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Diffusion Tensor Imaging of Healthy and Infarcted Porcine Hearts: Study on the Impact of Formalin Fixation

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Abstract

Background—Due to complexities of *in-vivo* cardiac diffusion tensor imaging (DTI), *ex-vivo* formalin-fixed specimens are used to investigate cardiac remodeling in diseases, and reported results have shown conflicting trends. This study investigates the impact of formalin-fixation on diffusion properties and optimizes tracking parameters based on controls to understand remodeling in myocardial-infarction (MI).

Methods—DTI was performed on 4 healthy (controls) and 4 MI induced formalin-fixed (PoMI) *ex-vivo* porcine hearts. Controls were scanned pre-fixation (PrCtrl) and re-scanned (PoCtrl) after formalin-fixation. Fractional anisotropy (FA) and apparent diffusion coefficient (ADC) were estimated in all hearts. Tracking parameters (FA, tract termination angle (TTA), fiber-length) were optimized in controls and then used to investigate structural remodeling in PoMI hearts.

Results—Fixation increased ADC and decreased FA. PoMI showed increased ADC but decreased FA in infarcted zone compared to remote zone. TTA showed sharp increase in slope from 5°–10°, which flattened after 25° in all groups. Mean fiber-length for different tracking length range showed that PoCtrl had shorter fibers compared to PrCtrl. Fibers around infarction were shorter in length and disarrayed compared to PoCtrl group.

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Author Declaration and Compliance with Ethical Standards

Competing interests: None declared

Conclusion—Formalin-fixation affects diffusion properties and hence DTI parametric trends observed in pathology may be influenced by the fixation process which can cause contradictory findings.

Keywords

Myocardial infarction (MI); diffusion tensor imaging (DTI); formalin-fixation; remodeling

Introduction

Cardiovascular diseases (CVD) continue to be the leading cause of mortality and morbidity worldwide. Among different types of CVD, myocardial infarction (MI), characterized by cellular necrosis of a segment of the heart muscle, is a major and well-recognized contributor. In US alone, approximately 6.4 million patients are hospitalized annually for initial occurrence of MI and 2.8 million for MI recurrence [1].

Histological evidence has shown that MI causes remodeling (change in shape, size and function) of the left ventricular (LV) myocardium [2–4]. This remodeling adversely affects cardiac function and, if untreated, eventually leads to heart failure [5, 6]. Therefore, there is a need to understand the effect of MI on LV remodeling associated with the changes in the fiber structure and orientation, which would potentially facilitate novel treatment for MI.

Conventional *ex-vivo* histological approaches to delineate MI have been replaced by advanced *in-vivo* imaging [7–11]. Cardiac magnetic resonance imaging (MRI) with delayed enhancement imaging [12–15] is highly accurate in detecting MI and myocardial tagging [16–18] can quantitatively determine the decrease in contractile function in an infarcted region. With the advent of cardiac diffusion tensor imaging (DTI) [19–27], a MRI-based technique, researchers have been able to non-invasively investigate the three dimensional (3D) myocardial fiber architecture in a MI model.

DTI is based on the Brownian motion exhibited by water molecules in biological tissues which is known to be impeded or facilitated based on the tissue structure and its orientation [28]. This inherent property of diffusion demonstrated by water molecules causes an exponential decay of the received MR signal which can be characterized using a 3×3 diffusion tensor $\mathbf{D}$ [29].

Diagonalization of $\mathbf{D}$ provides the eigenvalues ($\lambda_1, \lambda_2, \lambda_3$) and eigenvectors ($\varepsilon_1, \varepsilon_2, \varepsilon_3$) of the tensor. These eigenvalues and vectors completely define the anisotropic diffusion occurring at each imaging voxel. The principal eigenvector (ε_1) corresponds to the direction of maximum diffusivity which is aligned in the direction of the fiber; whereas the other two vectors ($\varepsilon_2, \varepsilon_3$) relate to the direction of radial diffusivity. $\mathbf{D}$ can be used to analyze the myocardial fiber architecture [30] and provide quantitative structural information using parameters such as fractional anisotropy (FA), trace values, apparent diffusion coefficient (ADC) [29] and helical angles [31]. The myocardial fiber architecture thus established with DTI has been validated against previously established histological models [31, 32].

Recently, cardiac DTI has been implemented in *in-vivo* humans [19] and in patients [27, 33, 34] to study normal and pathological fiber architecture. However, high resolution cardiac DTI is still under development due to difficulties associated with *in-vivo* imaging (sensitivity of DTI imaging parameters to cardiac and respiratory motion) and therefore, to date, majority of the studies performed to establish myocardial architecture using cardiac DTI, have been implemented on *ex-vivo* animal specimens. These *ex-vivo* specimens are generally fixed in formalin after extraction in order to preserve and maintain the tissue. Therefore, there is a need to understand the impact of formalin-fixation on the diffusion properties of the myocardium so as not to misinterpret the findings as being related to the pathology of concern. In addition, *ex-vivo* cardiac DTI has been explored by various groups to study remodeling following MI. FA, trace values, ADC and helical angles have been examined across the myocardial wall in regions remote from, adjacent to, and within the infarcted area in different animal models [35–42]. However, different study groups have reported different contradictory results with respect to the effect on DTI parameters as a result of MI [36, 38, 42]. The underlying ground truth behind how the actual pathology alters the DTI parameters and how much of it is a result of the fixation process remains arguable and needs to be verified before any substantial claim is made. We hypothesize that the conflicting results in terms of how diffusion parameters are affected due to MI can be attributed to the process of fixation and its effect on the diffusion properties of water molecules. The aim of the study is to investigate the effect of myocardial tissue fixation on DTI parameters (ADC, FA, tract termination angle (TTA) and fiber length) by comparing healthy *ex-vivo* pre-fixed (PrCtrl) hearts to post-fixed (PoCtrl) hearts in a porcine model. Furthermore, the study also investigates remodeling of the fiber architecture, with respect to fiber length, in fixed infarcted (PoMI) porcine model, by employing optimized DTI tracking parameters obtained from comparison of the control groups pre and post fixation.

