Research article

NMR IN BIOMEDICINE

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^{1}H nuclear magnetic resonance (NMR) as a tool to measure dehydration in mice

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Dehydration is a prevalent pathology, where loss of bodily water can result in variable symptoms. Symptoms can range from simple thirst to dire scenarios involving loss of consciousness. Clinical methods exist that assess dehydration from qualitative weight changes to more quantitative osmolality measurements. These methods are imprecise, invasive, and/or easily confounded, despite being practiced clinically. We investigate a non-invasive, non-imaging ^{1}H NMR method of assessing dehydration that attempts to address issues with existing clinical methods. Dehydration was achieved by exposing mice ($n = 16$) to a thermally elevated environment (37 °C) for up to 7.5 h (0.11–13% weight loss). Whole body NMR measurements were made using a Bruker LF50 BCA-Analyzer before and after dehydration. Physical lean tissue, adipose, and free water compartment approximations had NMR values extracted from relaxation data through a multi-exponential fitting method. Changes in before/after NMR values were compared with clinically practiced metrics of weight loss (percent dehydration) as well as blood and urine osmolality. A linear correlation between tissue relaxometry and both animal percent dehydration and urine osmolality was observed in lean tissue, but not adipose or free fluids. Calculated R^2 values for percent dehydration were 0.8619 (lean, $P < 0.0001$), 0.5609 (adipose, $P = 0.0008$), and 0.0644 (free fluids, $P = 0.3445$). R^2 values for urine osmolality were 0.7760 (lean, $P < 0.0001$), 0.5005 (adipose, $P = 0.0022$), and 0.0568 (free fluids, $P = 0.3739$). These results suggest that non-imaging ^{1}H NMR methods are capable of non-invasively assessing dehydration in live animals. Copyright © 2015 John Wiley & Sons, Ltd.
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Keywords: NMR; ^{1}H; dehydration; mice; multi-exponential; relaxometry

INTRODUCTION

Dehydration can lead to decreased cardiovascular function, disrupted thermoregulation, and hospitalization (1). The etiology of dehydration varies, and includes exercise without proper fluid replacement and scenarios such as sickness or reduced thirst perception, in the elderly for example. There are roughly half a million annual hospitalizations directly related to dehydration and innumerable affected individuals not needing or seeking clinical care (2). Manifestations are broad and can be as benign as thirst and dry mouth or as severe as renal failure or hypovolemic shock. Not all incidences require immediate clinical care. A direct impact on physical and cognitive performance, quality of life, and hospitalization prevention can, however, be made by diagnostic guidance towards proper fluid intake and fluid status awareness.

Dehydration is resultant from a physiological cascade of water movement in and between multiple bodily compartments. This redistribution of water can be arranged into three major compartments: intracellular, extracellular, and intravascular. It has been empirically demonstrated that intracellular and extracellular water contributes the most to overall water loss, whereas intravascular water is typically less affected to preserve cardiac output and critical organ function (3,4). Tissue and organ level stratification also exists. It has been shown that more highly hydrated tissues, such as muscle and skin, contribute greater amounts of water towards overall bodily water loss compared with those tissues that are less hydrated, such as adipose tissue (3).

Dehydration is not easily characterized clinically. The mild to moderate states that precede critical hospitalization are especially challenging. There are numerous qualitative and quantitative clinical methods aimed at assessing dehydration that suffer from being imprecise, invasive, and/or being easily confounded (5). Thirst is one such qualitative metric that most individuals experience. There is a well-documented phenomenon known as "voluntary dehydration" that often occurs during physical and/or environmental stress. The physiological responses to exercise and temperature dampen the body's thirst regulation, and the amount of fluid intake triggered by thirst is at a deficit to what is actually lost (6). Certain populations, such

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Abbreviation used: *DIA, dehydration induced anorexia.*

as the elderly or those with hypothalamic lesions, have decreased thirst perception. Thirst is therefore often not sufficient to stimulate a full recoup of total fluid loss and it is difficult to translate a subjective thirst level into an objective measure of dehydration. Other simple qualitative metrics include probing an individual's level of consciousness or skin turgor, where decreased responsiveness and increased skin tenting may be indicative of dehydration (1). These methods are rapid and non-invasive but can be highly imprecise. More quantitative approaches, such as plasma and urine osmolality measurements, can be more diagnostic of dehydration in a controlled setting. They are, unfortunately, easily confounded by factors such as food and fluid intake. There are instrumentation-based methods undergoing evaluation such as electrical bioimpedance methods but these have yet to be fully vetted and accepted in the clinic (7). No standalone method can reliably and unambiguously inform states of dehydration. The aforementioned measurements must typically be made in conjunction with one another for diagnostic cross-validation (5,8).

