Oxidative stress measured \textit{in vivo} without an exogenous contrast agent using QUEST MRI

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Abstract

Decades of experimental studies have implicated excessive generation of reactive oxygen species (ROS) in the decline of tissue function during normal aging, and as a pathogenic factor in a vast array of fatal or debilitating morbidities. This massive body of work has important clinical implications since many antioxidants are FDA approved, readily cross blood-tissue barriers, and are effective at improving disease outcomes. Yet, the potential benefits of antioxidants have remained largely unrealized in patients because conventional methods cannot determine the dose, timing, and drug combinations to be used in clinical trials to localize and decrease oxidative stress. To address this major problem and improve translational success, new methods are urgently needed that non-invasively measure the same ROS bio-marker both in animal models and patients with high spatial resolution. Here, we summarize a transformative solution based on a novel method: QUEnch-assiSTed MRI (QUEST MRI). The QUEST MRI index is a significant antioxidant-induced improvement in pathophysiology, or a reduction in 1/T1 (i.e., R1). The latter form of QUEST MRI provides a unique measure of uncontrolled production of endogenous, paramagnetic reactive oxygen species (ROS). QUEST MRI results to-date have been validated by gold standard oxidative stress assays. QUEST MRI has high translational potential because it does not use an exogenous contrast agent and requires only standard MRI equipment. Summarizing, QUEST MRI is a powerful noninvasive approach with unprecedented potential for (i) bridging antioxidant treatment in animal models and patients, (ii) identifying tissue subregions exhibiting oxidative stress, and (iii) coupling oxidative stress localization with behavioral dysfunction, disease pathology, and genetic vulnerabilities to serve as a marker of susceptibility.

Keywords  
Brain; Reactive oxygen species; Free radical; Oxidative stress; Retina
1. Introduction

Oxidative stress is defined as an uncontrolled and sustained production of reactive oxygen species (ROS) that overwhelms endogenous antioxidant defenses producing damage to many parts of cells, such as their carbohydrates, DNA, lipids, and proteins. This damage triggers inflammation and age- or pathology-related declines in cell function that ultimately leads to many fatal or debilitating diseases (Fig. 1) [1]. The range of oxidative stress-based morbidities is vast and includes (but is not limited to) cardiovascular disease, diabetes and its complications, cancer, inflammation, neurodegenerative diseases (e.g., Alzheimer's and Parkinson's diseases), traumatic brain injury, and severe mental disorders such as schizophrenia. Critically, early antioxidant treatment reduces oxidative stress and can modify the course of aging and progression of many diseases [for example, [1,2]]. Typically, experimental studies use relatively unambiguous ROS assays, including electron paramagnetic resonance (EPR) [3], dynamic nuclear polarization-magnetic resonance imaging (MRI, i.e., proton-electron double MRI or overhauser-enhanced MRI) [4], immune-spin trapping MRI [5], dichlorofluorescein or dihydroethidium (DHE) fluorescent staining of histologic sections, high-performance liquid chromatography (HPLC), or abnormally high levels of ROS (i.e., oxidative damage) measured as changes in gene expression, DNA, RNA, lipids, and proteins. These methods have been previously reviewed and are not the focus of this perspective [for example, [6]].

It is highly desirable to measure the unregulated and continuous production of abnormally high levels of ROS in patients to improve real time diagnosis early in the course of disease, to better predict progression, and to advance treatment efficacy. Unfortunately, achieving these goals is not currently possible. The above methods used in preclinical studies are not readily translatable into patients because they measure ROS in post-mortem tissue, or require, for example, non-standard MRI equipment. This major technological “blind spot” has greatly limited bench-to-bedside translation of promising therapies. Instead, clinical metrics evaluate blood or cerebral spinal fluid biomarkers of oxidative stress [7]. These “wet indices” contain no spatial information making them inadequate for interrogating specific localized regions of oxidative stress that underpin the beginning of disease. As a result, clinicians have to work with incomplete information and make educated guesses regarding antioxidant dose, timing, drug combinations, and whether or not the selected treatment strategy indeed reduced oxidative stress in the target tissue [8,9]. Also, clinical studies often rely on a one-antioxidant-solves-all approach that may be too simple or started too late to be effective in changing disease specific outcomes. Not surprisingly, many clinical trials find unclear medical benefits from “guesstimated” antioxidant treatments, leaving the role of antioxidant treatment uncertain.