Methods

Eight juvenile Yorkshire pigs (90–110 lbs) were used in this study in compliance with The Ohio State University's Institutional Animal Care and Use Committee. Four pigs were used as controls (PrCtrl and PoCtrl) to study the effect of formalin-fixation on the diffusion parameters and fiber architecture. MI was induced in the other four pigs (PoMI) to study the remodeling of fiber architecture.

Animal Preparation

Control Group—In the control group, the animals were euthanized and the hearts were extracted and stored in ice. A balloon was inserted through the aortic opening into the LV chamber and inflated with air using a syringe in order to prevent the LV from collapsing. Then, MRI was performed on these specimens (PrCtrl) within 5 to 48 hours of extraction (depending on the availability of the scanner). Post scanning, the hearts were placed in a 10% neutral buffered formalin solution (4% formaldehyde solution) and stored at room temperature for approximately two weeks (a window of 12 – 18 days) and then they (PoCtrl) were re-scanned.

MI Group—The animals were anesthetized by using ketamine and acepromazine and anesthesia was maintained using isoflurane (1–2%). A 50 mg bolus of amiodarone was administered as an anti-arrhythmic. A 7 french guiding catheter was advanced through the descending aorta into the left coronary ostia and a pre-surgical baseline coronary angiogram was recorded to identify the size of the vessel, location of diagonal branches, and to select an infusion site (Figure 1(a)). A 0.014” guide wire was advanced through the guide catheter and into the distal portion of the left anterior descending artery (LAD). An appropriately sized over-the wire balloon catheter (Maverick, Boston Scientific, Natick, MA) was advanced over the wire, and the balloon was inflated to occlude blood flow through the LAD. Complete occlusion was confirmed by a postsurgical coronary angiogram (Figure 1(b)). After 5 minutes of ischemia the guide wire was removed from the balloon catheter and 2 mL of absolute ethanol was injected through the guide wire lumen of the balloon catheter over 1 minute, followed by a 2 mL saline flush. The balloon was left inflated for an additional 3 minutes to prevent retrograde flow of the ethanol back up into the proximal LAD. The balloon was deflated and complete occlusion of the targeted portion of the LAD was confirmed with another coronary angiogram. The animals were monitored for three weeks to allow for maximum fibrosis and remodeling in the infarcted region [3] as indicated by T1-weighted MRI (Figure 1(c)). After 22 days, the animals were euthanized and the hearts were removed, flushed with saline and stained using tetrazolium chloride to delineate the area of the infarction. Then they were placed in a 10% neutral buffered formalin solution and stored at room temperature for six months. The PoMI group was obtained from another study which caused the difference in the fixation period between the PoCtrl (a window of 12 – 18 days) group and the PoMI group (6 months).

Image Acquisition

Ex-vivo cardiac DTI was performed on the eight porcine hearts in a 3T MRI scanner (Tim Trio, Siemens Healthcare, Erlangen, Germany). A bipolar 2 dimensional diffusion-weighted single shot spin-echo echo-planar imaging sequence was used to acquire multi-slice short axis views of the heart covering the entire LV. Imaging parameters included: diffusion encoding directions = 256; TE = 90 ms; TR=7000; slice thickness = 2 mm; matrix = 128×128; FOV = 256×256mm²; b values = 0,1000 s/mm²; acquisition voxel = 2×2×2mm³; number of averages = 1; acquisition time for 40 slices ~ 30 mins; GRAPPA acceleration factor of 2. Additionally, for the PoMI group, high resolution T1-weighted scout images were obtained to delineate the infarcted region from the rest of the myocardium (Figure 1(c)).

Image Analysis

The acquired images were corrected using FSL 4.1.6 (FMRIB Software Library, Oxford, UK) for eddy current induced artifacts [43] that are caused by rapid switching of the diffusion gradients. The eddy current corrected images were then masked to segment the LV myocardium (papillary muscles were included in the mask). The masked myocardium was processed to generate the diffusion tensors for the entire heart which were then used to estimate eigenvalues, eigenvectors and generate ADC and FA maps for the PrCtrl, PoCtrl and PoMI groups. Based on wall-thinning observed on the high resolution T1-weighted scout images in the PoMI group, regions of interests (ROI) were drawn to segment the

infarcted region from the remote region (Figure 1(d)). A comparison of mean ADC and FA was performed between the infarcted and remote regions in the PoMI group to analyze any discernible changes in the DTI parameter values caused due to MI. Next, tracking parameters were optimized to generate fiber tracts to assess the change in fiber architecture as a result of formalin-fixation and MI remodeling. A flow chart demonstrating the steps involved in fiber tracking following optimization of the tracking parameters is provided in Figure 2.

Histograms of the FA maps were created to determine the optimal FA range to be used for fibertracking based on the bins that corresponded to the highest voxel density. Once the optimal FA range was fixed, ExploreDTI v4.8.2 (a freely available non-commercial software package for academic institutions, ExploreDTI is capable of pre-/post processing and analyzing DTI data as well performing fiber tractography) [44], was used to perform tractography across the entire heart, using a deterministic streamlined approach [30]. TTA (the maximum angle between the principle eigenvectors (ϵ_1) in two successive voxels) was varied and the corresponding total number of fiber tracts was recorded to analyze the effect of formalin-fixation on these angles. Based on our initial experience, TTA was varied from 5° to 60° in increments of 5° and the corresponding total number of tracts for each TTA value was recorded. For this analysis, the length range parameter used for tractography was kept constant fixed (2 – 500 mm) so that the total number of tracts identified was independent of the effect of fiber length.