The majority of these methods are widely available for clinic and field use. Their shortcomings demonstrate a need for a hydration state measurement that unambiguously informs fluid therapy. We have performed an investigational study using non-imaging and non-invasive ^{1}H NMR to track fluid changes in a murine model of thermal dehydration.

Physiologic NMR measurements of water content are not new. *Ex vivo* experiments dehydrating rat muscle demonstrated a linear relationship between NMR signal and percent dehydration (9). Non-invasive MRI studies have also shown hydration dependent changes in NMR signals of rabbit kidneys and use as a diagnostic tool for brain edema in humans (10,11). More advanced spectroscopic imaging modalities have also been used to assess neurochemical profile shifts in models of dehydration induced anorexia (DIA) (12,13). These studies have assuredly demonstrated the utility of NMR in assessing tissue hydration; however, their shortcomings include imaging methods that are impractical for routine use, indirect measurements of dehydration, or measurements made on post-mortem tissues that ignore the complex interactions seen in a dynamic *in vivo* system.

Non-invasive NMR dehydration studies that do not utilize *in vivo* imaging are lacking. One indication of such NMR as a tool for dehydration came from a recent study investigating body composition analysis in rats. The authors reported that the hydration state of their animals had a noticeable effect on body composition estimates, especially for lean tissue (14). Our group has also provided evidence that non-invasive and non-imaging NMR is capable of detecting hydration state changes through an extended water depravation mouse model. We demonstrated a correlation between weight losses believed to be mostly associated with dehydration and NMR signal changes for whole body and muscle tissue measurements (15). This previous study was useful for demonstrating a general proof of concept trend. The study was, however, lacking in several areas. An issue of NMR signal changes not due to water loss, potentially due to metabolic or catabolic losses that may ensue during such long-term water restriction, was not fully addressed. Another issue was the use of a single-exponential regression for whole body NMR data when a multi-exponential fit would have been more suitable. The study described below uses an acute thermal dehydration model. The nature of this model is ideal because it allows for cross-validation with clinically used methods that are typically unreliable in uncontrolled conditions (5).

METHODS

Animals and dehydration protocol

All animal work was reviewed and approved by the Massachusetts Institute of Technology Committee on Animal Care. Female, Swiss Webster mice (6–8 weeks old, 24–28 g, Charles River Laboratories, Wilmington, MA, USA) were used for all experiments. Animals had *ad libitum* access to food and water prior to initial baseline measurement, after which food and water were restricted for the remainder of the experiment. A total of 16 animals were used for this study.

Whole body NMR measurements were made using a 0.18 T benchtop relaxometer (LF50 BCA-Analyzer/minispec mq7.5, Bruker, Billerica, MA, USA). Animals were not anesthetized and were placed in the provided Bruker measurement chamber, which utilized a plunger mechanism to restrict excessive animal movement. A standard Carr–Purcell–Meiboom–Gill pulse sequence was used for all measurements (T_R 1000 ms; T_E 1 ms; no of echoes 2000; no of scans 4). Measurements for each time point were made in triplicate. Each point in the figures represents a single animal. Control ($t = 0$) animals were removed from the Bruker instrument for roughly 30 s between baseline and final measurements. Dehydration animals were transferred to a 37 °C thermally regulated environment following a baseline measurement (Heracell 150i, Thermo Scientific, Waltham, MA, USA) to undergo dehydration for up to 7.5 h. This thermally controlled environment was open to air and kept at an ambient humidity. Weight loss was assessed every 30 min, and weight loss due to excrement was also recorded. Animals were removed from the thermal environment after a sufficient amount of weight change had occurred (up to ~13%). The mice were then kept in a normothermic environment for half an hour before post-dehydration measurements were made for thermal equilibration. Dehydration percent was calculated as non-fecal weight loss divided by initial animal weight multiplied by 100.

Animals were euthanized using standard CO_2 procedures after measurements were made. Blood and urine were then collected for osmolality (model 3250 osmometer, Advanced Instruments, Norwood, MA, USA) and NMR assessment. Blood and urine volumes ranged from 200 to 500 μL. Lean and adipose tissues were also excised post mortem for *ex vivo* NMR measurements. Lean tissue was taken from the hind limbs. Adipose tissue was retrieved from the lower intraperitoneal space. Lean and adipose samples were roughly 2–4 g. There were time delays of 5–15 min between animal euthanasia and tissue sample measurement.