Summarizing, poor outcomes in antioxidant clinical trials are insufficient for ruling out a pathogenic role of oxidative stress in patients, but rather highlight the need for non-invasive measurements of excessive ROS production with high spatial resolution.
2. Current oxidative stress-sensitive MR methods

The above considerations suggest that translational success of antioxidant therapy will be substantially improved by measuring pathogenic ROS levels using the same method in animals and in patients. Different MR methods have been put forward to address this problem. For example, localized MR spectroscopy can measure endogenous levels of ROS scavengers such as Vitamin C (ascorbate) and glutathione in both experimental models and in patients [10]. This approach is somewhat limited because spectroscopy (i) requires a large region-of-interest, causing an integration of signals from a vast array of cell types and preventing interrogation of the very focal regions of interest that are experiencing oxidative stress; (ii) measures only a small portion of a tissue's anti-oxidative stress defenses leaving unevaluated many other essential scavengers of pathogenic ROS, such as copper/zinc-containing superoxide dismutase in the cytosol, manganese-containing superoxide dismutase in the mitochondria, α-lipoic acid in the mitochondria, and catalase in peroxisomes; and, critically, (iii) does not measure the production of excessive ROS from sources such as mitochondria, xanthine oxidase, or NADPH oxidase (Fig. 1).

Susceptibility weighted imaging (SWI) evaluates tissue content of metals, such as copper and non-heme iron, that may be used as a possible oxidative stress biomarker since these metals are a potential sources of ROS via the Fenton reaction (Fig. 1) [11–13]. SWI measures local field inhomogeneities thought to arise primarily from ferritin iron and other paramagnetic substances. However, many regions that accumulate these elements do not generate excessive free radicals, making a positive reading on SWI insufficient for identifying a region experiencing oxidative stress [14]. In addition, antioxidant therapy usually scavenges abnormally high levels of ROS production without changing copper or iron levels, so that SWI is unable to address the problem of evaluating treatment efficacy.

Summarizing, conventional MR indices are insensitive to – and provide equivocal indices of – oxidative stress at the cellular level, and are not well suited for evaluating antioxidant treatment efficacy.

3. Indirect QUEnch-assiSTed (QUEST) MRI

The above problems motivated the search for better MR metrics of oxidative stress. One promising approach takes advantage of the fact that oxidative stress produces substantial downstream changes in, for example, ion regulation. This suggested the hypothesis that functional MRI indices of ion homeostasis can be used to detect the presence of oxidative stress if measured before and after acute antioxidant treatment (i.e., a quench). [15–21]. For example, many anti-oxidant approaches correct early diabetes-induced MRI functional abnormalities in the retina (summarized in Table 1 of [22]), and in several other studies [23–27]. In one example, apparent diffusion coefficient (ADC) MRI data measures ion regulation-dependent light-evoked expansion of the extracellular space surrounding the outer segments of rod photoreceptors in mice in vivo (Fig. 2) [28]. Notably, this light-stimulated expansion is absent in diabetic mice, and is corrected by the antioxidant α-lipoic acid systemically injected just 30 min before placing the mouse into the MRI machine (Fig. 2)
These data demonstrate oxidative stress in the outer retina of diabetic mice confirming \textit{in vivo} results from \textit{ex vivo} assay's [29].

Summarizing, QUEST MRI is a powerful approach for detecting (with high spatial resolution) oxidative stress based on its negative impact on function and correction with an antioxidant.