From the results obtained, critical TTA (see results) values were selected to investigate the effect of formalin-fixation on fiber lengths. Fiber tracking (across the entire heart) was performed by employing the optimal FA range and critical TTA. The length range used for tracking was varied by altering the lower limit (2, 10, 20, 30, 40 and 50 mm) and fixing the upper limit (500mm). For each iteration (altering the lower limit of fiber length), the total number of tracts obtained and the mean total length of the tracts were recorded. Mean total length was defined as the average of the total length contributed by all the tracts per tracking condition.

Finally, a qualitative analysis of the fiber architecture in the PoCtrl and PoMI was implemented to investigate the remodeling of fiber tracts based on fiber length and visual orientation. The analysis was performed by employing the optimal FA and critical TTA obtained from the control group to obtain whole heart tractography. Following tractography, small regions of interest (ROI) were defined in the infarcted region of the PoMI group and approximately in the same region of the PoCtrl group to qualitatively analyze fibers passing through the defined ROI.

Statistical Analysis

Differences in mean ADC, mean FA and the mode of FA between the PrCtrl and PoCtrl groups were tested for significance ($p < 0.05$) using a 2-tailed paired student's t-test. Additionally, a significant difference ($p < 0.05$) of the mean ADC and FA values between the remote and infarcted region in the PoMI group was also determined with a 2-tailed paired student's t-test. Furthermore, the mean ADC and FA values between the 4 groups (PrCtrl, PoCtrl, infarcted region in PoMI, remote region in PoMI) were tested with a one-way

analysis of variance (ANOVA). A 2-tailed unpaired student's t-test was performed to determine the significant difference ($p < 0.05$) in the modes of the FA between the PoCtrl and PoMI groups.

Results

ADC and FA

Mean ADC and FA were estimated for the entire heart in the PrCtrl and PoCtrl groups and in the infarcted and remote myocardium in the PoMI group as shown in Figure 3. Both ADC and FA showed a significant difference between pre and post formalin-fixation. Fixation increased (p -value = 0.0018) the mean ADC (Figure 3 (a)) from $0.52 \times 10^{-3} \pm 0.026 \times 10^{-3} \text{ mm/s}^2$ in PrCtrl to $0.80 \times 10^{-3} \pm 0.072 \times 10^{-3} \text{ mm/s}^2$ in PoCtrl. Whereas, fixation decreased (p -value=0.0090) mean FA (Figure 3 (c)) from 0.42 ± 0.028 in PrCtrl to 0.26 ± 0.034 in PoCtrl. A comparison between the infarcted and remote regions in the PoMI group indicated that the mean ADC (Figure 3 (b)) in the infarcted region ($0.95 \times 10^{-3} \pm 0.12 \times 10^{-3} \text{ mm/s}^2$) was significantly (p -value = 0.0006) higher than the remote region ($0.63 \times 10^{-3} \pm 0.052 \times 10^{-3} \text{ mm/s}^2$). Although, the mean FA (Figure 3 (d)) in the infarcted region (0.22 ± 0.023) was lower compared to the remote region (0.25 ± 0.031), the decrease in FA value was not significant (p -value = 0.0557). A oneway ANOVA showed significant difference in both the ADC (p -value < 0.00001) and the FA (p -value < 0.00001) between the 4 groups (PrCtrl, PoCtrl, infarcted region in PoMI, remote region in PoMI).

Optimal FA

Next, optimal FA range to be used for fiber tracking was determined for all the three groups. A distribution of the histogram showed that the mode of FA value was lower in post-fixation (PoCtrl and PoMI) group as compared to pre-fixation (PrCtrl) (Figure 4(a)). The mean and the SD of the mode of the FA values within the PrCtrl, PoCtrl and the PoMI groups were 0.27 ± 0.014 , 0.17 ± 0.010 and 0.16 ± 0.011 respectively (Figure 4(b)). A significant difference in the mode of the FA value was observed between the PrCtrl and PoCtrl group (p -value = 0.0013); and between the PrCtrl and PoMI group (p -value = 0.0007). However, no significant difference (p -value = 0.2) in the mode of the FA value was observed between the PoCtrl and PoMI group. Based on this observation, the same optimal FA range was used for fiber tracking in the PoCtrl and PoMI group and a different range was determined for the PrCtrl group. The upper limit of the range was fixed at 1 and the lower limit of the FA range was calculated based on a 25% drop from the peak value (refer to the lines on Figure 4(c)). This optimal FA range was determined to be [0.2 1] for PrCtrl and [0.1 1] for PoCtrl and PoMI for all further processing.

TTA

Critical TTA was determined by employing the optimal FA values in the fiber tracking algorithm (Figure 5). The plot of number of tracts (normalized by the total number of voxels in the LV mask) versus the TTA (maximum allowable turn angle between two successive voxels in degrees) showed a sharp increase in the slope from 5° to 10° , which gradually decreased after 10° and flattened after 25° . This trend was consistent for all the three groups

(PrCtrl, PoCtrl and PoMI). The plot provided two critical TTA (5° and 10°) to be examined for our next optimization of fiber length.

Fiber Length

The normalized mean fiber length decreased in the PoCtrl group when compared to the PrCtrl group (Figure 6). In the both groups (PrCtrl and PoCtrl), for both the angles (5° and 10°) as the lower limit of the length range increased from 2 mm to 50 mm (tracking conditions became more conservative) the mean length of the fibers increased and the total number of fiber tracts decreased. This is because shorter fibers were excluded from the tracking conditions which reduced the mean fiber length measurements.

MI Fiber Orientation

Qualitative differences were observed between the PoCtrl (Figure 7) and PoMI (Figure 8) groups with respect to orientation and length of fibers. Fiber tracts in the infarcted region of a heart from the PoMI group (shown in the Figure 8) demonstrate that the tracts were shorter in length, less dense and disarrayed as compared to healthy heart from the PoCtrl group. Similar patterns were observed in all the other hearts.

