears to decrease Gd concentration in bone and skin approximately 30-40% on average. The largest decrease occurs within the first 30 days of fixation followed by a considerably smaller decrease at 60 days.

Keywords:

MRI, Gadolinium, Deposition, ICP-MS, Formalin Fixation, Soluble,

Macrocyclic, Linear, Decreased Renal Function, GBCA, contrast agents

Introduction:

Gadolinium (Gd) based contrast agents (GBCAs) are used during clinical magnetic resonance imaging (MRI) to increase the diagnostic performance of examinations. The T1 shortening effects of Gd enhance soft tissue contrast for some disease entities, which helps the sensitivity of detection and characterization of these pathologies.

Elemental Gd is a rare earth heavy metal of the lanthanide series that is toxic to humans. The Gd in GBCAs is bound to a ligand or chelating agent to provide a chemical form that can be used safely in patients with normal renal function. There are two general classes of GBCAs, linear and macrocyclic, which are categorized based on their ligand structure.(1) Macrocyclic GBCAs are considered more stable compared to linear agents. As a possible consequence of this, some linear GBCAs have been associated with a rare systemic disease, nephrogenic systemic fibrosis (NSF), in patients with severe renal failure.(2) In addition, multiple reports have suggested that very small amounts of Gd are deposited in tissues including brain, bone and skin with apparently greater amounts resulting from linear as compared with macrocyclic GBCAs.(3-5) To quantify the amount present in tissues, studies have analyzed postmortem tissue samples from cases with history of MRI with GBCA injections during life. (3,6,7)

Tissue samples collected at autopsy are usually preserved or “fixed” in a solution of 10% neutral buffered formalin prior to analysis with inductively coupled plasma - mass spectrometry (ICP-MS). GBCAs are water-soluble and, although little is known about the chemical forms of deposited Gd, it can be presumed that some water solubility is preserved. To date, little is known about the effect of formalin fixation on Gd concentration in postmortem tissue. The purpose of this study is to investigate the effect of formalin fixation on measured Gd concentrations through analysis of fresh (unfixed) tissue as compared to samples fixed in formalin for one month and three months.

Materials and methods:

Autopsy Cases and Sample Selection:

This study was approved by the Human Subjects Review Board at our institution for retrospective review of medical records and was Health Insurance Portability and Accountability Act compliant. Additionally, autopsy consent in each case granted authorization for use of tissues for research purposes.

Study subjects were identified from cases undergoing autopsy between April and September 2017. Electronic medical records were thoroughly searched for exposure to GBCA. Fresh tissue samples of bone and skin were collected during autopsy from cases with a history of GBCA injection and divided into 3 sections, one of which was stored unfixed, frozen. Fresh brain tissue was not available because at our institution the whole brain is placed in formalin immediately upon removal at autopsy for 1-2 weeks prior to sectioning. The types of GBCA administered along with dose, number and dates of injections were recorded for all patients. Patient age, sex, renal function status (eGFR) were also recorded. Cases were acquired serially and included all autopsy cases during this period with exposure to GBCAs.

Sample Preparation and Formalin Fixation:

Fresh bone and skin samples were divided into three approximately equal-sized aliquots of approximately 60-200mg and assigned to one of three individual groups (Fresh, Fixed for 1 month [1M], Fixed for 3 months [3M]). Associated soft tissues were removed from all bone samples and subcutaneous fat was removed from all skin samples(which included only the epidermis and dermis). All aliquots were stored in sterile 15cc centrifuge tubes. From the 3 aliquots, one was stored in a freezer until ICP-MS analysis (Fresh group), and the two remaining aliquots were stored at room temperature immersed in 10 mL of 10% neutral buffered formalin. The formalin fixed aliquots were stored and sampled after one month (Fixed 1M group) and after three months (Fixed 3M group).

All tissue samples were cut using stainless steel surgical scissors which were cleaned between each sample to prevent cross contamination of gadolinium. All tissue samples were cut, weighed using an analytical balance (AG104; Mettler Toledo, Greifensee, Switzerland) and placed in new polypropylene centrifuge tubes before ICP-MS analysis. All samples were sent and analyzed at the Environmental Health Laboratory and Trace Organics Analysis Center (ISO/IEC 17025 accredited) at our institute.

In addition to bone and skin samples, 1ml of formalin from the fixed samples was collected from three cases with exposure to gadoteridol, a macrocyclic GBCA, and three

cases with exposure to linear agents. These formalin samples were also analyzed for Gd with ICP-MS.