4. Moving beyond detection

The above QUEST MRI paradigm is useful for evaluating antioxidant treatment efficacy in disease \textit{in vivo} but is limited to use only in regions demonstrating oxidative-stress-induced dysfunction and then, only indirectly regarding whether oxidative stress is present or not (i.e., its incidence) but not how much oxidative stress is present (i.e., its severity). To better map the spatial distribution of oxidative stress severity, a more direct measure of endogenous ROS levels is needed. Intriguingly, ROS are inherently paramagnetic, suggesting a quenchable contrast mechanism [30–32]. On the other hand, it is often argued that this contrast mechanism is not measureable because ROS have very short lifetimes ($\mu$s), and modest relaxivity based on that of stable free radicals ($\sim 0.17 \text{ mM}^{-1} \text{s}^{-1}$) compared to Gd-DTPA or manganese [4]. Instead, many labs have focused on prolonging and amplifying the endogenous ROS signal with exogenously administered, stable free radicals (e.g., mito-tempo) providing redox sensitive contrast [33,34]. This approach has been useful in animal studies, yet its potential application in patients is uncertain because exogenous MRI redox contrast agents are non-FDA approved, have difficulty crossing blood-brain barriers, require careful attention to timing based on their pharmacokinetics, and can change the environment being studied [5,33,35–40]. For example, mito-tempo is an antioxidant [41].

5. Key insights regarding the detection of excessive endogenous ROS using MRI

Here, we present new insights for MR detection of ROS by re-examining the above assumptions in the special case of excessive endogenous ROS production (i.e., oxidative stress). To start, remember that oxidative stress is defined as an uncontrolled production of a very large number of ROS in a sustained manner. Although the lifetime of any particular ROS free radical is not long enough to be detected by standard MRI, sustained ROS production is staggered (i.e., not synchronized), raising the possibility that there is a net residual level of paramagnetic ROS present at any given moment. We further speculate that during oxidative stress net ROS levels remain high enough for long enough to influence surrounding water relaxation such as $1/T1$ (i.e., $R1$) or $1/T2$. In addition, production of one species of ROS typically leads to generation of other ROS species (Fig. 1). This free radical cascade can amplify ROS detection sensitivity since paramagnetic contrast is additive, and relaxation rates, such as $R1$, reflect a summed contribution from all endogenous ROS species, each with potentially different relaxivities [3,42]. Unfortunately, the relaxivity of endogenous ROS has not been well studied. Nonetheless, it appears that they are substantially greater than previously thought based on work involving stable free radicals [43]. For example, a recent report claimed a remarkable 8-fold increase in magnitude between the T1 relaxivity of endogenous ROS produced in an egg white phantom, and that
of Gd-DTPA [31]. The reason for this surprisingly large difference in T1 relaxivity is unclear, and more work is needed to confirm and understand the nature of this dramatic improvement. It remains to be seen if different species of ROS have different relaxivities.

Summarizing, during oxidative stress previous assumptions that argue against R1 detection of continuous production of endogenous ROS appear to be incorrect.

6. Limitations of using R1 alone to detect excessive ROS

Interpreting an increase in relaxation rates in terms of abnormally high endogenous ROS levels alone is problematic because these rates can be increased by non-ROS factors, such as changes in oxygen content [44], water content [45], or flow [46]. To our knowledge, the few studies that have investigated these factors do not find evidence that they are major confounds. For example, in a model of brain anoxic-anoxia hyperoxia treated animals, T2 changes occurred but without substantial changes in water content suggesting that the changes were due to excessive production of free radicals [30]. Nonetheless, it is important to provide confidence that a change in relaxation rate is due to an uncontrolled generation of ROS. This can be achieved using the above QUENCH MRI paradigm. For example, a preliminary report that studied liver R1 of NADH:ubiquinone oxidoreductase iron-sulfur protein 4 homozygous knock-out mice found evidence that oxidative stress could be detected if R1 was measured before and up to a week after antioxidant treatment [47]. Since QUEST images are obtained soon after antioxidant administration, ROS reduction is likely to occur faster than water content can change. In this case, QUEST MRI would be heavily weighted toward a measurement of ROS; more work in this area is needed to test this hypothesis.