Multi-exponential analysis

Relaxometry analysis was performed using a multi-exponential fit method using the following equation:

$$A_{\text{total}} = A_1 e^{\left(-\frac{t}{T_{2,1}}\right)} + A_2 e^{\left(-\frac{t}{T_{2,2}}\right)} + A_3 e^{\left(-\frac{t}{T_{2,3}}\right)} + A_4 e^{\left(-\frac{t}{T_{2,4}}\right)}$$

in which A_x is the individual component amplitude, t is time, and $T_{2,x}$ is the relaxation time of each component. Analysis was performed using custom software written in MATLAB (MathWorks, Natick, MA, USA). The number of terms in the model was determined based on specific tissues of interest and the number of physiological compartments that the Bruker software reported. This software was used as a starting point, because the primary commercial purpose of the relaxometer/software combination

is body composition analysis (namely lean, adipose, and free fluids). This type of analysis falls in line with our end goals. It is unclear what specific algorithms Bruker used, as they are considered proprietary and we did not have the ability to directly investigate or alter them. Custom multi-exponential fitting methods were preferred to the commercial software because we were able to fully understand and customize such methods. Linear regression parameters were calculated using STATA (StataCorp LP, College Station, TX, USA).

Comparison between single-exponential modeling of discrete tissues and whole body measurements yielded the best correlation with a four-exponential model, in which we were able to capture lean tissue, adipose, and free fluid compartments (Table 1). It should be noted that the defined physiological compartments are approximations for fundamental physical compartments that have uniform relaxivity. Physiologically, the majority of what is considered lean tissue is skeletal muscle. We were not able to empirically establish the fourth component in our model. There was, however, no trend seen with dehydration and the fourth component was deemed unimportant.

Relative amplitudes from the multi-exponential fittings were also compared with published empirical values to further validate our analytical methods. We were able to gender match our values with published data on female Swiss Webster mice, but were unable to age match. Relative amplitudes were obtained by dividing the calculated amplitude for a specific physiological compartment by the sum of all amplitudes and multiplying by 100. The literature unfortunately did not present errors on data and these are hence missing from our analysis.

There did not exist literature that directly provided information on fluid percentages for Swiss Webster mice to compare with our calculated values. Fluids were broken down into two major constituents, blood and urine. Calculations were performed using generalized mouse values found in literature. Blood volume was calculated by multiplying animal weight by 72 mL/kg (16). We did not perform density compensation because blood and water are nearly equivalent (1060 kg/m^3 for blood versus 1000 kg/m^3 for water). Urine was calculated by assuming a full bladder as 0.15 mL (regardless of animal weight) (17). We did not take urine production into account. Total free fluids were calculated by summing blood and urine percentages. It is important to note that our experimental free fluid value was calculated from our multi-exponential method and not as a sum of calculated blood and urine values.

Table 1. Comparison between discrete single-exponential tissue T_2 NMR measurements and multi-exponential fitted whole body measurements using a four-exponential model. There is correlation between lean and adipose compartments. Blood and urine categories were lumped together as "free fluids" in the multi-exponential fit to parallel the output given by the commercial Bruker software. It is reasonable that there is a high level of variability in this compartment, given that there is likely a high variance in the amount of urine between animals. The unknown component was found to have a relatively fast relaxation time. We were not able to empirically establish the fourth component in our model; however, there was no trend seen with dehydration and the term was deemed unimportant for our objectives

	Ex vivo measurement (ms)	Multi-exponential fit (ms)
Unknown	N/A	10.6 ± 1.1
Lean	54.3 ± 1.6	45.7 ± 2.4
Adipose	170.0 ± 1.9	166.6 ± 11.6
Free fluids	276.8 ± 19.8 (blood) 2770.2 ± 56.9 (urine)	677 ± 143.3

RESULTS

Multi-exponential compartment validation

Table 1 presents the relaxation times from *ex vivo* measurements of individual tissues and the multi exponential fits for whole body measurements. The three major components of interest were lean tissue, adipose, and free fluids, with an additional, unknown fourth component. The three identified physiological compartments correspond to those reported by the commercial Bruker software. *Ex vivo* measurements were analyzed using a single-exponential fit ($R^2 \geq 0.98$ for all measurements).