Discussion

Our study demonstrated that formalin-fixation alters the diffusion properties of cardiac muscle fibers. Post-fixed hearts had higher ADC values, lower FA and reduced total mean length as compared to pre-fixed hearts. However, the trend in TTA was consistent in both the control groups and the diseased myocardium. PoMI hearts had significantly higher ADC in infarcted region as compared to the remote regions. FA showed an insignificant decrease in the infarcted zone compared to the remote normal tissue. Additionally, fibers in the infarcted region were short and disarrayed when compared to the PoCtrl group.

The results from our study and other previous studies [35–42] demonstrate that cardiac DTI can be used to study fiber architecture remodeling as a result of MI. Therefore, cardiac DTI can potentially be used as a diagnostic tool for patients having contraindications to Gadolinium based contrast agents (since delayed enhancement based MRI is not feasible in such patients). However, *in-vivo* cardiac DTI [19, 20, 27, 33, 34] is still under investigation due to the challenges associated with cardiac motion, respiratory motion and long breathhold times. Hence, majority of the current research for technique establishment and optimization is based on experiments performed on formalin-fixed *ex-vivo* specimens. It is evident from our results that the fixation process affects diffusivity of water molecules. Therefore, when standardizing quantitative measurements (FA, ADC) for *in-vivo* applications based on information from formalin-fixed *ex-vivo* hearts, in either healthy or diseased myocardium, the effects of fixation on the measurements should be considered.

Fixation caused an increase in the ADC values in the controls which was consistent with a study performed on the nervous tissues of rat brains [45]. Formalin-fixation prevents degeneration of tissues by reacting with primary amines on proteins and nucleic acids to form partially-reversible methylene bridges. During this process the cells tend to undergo i) a period of shrinkage followed by ii) a prolonged period of swelling followed by iii) a period

of secondary shrinkage. It has been shown that when the concentration of formaldehyde is below 5% (4 % in this study) the initial shrinkage is very low and it is followed by a period of extensive swelling [46]. This extreme swelling in cellular structures impacts the diffusion properties and could be reflected with increased ADC estimates. Since the PoCtrl hearts were fixed for approximately 2 weeks, the hearts could be in the stage of cellular swelling which led to the observation of higher ADC values in PoCtrl hearts when compared to the PrCtrl hearts.

Furthermore, a decrease in ADC values was observed in the remote normal tissue in the PoMI model when compared to the PoCtrl model. We speculate that because the PoMI specimens were fixed for nearly 6 months before being scanned it provided the tissues enough time to migrate to the final stage of the fixation process which involves a period of cellular shrinkage. This hypothesis is further supported by another study where 4 weeks of formalin-fixation with a 10% neutral buffered solution resulted in decreased diffusivity in mouse brains [47].

However, there are other studies which have observed contradictory results [48, 49]. Since there is insufficient literature on the effect of diffusion properties of the heart as a result of fixation our comparison with earlier work is based mainly on brain studies, and the type of tissue being investigated can play a role in the contradictions observed. Additionally inconsistencies and contradictions with previous studies can be associated with the fixation process in general [50]. Factors, such as the type of fixation (perfusion fixation, immersion fixation or both), thickness of tissue, preparation of the fixing agent, time interval between tissue extraction and fixation, the ratio of the volume of the specimen to the amount of formalin solution, extent of fixation and temperature of fixation, play a role in the fixation process which can eventually affect the diffusion properties of the heart differently. Therefore for any substantial standardization of diffusion parameters in normal tissues, and comparison between different studies using *ex-vivo* formalin-fixed specimens, a standard protocol for the fixation procedure should be followed. In the PoMI model, ADC in the infarcted region was significantly higher compared to the remote regions. This observation of higher ADC in the infarcted zone is consistent with results obtained from other small animals (rats [35]) large animals (pigs [38, 51]) and humans ([34, 38]). It is well-known that in the infarcted myocardium, the ruptured cell membranes cause an increase in the extra-cellular space thereby assisting unrestricted diffusion. This is well-reflected by the significant increase in the ADC values in the infarcted myocardium as opposed to the remote normal myocardium.

Fixation caused a reduction in the FA value, which was consistent with results obtained from studies performed in the brain [52] and spinal cord [53]. This can be attributed to the observation of reduced total mean fiber length in the post-fixed specimens. Shorter fibers in the PoCtrl group imply reduced available length for unidirectional channelized diffusion resulting in lower FA values. Furthermore, as mentioned earlier, fixation results in a stage of extensive swelling and a subsequent increase in water diffusion which allows increased random movement of water molecules. Increased unguided random motion contributes to reduced anisotropy and hence decreased FA in the PoCtrl model as compared to the PrCtrl model.

Our results did not demonstrate a significant difference in the FA value within the PoMI group when comparing the infarcted region to the remote normal tissue which is consistent with results obtained by Wu Y. et al. (post-infarct porcine models) [42, 54]. However, as mentioned earlier, there are contradictory conclusions about FA values in MI induced hearts. Researchers have shown that a marked decrease in FA occurred in infarcted hearts in humans [34], rats [35] and pigs [38] when compared to the controls. In contrast, another study in rat models [36] showed increased FA due to infarction. Therefore, it might not be sufficient to diagnose infarction in fixed *ex-vivo* hearts based only on FA values because the integrity of the results might be affected by the fixation process. Additionally since the infarcted region demonstrated extreme wall thinning, partial volume effects could be a cause for insignificant difference in FA values between the infarcted and normal myocardium.

TTA did not show a noticeable discrepancy in the trend of the slope followed by the three groups (PrCtrl, PoCtrl and PoMI). Limited literature is available on TTA and its variation as a result of infarction. To the best of our knowledge, only one group has reported change in propagation angle (the average angle between adjacent eigenvectors along myofiber tracts) with MI [55, 56]. However, they have not mentioned any change in TTA with MI and therefore, we cannot directly compare our results to theirs.