Summarizing, during oxidative stress, QUEST MRI appears useful for unmasking a ROS contribution to an increased relaxation rate, such as R1.

7. Biophysical validation of direct QUEST MRI and its assumptions

To test whether R1 ± acute antioxidant measures uncontrolled and continuous production of ROS, we examined the xanthine/xanthine oxidase reaction in a simple phantom experiment [48–50]. Xanthine/xanthine oxidase is commonly used to generate a sustained production of superoxide free radicals in vitro [48,51]. We first confirmed (absorbance spectroscopy) that superoxide levels were constant over a 20 min examination period. Consistent with this, as shown in Fig. 3, a steady ∼6% increase over baseline R1 values was observed [50]. This R1 increase suggest that production of superoxide free radical dominated the R1 change. The addition of the superoxide free radical scavenger superoxide dismutase (SOD) to the phantom prevented the R1 increase, thus validating the QUEST MRI paradigm (Fig. 3) [49,50].

Summarizing, results from a simple phantom demonstrate for the first time, unambiguous measurement of sustained ROS production via its net detectable impact as an effective paramagnetic R1 contrast mechanism in the absence of a spin trap contrast agent and potentially confounding variables that are present in vivo [4,47,50,52–54].
8. Detection sensitivity of direct QUEST MRI

We next asked if the ROS concentration generated in the xanthine/xanthine oxidase phantom and detected by QUEST MRI was comparable to that in vivo during oxidative stress, which has been measured to be in the range of 13–76 μM [3,42]. A simple calculation based on enzyme activity and substrate concentration suggested that the xanthine/xanthine oxidase phantom produced a steady state superoxide free radical concentration of ~60 μM. This calculation is somewhat uncertain since a variable level of superoxide is produced depending on the oxygen content of the solution and enzymatic activity of a particular batch of xanthine oxidase [48,51,55]. Also, converting the 6% change back to a concentration was not feasible because the relaxivity of superoxide radicals is not known. In addition, differences in viscosity between a simple aqueous solution and the intracellular environment in vivo are anticipated to increase ROS relaxivity, further potentially complicating accurate estimation of the ROS concentration produced [43]. Nonetheless, paramagnetic ROS has a linear relationship with the percent change in R1 raising the possibility that ROS concentrations as low as 10 μM (i.e., a 1% change) can be measured using QUEST MRI although more work is needed to validate this conjecture.

Summarizing, QUEST MRI appears to have adequate sensitivity to detect in vivo oxidative stress-level ROS concentrations.

9. Validating QUEST MRI in vivo

The above theoretical, biophysical, and detection sensitivity considerations all support attempts to directly measure excessive endogenous ROS production in vivo using QUEST MRI. At first, we tested the reproducibility of a simple 2D progressive saturation recovery measurement of R1 in 2 mo control mouse groups [53]. Surprisingly, mice studied on the same day had R1 measurements that were largely similar in all regions-of-interest; in contrast, mice studied on different days had R1 that could vary substantially (vide infra). Thus, in the initial studies, day-to-day variability was minimized by measuring R1 from both control mice and experimental mice on the same day. Then correction factors were calculated to adjust the mean control R1 values of the same-day controls to a reference set of control R1 values. These correction factors were then applied to the experimental data from that day.

To test this approach in vivo, we first examined in vivo rod photoreceptor cells and retinal pigment epithelium from sodium iodate (SI)-treated mice [22]. SI is a selective toxin of retinal pigment epithelium that causes outer retinal oxidative stress before later degeneration of photoreceptors [53]. In the SI model, retinal ROS (measured using a gold standard lucigenin assay of superoxide production), and outer retina-specific R1 values were both significantly greater than normal and corrected to baseline with a combination of methylene blue (an alternate electron transporter that effectively inhibits superoxide generation by mitochondria) and α-lipoic acid therapy (a potent free radical scavenger endogenously found in the mitochondria) (Fig. 4) [53,56]. In two other “proof-of-concept” experiments using the correction factor approach agreement was also found between gold standard oxidative stress assays and QUEST MRI in diabetic mice (before the appearance of vascular
histopathology), and in retinal pigment epithelium Mn-SOD knockout mice (data not shown) [53].