ICP-MS:

Samples were prepared by microwave-assisted (MARS 5, CEM Matthews, NC), open-vessel, nitric acid digestion (1 mL concentrated; trace metal grade, Fisher) with terbium (Tb) (100 ng) added as recovery internal standard. Two rounds of a ramped microwave program were used (first round: 400 W 50% power, 10min ramp to 40˚C, hold 10min at 40˚C; 800 W 75%, 10min ramp to 60˚C, hold 10min at 60˚C; 800 W, 100%, 10min ramp to 90˚C, 30min hold at 90˚C; second round, the last step was 60min hold at 90˚C), with inspection between rounds to insure tissue/bone fragments were submerged. Digestate was brought to 10 mL with deionized water (≥18 MΩ). Bone digestate was further diluted 1:10 with 10% nitric acid before analysis to reduce matrix effects due to high-solute concentration.

The ICP-MS instrument (Agilent 7900-CE; Santa Clara, CA) has a collision reaction cell, which was used in He mode (4.3 mL/min, OctP RF 200V) to eliminate polyatomic interferences. The ICP-MS conditions were as follows: radiofrequency power - 1550W;

sampling depth - 8 mm; carrier gas - 1.03 L/min; no makeup gas; instrument internal standards - ^{193}Ir. Gd and Tb calibrants (0.01–100 ng/mL in 10% nitric acid) were prepared from commercial stock (certified reference material, Aristar-BDH; VWR, Radnor, PA) and confirmed with an independent check standard (Ultra Scientific, N. Kingstown, RI). The instrument limit of detection (ILOD) for ^{157}Gd was 0.1 ng/sample (0.001 μg/g tissue assuming 100 mg sample; 0.006 nmol/g tissue). The lowest valid calibrant (relative standard error ≤10%) varied between batches of samples. The reporting limit was set to the higher of the lowest valid calibrant or the ILOD. Thus, the reporting limit of the assay - at and above which a numerical value was provided - ranged between 0.1-0.5 ng/sample (0.005 μg/g; 0.032 nmol/g). Tb has historically demonstrated good percent recovery (Mean - 97±5% for analytical batches discussed here) and tracking - when quantifying Gd in similar matrices (Murata et al., 2016). Mean Gd spike recovery efficiencies (corrected for Tb recovery) in sample batches ranged from 96-104%. All method blanks demonstrated historically consistent background levels significantly below the ILOD (<0.01ng).

Data Analysis:

The percent change in Gd concentration between the fresh, fixed 1M and fixed 3M

samples was calculated for bone and skin. The paired t-test was used to test changes in Gd concentration after log-transformation to reduce right-skewness. Welch's two-sample t-test was used to explore differences in Gd concentration and changes in concentration between subgroups defined by kidney function and the type of GBCA the decedent was exposed to prior to death. Spearman's rank correlation was used to test for trends between the Gd concentration of the formalin sample and the change in Gd concentration from fresh to fixed samples. All statistical calculations were conducted with the statistical computing language R (version 3.1.1; R Foundation for Statistical Computing, Vienna, Austria). Two-sided tests were used with statistical significance defined as $p<0.05$.

Results:

Our study describes the effects of formalin fixation on postmortem Gd tissue concentrations in a cohort of 18 subjects with prior history of GBCA exposure. Twelve (67%) cases were exposed to only macrocyclic GBCAs, one (6%) case was exposed to only linear GBCAs, and 5 (28%) cases were exposed to both linear and macrocyclic GBCAs. Five (28%) cases had eGFR <60, of which three were only exposed to macrocyclic GBCAs.

Tissue Gd concentration in fresh and fixed tissue is summarized in Table 1 and Figure 1. The Gd concentration in all samples exceeded the instrument limit of quantification. On average, Gd concentration decreased 30.7% (95% CI: 1.2, 51.4%; p=0.043) in fresh bone samples when compared to the fixed 1M bone samples (Table 2). There appeared to be minimal, if any change in concentration between the fixed 1M and fixed 3M bone samples, with the average decrease estimated at 1.5% (95% CI: -24.2, 21.9; p=0.89). The average change in concentration from the fresh sample to the average of the 1M and 3M fixed samples was -31.2% (95 CI: -48.9, -7.3%; p=0.017).