Multi-exponential relative compartment amplitude validation

Table 2 compares the relative amplitudes of relevant physiological compartments from our multi-exponential fit versus values obtained or derived from literature. The fourth "unknown" component was omitted from analysis because there would be no literature value to compare it with. Values for lean and adipose tissue correlate very well between our experimentally derived values and those obtained from literature. Free fluid percentage values differ by roughly twofold. The order of magnitude for free fluids in relation to the other compartments, however, is comparable between experimental and literature values.

Table 2. Comparison between relative physiological compartment amplitudes from empirical data found in literature and values derived from our four-exponential fit for female Swiss Webster mice. There is good agreement between lean and adipose compartments. Free fluids, calculated through exponential fitting, are roughly half what empirical values estimate. We posit that this is due to differences in relaxometry values in oxygenated and deoxygenated blood. There is no value for "unknown" literature values given the ambiguity of anatomic signal origin

	Unknown (%)	Lean (%)	Adipose (%)	Free fluids (%)	Calculated free fluids	
					Blood (%)	Urine (%)
Literature	N/A	64.7	22.2	8.0	7.2	0.8
Experimental	11.9 ± 1.5	57.6 ± 2.3	26.6 ± 2.8	3.9 ± 1.3		

Validation of mouse dehydration

Dehydration was assessed by measuring weight loss, and plasma and urine osmolality. Figure 1(a) demonstrates a clear linear correlation between dehydration time and percent weight loss ($R^2 = 0.9418$, $P < 0.0001$). Weight loss due fecal matter was removed from this calculation to isolate the predominant loss due to water. Exposure to the elevated thermal environment ranging from 0 to 7.5 h induced water-associated weight loss ranging from 0.14–13% of initial weight.

Figure 1(b) demonstrates an expected linear correlation between urine osmolality and percent dehydration ($R^2 = 0.9188$, $P < 0.0001$). Complementing the weight loss versus dehydration time data, this increase in urine osmolality as a function of weight loss further confirms that the animals are in fact undergoing dehydration.

Plasma osmolality, on the other hand, is held relatively constant and without correlation with dehydration level ($R^2 = 0.0010$, 0.9070) (Fig. 1(c)).

Relaxometry analysis of mouse dehydration

We were able to measure three approximated physiological compartments (which matched those reported by the commercial Bruker software), lean tissue, adipose, and free fluids, with a fourth unknown compartment that exhibited a relatively fast relaxation time. Figure 2(c), (d) shows a negative linear correlation between percent dehydration and urine osmolality and percent change in amplitude, with a range of roughly 1 to −14% change in signal ($R^2 = 0.8619$, $P < 0.0001$, and $R^2 = 0.7760$, $P < 0.0001$, for percent dehydration and urine osmolality respectively).

Figure 2(a), (b), (e), (f), and (g), (h) demonstrates weak correlation between signal amplitude for the unknown component ($R^2 = 0.0235$, $P = 0.5705$, and $R^2 = 0.6186$, $P = 0.0182$, respectively), adipose tissue ($R^2 = 0.5609$, $P = 0.0008$, and $R^2 = 0.5005$, $P = 0.0022$, respectively), and free fluids ($R^2 = 0.0640$, $P = 0.3445$, and $R^2 = 0.0568$, $P = 0.3739$, respectively) and percent dehydration and urine osmolality respectively.

Figure 3(a)–(f) demonstrates weak correlation between percent dehydration and urine osmolality and percent change in relaxation time for unknown, lean, adipose, and free fluids. Multi-exponential fit characteristics for percent dehydration and urine osmolality respectively were the following: unknown component, $R^2 = 0.1056$, $P = 0.2194$, and $R^2 = 0.0798$, $P = 0.2892$; lean tissue, $R^2 = 0.0100$, $P = 0.7131$, and $R^2 = 0.0219$, $P = 0.5843$; adipose tissue, $R^2 = 0.0006$, $P = 0.9291$, and $R^2 = 0.0083$, $P = 0.7371$; free fluids, $R^2 = 0.0086$, $P = 0.7324$, and $R^2 = 0.0215$, $P = 0.5877$.

DISCUSSION

This study demonstrates that (a) relaxation times derived from a four-exponential fit of whole body mouse NMR closely correlate with discrete *ex vivo* measurements, (b) relative amplitudes of relevant physiological compartments from a four-exponential fit of whole body mouse NMR closely correlates with relative amplitudes found in literature, and, most interestingly, (c) NMR signal amplitude changes of lean tissue closely correlate with clinical metrics of weight loss and urine osmolality in a murine model of acute thermal dehydration.