Summarizing, QUEST MRI is a feasible method for noninvasively measuring pathologic production of ROS in retinal tissue in vivo.

10. Direct QUEST MRI

In a clinical setting, the above between-subject correction factor method will not be useful since, unlike mice, each patient has a unique medical history. Instead, a within-subject design is best in which each patient acts as their own baseline before giving the antioxidant. To minimize imprecision on a day-to-day basis, we considered both (i) slice bias in the 2D data, and (ii) low signal-to-noise ratio at the smaller TR values as important factors in making the T1 estimate highly dependent on the signal intensity of the TR 150 ms image. Based on a suggestion by Dr. Mark Haacke, we investigated normalizing the T1 data set to the TR 150 ms image in order to reduce the slice bias and produce a more precise estimate for T1. To do this, we first smoothed the lowest signal-to-noise ratio image (e.g., TR 150 ms in our progressive saturation studies) with a 3 × 3 Gaussian filter 3 times to minimize noise and emphasize signal. This smoothed image was then divided into the rest of the images in that T1 data set. In preliminary experiments (data not shown) this procedure indeed minimized the day-to-day variation in the R1 profile of simple control mice, largely abolishing the need for a same-day group of control mice and a reference set of control mice [57].

We tested this normalization approach in different models of oxidative stress. In a retina study, an intraperitoneal line containing either saline or α-lipoic acid was secured in place and T1 data sets collected before and ~30 min post injection into control or SI-treated mice [57]. As expected, control mice did not show a change in R1 profiles through the retina from baseline following saline injection (data not shown). However, SI treated mice showed a significant transretinal decrease after α-lipoic acid injection, but not saline, indicative of oxidative stress (Fig. 5). Moreover, SI-treated C57Bl/6 mice retinas had significantly more oxidative stress than SI-treated 129S6 mice (Fig. 5). QUEST MRI revealed that this difference was largely due to the retinal region of highest mitochondrial concentration [58], the inner segment layer, where R1 was responsive to α-lipoic acid in C57Bl/6 mice but not in 129S6 mice (Fig. 5) [57]. Importantly, agreement was found in the strain-specific severity of oxidative stress measured by QUEST MRI and a gold standard ex vivo method. Identifying the mechanisms underlying genetic vulnerabilities to oxidative stress is expected to help in understanding the pathogenesis of disease.

Summarizing, endogenous ROS can be readily detected by MRI in vivo in neuronal tissue with oxidative stress [31,47,54, 56,57,59]. Robust within-subject detection of R1 reduction by antioxidants is possible using a simple post hoc image analysis procedure that allows measurement of incidence and severity of oxidative stress in a protocol that anticipates future human studies.
11. Which ROS species are measured by QUEST MRI?

We hypothesize that QUEST MRI measures the summed contribution of all species of continuously produced ROS in vivo (Fig. 1) although this idea requires further testing. Future studies are envisioned in which the contribution of each ROS source (e.g., mitochondria, peroxisome, nicotinamide adenine dinucleotide phosphate oxidase) or ROS species (e.g., superoxide, peroxide, nitric oxide) is selectively targeted with a combination of targeted AO therapies and genetically modified animal models. Nonetheless, an integrated readout of ROS burden is viewed as an advantage to ensure antioxidant targeting of as many ROS species as possible in different diseases processes, and to best monitor and treat how ROS species vary over time.

12. Measuring R1 in patients

Moving QUEST MRI into clinical studies will likely depend on fast and precise T1 measurements. Several methods that address this problem are available but each has its pro’s and con’s, and more work is needed to identify the most promising methods for QUEST MRI [60–63]. At present, variable flip angle methods that correct for radio-frequency transmit and receive variations appear promising for achieving the goal of detecting differences after scanning a given individual before and after antioxidant administration. For example, scanning the same person 10 times with STAGE produced an error of ~3% measured over about 100 pixels (or 0.3% per voxel) [60].