The changes in Gd concentration in skin from the fresh sample to fixed 1M sample

(mean: -36.9%, [95% CI: -64.3, 11.4%], p=0.11) and from the fixed 1M sample to the fixed 3M sample (mean: 6.0%, [95% CI: -25.5, 50.7%], p=0.73) were numerically similar to the pattern observed in bone (Table 2). However, changes were more variable between samples, as evidenced by the wider CIs, so the changes did not reach statistical significance. The average change in concentration from the fresh sample to the average of the 1M and 3M fixed samples was -35.1% (95 CI: -59.9, 5.2%; p=0.076).

In the fresh samples, the Gd concentration was significantly higher in bone (median: 3.59 vs. 0.51 μg/g, p=0.035) and (median: 1.46 vs. 0.21 μg/g, p=0.002) in the cases with compromised renal function (eGFR < 60; n=5) compared to those with normal renal function (n=13). Similarly, those with compromised renal function tended to have greater decreases in Gd concentration from the fresh samples to the fixed samples (average of 1M and 3M) in both bone (mean: -54.5% vs. -19.4%, 0.074) and skin (mean: -75.2% vs. 6.0%, p=0.006).

In the fresh samples, the cases exposed only to macrocyclic GBCAs (n=12) had significantly lower concentrations of Gd in bone (median: 0.43 vs. 10.1 μg/g, p<0.001) but not in skin (median: 0.21 vs. 0.34 μg/g, p=0.51) compared to cases exposed to linear

GBCAs (alone or mixed; n=6). The changes in concentration from fresh to fixed (average of 1M and 3M) were not statistically significantly different between the macrocyclic and linear/mixed group for bone (mean: -38.6 vs. -13.6%, p=0.15) or skin (mean: -31.5 vs. -41.6%, p=0.74).

Gd concentration of the formalin used to fix the 1M and 3M samples was measured in 6 cases. At 1M, the Gd concentration of formalin tended to be inversely related to the change in concentration from fresh to fixed 1M samples of bone (rho = -0.74, p=0.14) and skin (rho = -0.74, p=0.14). The pattern was similar at 3M, where greater Gd concentrations of formalin tended to be associated with a greater decrease in concentration in bone (rho = -0.43, p=0.42) and skin (rho = -0.94, p=0.017) from fresh to fixed 3M samples.

Discussion:

Since the recognition of the association of GBCAs and NSF in patients with renal compromise was established there has been considerable interest focused on Gd tissue deposition in patients undergoing contrast enhanced MRI. This interest has been further heightened following the report by Kanda et al. (2014) (8) which described high T1 signal intensity in the dentate nucleus and globus pallidus in patients with normal renal function who received multiple injections of GBCA indicating Gd retention. Research is currently ongoing to explore the sites of tissue deposition, the amount of Gd deposited by different contrast agents, the natural history and fate of the deposited Gd as well as attempting to define any possible adverse events that may result from this deposition. A number of studies have utilized postmortem tissue analyses from human autopsy cases and from animal studies using ICP-MS. (3,7,9-12) Many postmortem tissue analysis studies involve fixation with formalin-based solutions, especially when processing brain tissue. Thus, it is important to know if tissue Gd concentration is reduced through formalin fixation and, if so, the approximate magnitude of such decrease.

This study addresses this issue by comparing levels of Gd in fresh frozen tissue obtained at autopsy to the same tissues immersed in 10% neutral buffered formalin for 1 month and for 3 months. While it would have been desirable to include tissue from deep

brain nuclei (globus pallidus and dentate nucleus), this was not possible due to neuropathology processing methods that require formalin fixation of the brain prior to sectioning. Therefore, skin and bone tissue served as potential representative surrogates for these observations.

There have been few reports describing the effects of tissue fixation on heavy metals following fixation and even fewer have included Gd in their analysis. A report by Sarafanov et al.(2008) which compared some metals in prostate tissue found that formalin fixed, paraffin embedded tissue had decreased concentrations of several metals compared to fresh tissue. In some metals such as Zn, only 24±11% of the total amount was recovered from the formalin fixed, paraffin embedded tissue compared to fresh tissue (13). On the other hand, Gambino et al. (2018) have reported that fixation did not affect Gd concentration in dog brains exposed to GBCAs at up to 69 days of fixation (14). However, the sample size of this study was small, and the result of experimental animal studies may not directly mimic the physiologic behavior of Gd in postmortem human tissue.