Our results showed a reduction in the total mean length per tracking condition (where, the tracking condition was dependent on the lower limit of the fiber length range) in the PoCtrl group when compared to the PrCtrl group. As mentioned earlier, the process of fixation reduces diffusion of water molecules as demonstrated by our FA values thereby causing reducing in the total mean length. Due to reduced diffusivity the tracking algorithm considered lesser number of pixels as part of the same fiber. As a result, a single long fiber pre-fixation appeared to be composed of multiple short fibers post-fixation thereby reducing the total mean length. Additionally, the interval between euthanizing the animal and fixing the specimen resulted in partial autolysis of the fibers as suggested by D'Arceuil et al. [52], thereby further reducing the total mean length.

Finally, a qualitative analysis of the fiber architecture demonstrated that the number of fibers passing through a defined ROI in the PoMI group was less dense compared to the PoCtrl group proving that the infarcted region had undergone structural changes. Histological evidence shows that immediately post-infarction the infarcted region undergoes necrosis which progresses to a stage of fibrosis followed by remodeling [3]. In porcine models, three weeks post-infarction, the animal is towards the end of the fibrotic stage which is classified by deposition of collagen and shrinkage of the myocardium at the site of the infarction [2, 3, 57]. The loss of the muscle mass along with the deposition of collagen reduces molecular diffusivity and results in fewer fiber tracts being identified in the infarcted region. Additionally, fibers passing through the infarcted region were disarrayed and not as smoothly oriented as the control group.

Our study includes several limitations. First, there was a gap (5 to 48 hours) between euthanizing the animal and data acquisition due to unavailability of the scanner immediately post-sacrifice. Nonetheless, the tissue was stored in an ice bath during this period to prevent decomposition. Second, in PrCtrl group, the hearts were inflated to an arbitrary pressure to

prevent the LV from collapsing and to ensure proper imaging. The pressure variation might have an effect on the DTI parameters. However, we did not observe any major variations in the DTI parameters within the PrCtrl group. Third, since the PoMI hearts were from a different study it induced a discrepancy of the fixation period between the controls and the diseased myocardium. Finally, the image resolution ($2 \times 2 \times 2 \text{ mm}^3$) may have an influence on the DTI parameters. Despite of these limitations, our results were consistent with previously reported results.

Conclusions

In conclusion, we have demonstrated that formalin-fixation affects molecular diffusivity by reducing FA and mean fiber length and increasing ADC. Therefore, the fixation process and its influence on the diffusion parameters need to be taken into account when interpreting impact of diseases on these parameters. Further, when extrapolating the changes observed in diffusion parameters in fixed *ex-vivo* specimens with *in-vivo* studies, inconsistencies might be observed due to the fixation process. However, fixation does not alter the structural orientation of the fibers which was evident from the TTA graphs. Therefore, structural remodeling of the fiber architecture needs to be considered when investigating the effect of MI on the fixed myocardium. This is established by the distinct sparse disarrayed short fibers observed near the infarcted myocardium which is absent in the control group.

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References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, et al. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. 2013; 127(1):e6–e245. [PubMed: 23239837]
2. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation*. 2000; 101(25):2981–2988. [PubMed: 10869273]
3. Holmes JW, Borg TK, Covell JW. Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng*. 2005; 7:223–253. [PubMed: 16004571]
4. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation*. 1990; 81(4):1161–1172. [PubMed: 2138525]
5. Buckberg GD, Weisfeldt ML, Ballester M, Beyar R, Burkhoff D, Coghlan HC, Doyle M, Epstein ND, Gharib M, Ideker RE, et al. Left ventricular form and function: scientific priorities and strategic planning for development of new views of disease. *Circulation*. 2004; 110(14):e333–e336. [PubMed: 15466651]
6. Konstam MA, Kramer DG, Patel AR, Maron MS, Udelson JE. Left ventricular remodeling in heart failure: current concepts in clinical significance and assessment. *JACC Cardiovasc Imaging*. 2011; 4(1):98–108. [PubMed: 21232712]
7. Arnett JH, Mohajer K, Okon SA. Evidence of acute myocardial infarction on CT. *Br J Radiol*. 2007; 80(957):e219–e221. [PubMed: 17928493]
8. Gosalia A, Haramati LB, Sheth MP, Spindola-Franco H. CT detection of acute myocardial infarction. *AJR Am J Roentgenol*. 2004; 182(6):1563–1566. [PubMed: 15150010]
9. Baroldi G, Bigi R, Cortigiani L. Ultrasound imaging versus morphopathology in cardiovascular diseases. Myocardial cell damage. *Cardiovasc Ultrasound*. 2005; 3:32. [PubMed: 16209715]