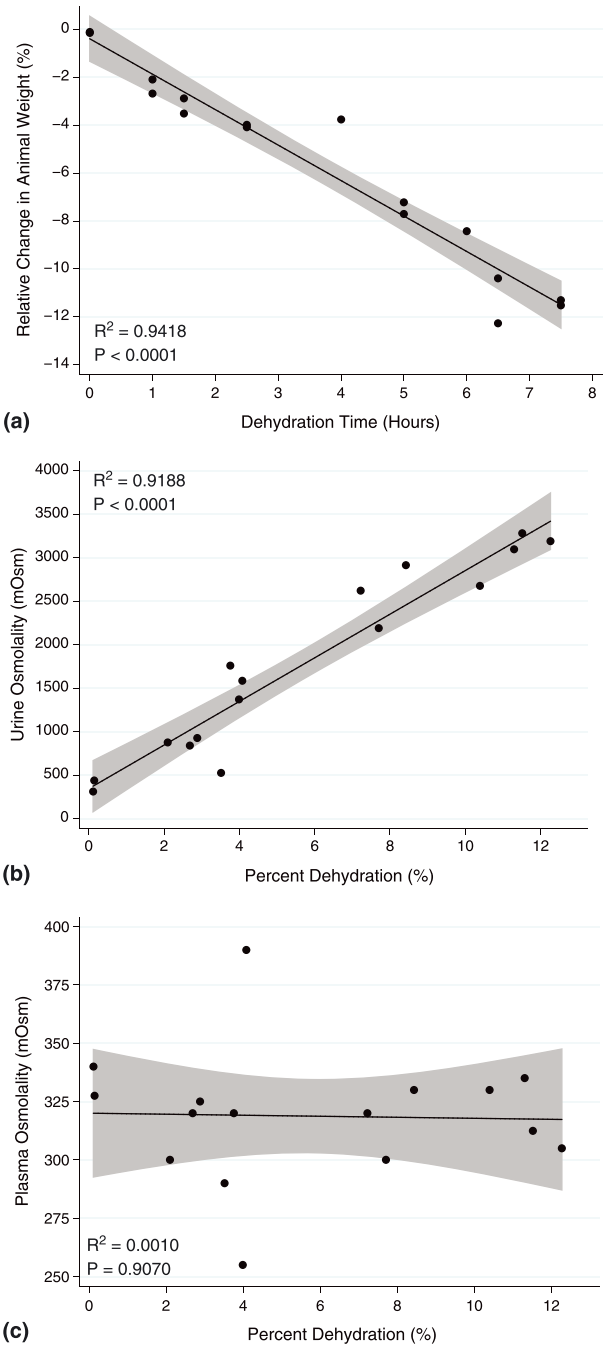

Figure 1. Body weight changes and plasma and urine osmolality as indicators for mouse dehydration. Shaded grey areas indicate 95% confidence intervals of the linear fit. (a) There is an observable increased loss of weight as a function of exposure time to the elevated thermal environment ($R^2 = 0.9418$, $P < 0.0001$). This is a preliminary indicator that the mice were indeed becoming dehydrated. (b) Urine osmolality also demonstrated a correlation with percent dehydration ($R^2 = 0.9188$, $P < 0.0001$). As an animal becomes more dehydrated, water stores in the bladder become reabsorbed in an attempt to restore lost fluids due to dehydration. (c) Plasma osmolality does not demonstrates correlation with percent dehydration ($R^2 = 0.0010$, $P = 0.9070$). Plasma osmolality is a tightly regulated homeostatic value and thus would only show significant changes under extreme and severe dehydration, a level that we did not achieve in this study. Percent dehydration was calculated as [(non-fecal weight change)/(initial weight)] × 100.