The results of our study show that in formalin fixed tissue there is a 30-40% lower Gd concentration compared to fresh tissue. This represents a modest decrease in Gd tissue concentration with fixation. In addition, in our study Gd concentration declines most

(approximately 30-36%) during the first 30 days of formalin fixation and then declines only slightly, if at all, after 2 additional months, approximately 1.5-6% on average. This suggests that studies done in different preservation times greater than 1 month are likely to be comparable.

The group of cases with exposure to linear agents (either exclusively or mixed) had a higher drop in concentration of Gd. However, they also had a higher baseline concentration of Gd in fresh tissue so the percent drop in Gd levels were not significantly different in linear vs. macrocyclic agents. The group which had renal failure at the time of GBCA injection did show considerably higher levels of Gd at baseline and also showed a considerably higher percent drop in Gd concentration with fixation (approximately 55-75%). It may be speculated the observed differences in the renally impaired subjects may be due to differences in the chemical forms of Gd deposited and that some of these chemical forms may be more soluble. Thus, these forms could be more susceptible to extraction by formalin-based solutions.

Limitations of our study include the relatively small sample size. While the changes in Gd concentration in skin from fresh to fixed did not reach statistical significance, the magnitude the average changes were similar to but slightly greater than those changes in bone. We only examined fixed time points at 1 month and at 3 months of fixation, so we

are unable to draw inferences about the rate that Gd concentration decreases during the initial days and weeks of fixation. Brain tissue could not be collected fresh and thus, only samples of the skin and bone were analyzed. Thus, rates of Gd concentration decrease may not generalize to the brain or other tissues.

In conclusion, formalin fixation decreased the gadolinium tissue concentration from human skin and bone samples collected at autopsy by about 30-40%. Most of this occurred during the first 30 days. A more pronounced drop was found in cases with renal failure, which may suggest that some different chemical forms of gadolinium may be retained compared to cases with normal renal function. Future studies should consider changes in concentrations linked to sample preservation.

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Table 1. Gd concentration in fresh and fixed tissue (N=18)

	State*		
Variable	**Fresh**	**Fixed 1M**	**Fixed 3M**
Bone sample	1.25 (0.35-5.34)	0.70 (0.39-4.71)	0.61 (0.33-4.59)
Formalin from bone sample†	n/a	0.0085 (0.0038-0.0422)	0.0032 (0.0015-0.0059)
Skin sample	0.24 (0.15-0.55)	0.21 (0.12-0.33)	0.19 (0.14-0.27)
Formalin from skin sample†	n/a	0.0015 (0.0007-0.0027)	0.0008 (0.0005-0.0011)

*Median (inter-quartile range) Gd concentration (μg/g)

†Available for 6 cases.

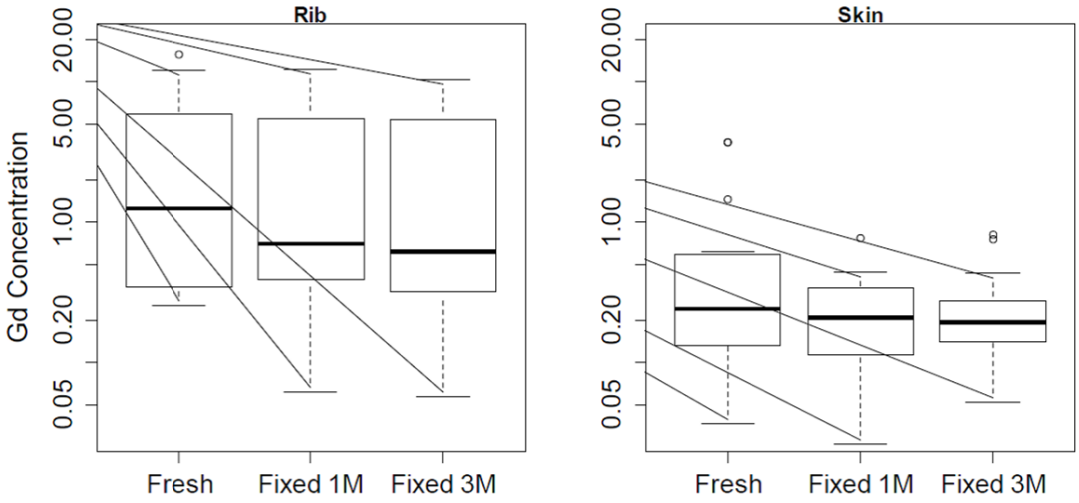
Rib
Skin
Gd Concentration
0.05
0.20
1.00
5.00
20.00
Fresh
Fixed 1M
Fixed 3M

Fig. 1