10. Klein C, Schmal TR, Nekolla SG, Schnackenburg B, Fleck E, Nagel E. Mechanism of late gadolinium enhancement in patients with acute myocardial infarction. *J Cardiovasc Magn Reson*. 2007; 9(4):653–658. [PubMed: 17578720]
11. Roes SD, Kelle S, Kaandorp TA, Kokocinski T, Poldermans D, Lamb HJ, Boersma E, van der Wall EE, Fleck E, de Roos A, et al. Comparison of myocardial infarct size assessed with contrast enhanced magnetic resonance imaging and left ventricular function and volumes to predict mortality in patients with healed myocardial infarction. *Am J Cardiol*. 2007; 100(6):930–936. [PubMed: 17826372]
12. Beek AM, Kuhl HP, Bondarenko O, Twisk JW, Hofman MB, van Dockum WG, Visser CA, van Rossum AC. Delayed contrast-enhanced magnetic resonance imaging for the prediction of regional functional improvement after acute myocardial infarction. *J Am Coll Cardiol*. 2003; 42(5):895–901. [PubMed: 12957439]
13. Ordovas KG, Higgins CB. Delayed contrast enhancement on MR images of myocardium: past, present, future. *Radiology*. 2011; 261(2):358–374. [PubMed: 22012903]
14. Vogel-Claussen J, Rochitte CE, Wu KC, Kamel IR, Foo TK, Lima JA, Bluemke DA. Delayed enhancement MR imaging: utility in myocardial assessment. *Radiographics*. 2006; 26(3):795–810. [PubMed: 16702455]
15. Kim RJ, Fieno DS, Parrish TB, Harris K, Chen EL, Simonetti O, Bundy J, Finn JP, Klocke FJ, Judd RM. Relationship of MRI delayed contrast enhancement to irreversible injury, infarct age, contractile function. *Circulation*. 1999; 100(19):1992–2002. [PubMed: 10556226]
16. Geskin G, Kramer CM, Rogers WJ, Theobald TM, Pakstis D, Hu YL, Reichek N. Quantitative assessment of myocardial viability after infarction by dobutamine magnetic resonance tagging. *Circulation*. 1998; 98(3):217–223. [PubMed: 9697821]
17. Mewton N, Croisille P, Revel D, Weber O, Higgins CB, Saeed M. Left ventricular postmyocardial infarction remodeling studied by combining MR-tagging with delayed MR contrast enhancement. *Invest Radiol*. 2008; 43(4):219–228. [PubMed: 18340245]
18. Gotte MJ, Germans T, Russel IK, Zwanenburg JJ, Marcus JT, van Rossum AC, van Veldhuisen DJ. Myocardial strain and torsion quantified by cardiovascular magnetic resonance tissue tagging: studies in normal and impaired left ventricular function. *J Am Coll Cardiol*. 2006; 48(10):2002–2011. [PubMed: 17112990]
19. Nielles-Vallespin S, Mekkaoui C, Gatehouse P, Reese TG, Keegan J, Ferreira PF, Collins S, Speier P, Feiweier T, de Silva R, et al. In vivo diffusion tensor MRI of the human heart: Reproducibility of breath-hold and navigator-based approaches. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2012
20. Tseng WY, Reese TG, Weisskoff RM, Wedeen VJ. Cardiac diffusion tensor MRI in vivo without strain correction. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 1999; 42(2):393–403.
21. Sosnovik DE, Wang R, Dai G, Reese TG, Wedeen VJ. Diffusion MR tractography of the heart. *J Cardiovasc Magn Reson*. 2009; 11:47. [PubMed: 19912654]
22. Dou J, Reese TG, Tseng WY, Wedeen VJ. Cardiac diffusion MRI without motion effects. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2002; 48(1):105–114.
23. Gamper U, Boesiger P, Kozerke S. Diffusion imaging of the in vivo heart using spin echoes--considerations on bulk motion sensitivity. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2007; 57(2):331–337.
24. Reese TG, Weisskoff RM, Smith RN, Rosen BR, Dinsmore RE, Wedeen VJ. Imaging myocardial fiber architecture in vivo with magnetic resonance. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 1995; 34(6):786–791.
25. Tseng WY, Reese TG, Weisskoff RM, Brady TJ, Wedeen VJ. Myocardial fiber shortening in humans: initial results of MR imaging. *Radiology*. 2000; 216(1):128–139. [PubMed: 10887238]

26. Wu EX, Wu Y, Tang H, Wang J, Yang J, Ng MC, Yang ES, Chan CW, Zhu S, Lau CP, et al. Study of myocardial fiber pathway using magnetic resonance diffusion tensor imaging. *Magn Reson Imaging*. 2007; 25(7):1048–1057. [PubMed: 17707167]
27. McGill LA, Ismail TF, Nielles-Vallespin S, Ferreira P, Scott AD, Roughton M, Kilner PJ, Ho SY, McCarthy KP, Gatehouse PD, et al. Reproducibility of in-vivo diffusion tensor cardiovascular magnetic resonance in hypertrophic cardiomyopathy. *J Cardiovasc Magn Reson*. 2012; 14:86. [PubMed: 23259835]
28. Garrido L, Wedeen VJ, Kwong KK, Spencer UM, Kantor HL. Anisotropy of water diffusion in the myocardium of the rat. *Circ Res*. 1994; 74(5):789–793. [PubMed: 8156627]
29. Le Bihan D, Mangin JF, Poupon C, Clark CA, Pappata S, Molko N, Chabriat H. Diffusion tensor imaging: concepts and applications. *J Magn Reson Imaging*. 2001; 13(4):534–546. [PubMed: 11276097]
30. Bassar PJ, Pajevic S, Pierpaoli C, Duda J, Aldroubi A. In vivo fiber tractography using DT-MRI data. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2000; 44(4):625–632.
31. Scollan DF, Holmes A, Winslow R, Forder J. Histological validation of myocardial microstructure obtained from diffusion tensor magnetic resonance imaging. *Am J Physiol*. 1998; 275(6 Pt 2):H2308–H2318. [PubMed: 9843833]
32. Holmes AA, Scollan DF, Winslow RL. Direct histological validation of diffusion tensor MRI in formaldehyde-fixed myocardium. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2000; 44(1): 157–161.
33. Wu MT, Su MY, Huang YL, Chiou KR, Yang P, Pan HB, Reese TG, Wedeen VJ, Tseng WY. Sequential changes of myocardial microstructure in patients postmyocardial infarction by diffusion-tensor cardiac MR: correlation with left ventricular structure and function. *Circ Cardiovasc Imaging*. 2009; 2(1):32–40. 36 p following 40. [PubMed: 19808562]
34. Wu MT, Tseng WY, Su MY, Liu CP, Chiou KR, Wedeen VJ, Reese TG, Yang CF. Diffusion tensor magnetic resonance imaging mapping the fiber architecture remodeling in human myocardium after infarction: correlation with viability and wall motion. *Circulation*. 2006; 114(10):1036–1045. [PubMed: 16940196]
35. Chen J, Song SK, Liu W, McLean M, Allen JS, Tan J, Wickline SA, Yu X. Remodeling of cardiac fiber structure after infarction in rats quantified with diffusion tensor MRI. *Am J Physiol Heart Circ Physiol*. 2003; 285(3):H946–H954. [PubMed: 12763752]
36. Strijkers GJ, Bouts A, Blankesteijn WM, Peeters TH, Vilanova A, van Prooijen MC, Sanders HM, Heijman E, Nicolay K. Diffusion tensor imaging of left ventricular remodeling in response to myocardial infarction in the mouse. *NMR Biomed*. 2009; 22(2):182–190. [PubMed: 18780284]
37. Mekkaoui C, Huang S, Chen HH, Dai G, Reese TG, Kostis WJ, Thiagalingam A, Maurovich-Horvat P, Ruskin JN, Hoffmann U, et al. Fiber architecture in remodeled myocardium revealed with a quantitative diffusion CMR tractography framework and histological validation. *J Cardiovasc Magn Reson*. 2012; 14:70. [PubMed: 23061749]
38. Wu EX, Wu Y, Nicholls JM, Wang J, Liao S, Zhu S, Lau CP, Tse HF. MR diffusion tensor imaging study of postinfarct myocardium structural remodeling in a porcine model. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2007; 58(4):687–695.
39. Wu Y, Chan CW, Nicholls JM, Liao S, Tse HF, Wu EX. MR study of the effect of infarct size and location on left ventricular functional and microstructural alterations in porcine models. *J Magn Reson Imaging*. 2009; 29(2):305–312. [PubMed: 19161181]
40. Sosnovik DE, Wang R, Dai G, Wang T, Aikawa E, Novikov M, Rosenzweig A, Gilbert RJ, Wedeen VJ. Diffusion spectrum MRI tractography reveals the presence of a complex network of residual myofibers in infarcted myocardium. *Circ Cardiovasc Imaging*. 2009; 2(3):206–212. [PubMed: 19808594]
41. Walker JC, Guccione JM, Jiang Y, Zhang P, Wallace AW, Hsu EW, Ratcliffe MB. Helical myofiber orientation after myocardial infarction and left ventricular surgical restoration in sheep. *J Thorac Cardiovasc Surg*. 2005; 129(2):382–390. [PubMed: 15678050]