Summarizing, new methods have the potential to precisely measure changes in R1 of 6% or less and thus achieve an important design goal for performing QUEST MRI in patients in future studies.

13. Overall perspective

QUEST MRI provides a transformational in vivo paradigm for assessing oxidative stress without an exogenous contrast agent in order to analytically measure how and when antioxidant treatment efficacy is best achieved during emerging disease. This new tool enables earlier evaluation of disease progression and anti-oxidant treatment efficacy than is currently possible with conventional methods. Measuring excessive ROS production and functional consequences using an endogenous contrast mechanism is anticipated to greatly facilitate translation of QUEST MRI into patients in the near future using quantitative T1 techniques.

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Fig. 1.
Major sources of the ROS cascade involving superoxide (\(O_2^{•−}\)), nitric oxide (NO\(•\)), hydrogen peroxide (\(H_2O_2\)), peroxynitrite,(ONOO\(^{−}\)) and hydroxyl radicals (OH\(•\)); NOS, nitric oxide synthase; SOD, superoxide dismutase (modified from [64])
Fig. 2.
Oxidative stress detection using functional ADC MRI. (A) Summary of central retinal ADC with retinal depth during dark (closed symbols, n = 23) and light (open symbols, n = 23) in untreated mice (WT). Approximate location of retinal layers is indicated (dotted lines and OCT). Profiles are spatially normalized to retinal thickness (0% = vitreous/retina border, 100% = vitreous/choroid border). Horizontal line, P < 0.05. B) Summary of paired data (filled = dark, open = light) of WT (n = 23), diabetic mice (STZ, n = 9), diabetic mice treated acutely with the anti-oxidant α-lipoic acid (STZ + ALA, n = 8) (modified from [28]).
Fig. 3.
Detection of sustained production of free radicals by QUEST MRI in vitro. Time course of 1/T1 from phantoms containing xanthine oxidase (black circles, n = 3) before (left of arrow, baseline) and after (right of arrow) in-magnet addition of xanthine (arrow), or containing xanthine oxidase and SOD (open squares, n = 3). Horizontal bar indicates significant difference range; error bars ± SEM (modified from [50]).
Fig. 4.
QUEST MRI measurement in vivo of outer retina oxidative stress. (A) Retina superoxide production measured ex vivo from dark-adapted controls (C, black, n = 10); sodium iodate–treated mice (IO3, green, n = 6); IO3 mice treated with AO (IO3 + AO, red, n = 6).
*Significant difference (P < 0.05). (B) QUEST MRI profiles measured in vivo from dark-adapted controls (black, n = 30); IO3 mice (green, n = 7); IO3 + AO mice (red, n = 9).
Optical coherence tomography images (control vs. IO3) show mostly unchanged laminar spacing within the retina; dashed vertical lines map outer plexiform layer (48%) and retina/choroid boundary (100%) onto MRI profiles; MRI insert shows regions studied (white boxes); visual inspection of each group’s MRI does not allow for easy appreciation of differences in the derived parameter 1/T1 and so only a representative image is presented.
**Retinal depth range with significant difference (P < 0.05). Adjusted 1/T1 data at each depth used factors that normalize same-day controls to a control reference data set (modified from [53]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5.

QUEST MRI measurements of SI-treated B6 mice. (A) SI-treated B6 (black, n = 8) and SI-treated B6 + ALA (red, n = 8), and (B) SI-treated S6 (black, n = 4) and SI-treated S6 + ALA (red, n = 4) groups. Representative OCT image (above [A]) illustrate laminar spacing within the retina; layer assignments (GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, rod inner segment layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, rod outer segment layer) are as reported previously. Dashed vertical lines map OPL (42%) and retina/choroid boundary (100%) onto MRI profiles (below). Retinal depth range with significant difference (P < 0.05). Each 1/T1 data set was normalized to its TR 150 ms image (“Normalized 1/T1”). (from [57]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)