42. Wu Y, Zhang LJ, Zou C, Tse HF, Wu EX. Transmural heterogeneity of left ventricular myocardium remodeling in postinfarct porcine model revealed by MR diffusion tensor imaging. *J Magn Reson Imaging*. 2011; 34(1):43–49. [PubMed: 21618331]
43. Basser PJ, Jones DK. Diffusion-tensor MRI: theory, experimental design and data analysis - a technical review. *NMR Biomed*. 2002; 15(7–8):456–467. [PubMed: 12489095]
44. Leemans, A.; Jeurissen, B.; Sijbers, J.; Jones, DK. ExploreDTI: a graphical toolbox for processing, analyzing, and visualizing diffusion MR data. *Proceedings of the 17th Annual Meeting of ISMRM; Hawaii, USA*. 2009. p. 3537
45. Shepherd TM, Thelwall PE, Stanisz GJ, Blackband SJ. Aldehyde fixative solutions alter the water relaxation and diffusion properties of nervous tissue. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2009; 62(1):26–34.
46. Crawford GN, Barer R. The Action of Formaldehyde on Living Cells as Studied by Phase-contrast Microscopy. *Journal of Microscopical Science*. 1951; 92(4):403–452.
47. D'Arceuil HE, Westmoreland S, de Crespigny AJ. An approach to high resolution diffusion tensor imaging in fixed primate brain. *Neuroimage*. 2007; 35(2):553–565. [PubMed: 17292630]
48. Pattany PM, Puckett WR, Klose KJ, Quencer RM, Bunge RP, Kasuboski L, Weaver RG. High-resolution diffusion-weighted MR of fresh and fixed cat spinal cords: evaluation of diffusion coefficients and anisotropy. *AJNR Am J Neuroradiol*. 1997; 18(6):1049–1056. [PubMed: 9194432]
49. Sun SW, Neil JJ, Song SK. Relative indices of water diffusion anisotropy are equivalent in live and formalin-fixed mouse brains. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2003; 50(4):743–748.
50. Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. *J Oral Maxillofac Pathol*. 2012; 16(3):400–405. [PubMed: 23248474]
51. Nguyen CT, Fan Z, Xie Y, Sharif B, Dawkins J, Tseliou E, Bi X, Dharamakumar R, Marban E, Li D. Preliminary application of in vivo cardiac diffusion weighted MRI in chronic myocardial infarction porcine model. *Journal of Cardiovascular Magnetic Resonance*. 2014; 16(Suppl 1):56. (Suppl 1). [PubMed: 25160814]
52. D'Arceuil H, de Crespigny A. The effects of brain tissue decomposition on diffusion tensor imaging and tractography. *Neuroimage*. 2007; 36(1):64–68. [PubMed: 17433879]
53. Shepherd TM, Flint JJ, Thelwall PE, Stanisz GJ, Mareci TH, Yachnis AT, Blackband SJ. Postmortem interval alters the water relaxation and diffusion properties of rat nervous tissue--implications for MRI studies of human autopsy samples. *Neuroimage*. 2009; 44(3):820–826. [PubMed: 18996206]
54. Wu Y, Tse HF, Wu EX. Diffusion tensor MRI study of myocardium structural remodeling after infarction in porcine model. 2006 28th Annual International Conference of the IEEE Engineering in Medicine and Biology Society. 2006; 1–15:3166–3169.
55. Mekkaoui C, Huang S, Dai G, Reese TG, Ruskin JN, Hoffmann U, Jackowski MP, Sosnovik DE. Myocardial infarct delineation *in vivo* using diffusion tensor MRI and the tractographic propagation angle. *Journal of Cardiovascular Magnetic Resonance*. 2013; 15(1)
56. Mekkaoui, C.; Huang, S.; Dai, G.; Reese, TG.; Hoffmann, U.; Jackowski, MP.; Sosnovik, DE. The Tractographic Propagation Angle: A Novel Tool to Detect Infarction and Characterize Myocardial Microstructure. *Proceedings of the 19th Annual Meeting of ISMRM; Montreal, Canada*. 2011. p. 3402
57. Zimmerman SD, Criscione J, Covell JW. Remodeling in myocardium adjacent to an infarction in the pig left ventricle. *Am J Physiol Heart Circ Physiol*. 2004; 287(6):H2697–H2704. [PubMed: 15319211]

Figure 1. Myocardial infarction

Coronary angiogram (a) pre-surgery and (b) post-surgery. The arrow in the right image shows the absence of blood flow in the LAD. (c) T1-weighted image of one of the infarcted hearts. The location of the infarcted region (thinner myocardium) near the apex is illustrated with a black circle.

Figure 2. Flow chart for image analysis

Figure shows the sequence of steps followed for tracking parameter optimization and fiber tracking.

Figure 3. Mean and standard deviation of ADC and FA in all the 8 porcine hearts

a) ADC in control group. The mean and SD of the ADC for the PrCtrl (green dashed line) and PoCtrl (blue dashed line) are $0.52 \times 10^{-3} \pm 0.026 \times 10^{-3} \text{ mm}^2/\text{s}$ and $0.80 \times 10^{-3} \pm 0.072 \times 10^{-3} \text{ mm}^2/\text{s}$ respectively. **b) ADC in PoMI.** The mean ADC across the infarcted (red dashed line) and remote regions (orange dashed line) are $0.95 \times 10^{-3} \pm 0.12 \times 10^{-3} \text{ mm}^2/\text{s}$ and $0.63 \times 10^{-3} \pm 0.052 \times 10^{-3} \text{ mm}^2/\text{s}$ respectively. **c) FA in control group.** The mean and SD of the FA for the PrCtrl (green dashed line) and PoCtrl (blue dashed line) are 0.42 ± 0.028 and 0.26 ± 0.034 respectively. **d) FA in PoMI.** The mean FA across the infarcted (red dashed line) and remote regions (orange dashed line) are 0.22 ± 0.023 and 0.25 ± 0.031 respectively.

Figure 4. Determination of optimal FA range for fiber tracking

a) FA Histogram. The FA histogram indicates a lower mode post-fixation (PoCtrl (dotted-blue) and PoMI (dashed-red)) as compared to pre-fixation (PrCtrl (solid-green)). **b) Mean and SD of FA mode.** Mean and SD of the FA mode for PrCtrl (green), PoCtrl (blue) and PoMI (red) groups are 0.27 ± 0.014 , 0.17 ± 0.010 and 0.16 ± 0.011 respectively. **c) Mean FA histogram (controls) to estimate optimal FA range for fiber tracking.** The peak of the normalized mean FA (normalization based on the total number of voxels) for the PrCtrl and PoCtrl group is 11.5 (corresponding to FA bin 0.15–0.175) and 8.4 (corresponding to FA bin 0.25–0.275), respectively. The lower limit of the FA range corresponding to 25% drop from the peak was determined to be 0.2 for the pre-fixation group and 0.1 for the post-fixation group.

Figure 5. TTA versus Normalized number of tracts

The figure shows the plot of TTA versus number of tracts. The x-axis corresponds to TTA increasing from 5^0 to 60^0 . The y-axis corresponds to the number of tracts normalized with respect to the total number of masked voxels. A sharp change in slope is observed when transitioning from 5^0 – 10^0 TTA.

Figure 6. Normalized mean length (in μm) versus normalized number of tracts in the control group with two different TTA's

a) 5° and b) 10° . The x-axis represents normalized number of tracts and the y-axis represents the normalized total mean length contributed by all the tracts in the entire heart. Normalization was based on the total number of masked voxels. Every point on the graph corresponds to fiber tracking with a specific fiber length range. As the lower limit (2, 10, 20, 30, 40, 50 mm) of the tracking range was increased (i.e., the markers on the plot shifts from right to left), the tracking conditions became conservative which increased the mean length

but decreased the total number of fibers tracked. The normalized mean length was higher in PrCtrl (solid green) group as compared to PoCtrl (dotted blue) group.

Figure 7. 3D fiber architecture in a healthy porcine myocardium

Figure shows fiber tracts passing through a defined region of interest (ROI). Tracking parameters include: FA [0.1 1], TTA 45° and tracking length range [2 500] mm. TTA was set to 45° to mitigate its effect on the number of tracts identified (refer to Figure 6). **Left Image:** The planes denote the two long axes and the short axis of the principle eigenvector. The white circle shows the ROI drawn to track the fibers. **Right Image:** Tracts without the planes. The color on the fiber and planes denote the direction of fiber orientation; Green, red and blue corresponds to the x, y and z directions of the image, respectively.

Figure 8. 3D fiber architecture in an infarcted porcine myocardium

Figure shows fiber tracts passing through a defined region of interest (ROI). Tracking parameters include: FA [0.1 1], TTA 45° and tracking length range [2 500] mm. TTA was set to 45° to mitigate its effect on the number of tracts identified (refer to Figure 6). **Left Image:** The planes denote the two long axes and the short axis of the principle eigenvector. The white circle shows the ROI drawn to track the fibers through the infarcted region. **Right Image:** Tracts without the planes. The color on the fiber and planes denote the direction of fiber orientation; Green, red and blue corresponds to the x, y and z directions of the image, respectively.