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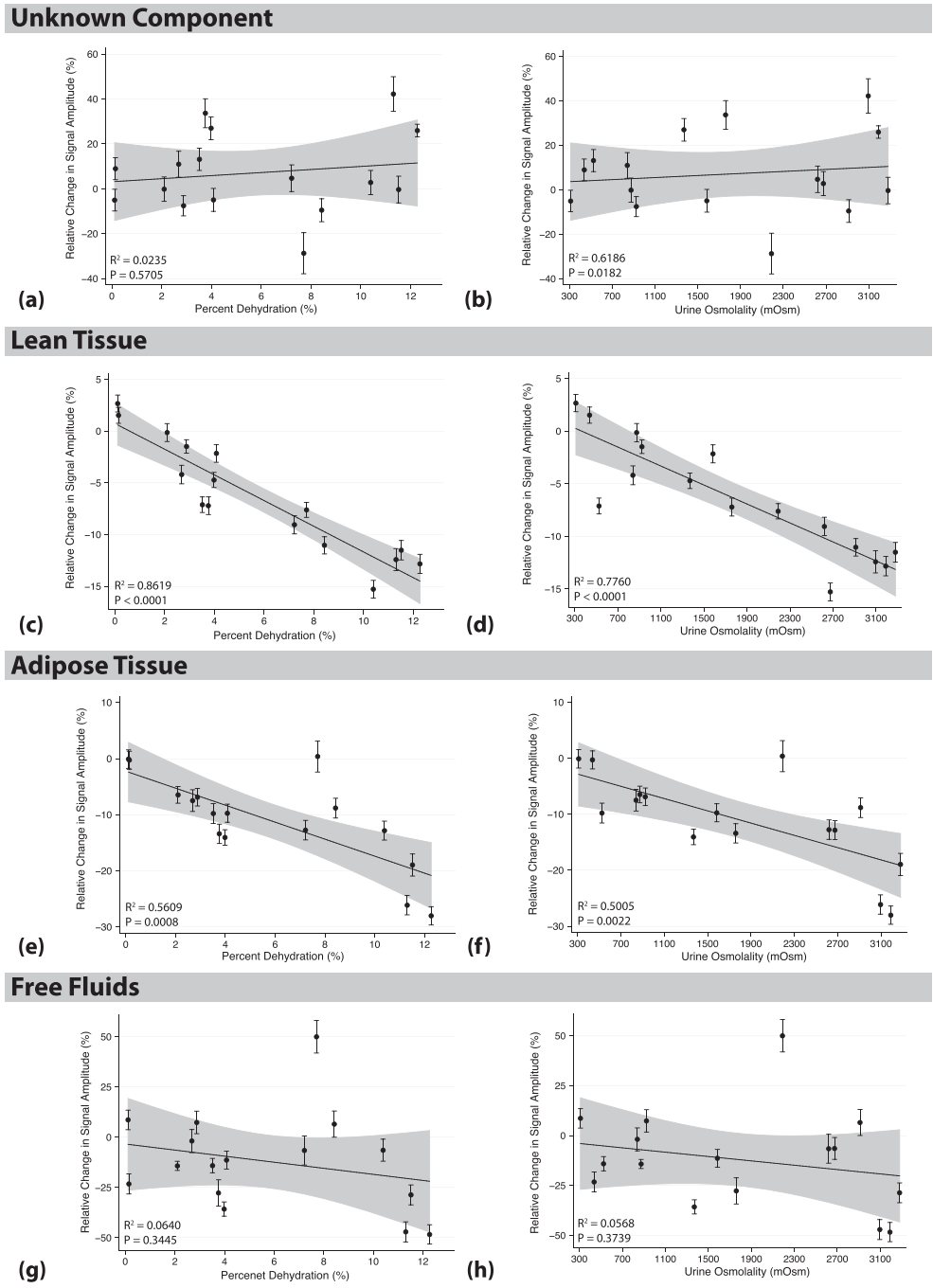

Figure 2. Multi-component NMR amplitude changes as a function of percent dehydration and urine osmolality. Shaded grey areas indicate 95% confidence intervals of the linear fit. (a), (b) There is weak correlation between the unknown component and percent dehydration ($R^2 = 0.0235$, $P = 0.5705$) and urine osmolality ($R^2 = 0.6186$, $P = 0.0182$). (c), (d) There is a clear negative linear correlation between lean tissue amplitude and percent dehydration ($R^2 = 0.8619$, $P < 0.0001$) and urine osmolality ($R^2 = 0.7760$, $P < 0.0001$). This trend is expected, since lean tissue has been reported to be a major source of water loss during dehydration. (e), (f) Adipose tissue demonstrated weak correlation between signal amplitude and percent dehydration ($R^2 = 0.5609$, $P = 0.0008$) and urine osmolality ($R^2 = 0.5005$, $P = 0.0022$). This result was not surprising, given that adipose is a minimally hydrated tissue and has shown non-significant mass loss in other studies. (g), (h) Free fluids also do not demonstrate correlation with percent dehydration ($R^2 = 0.0640$, $P = 0.3445$) or urine osmolality ($R^2 = 0.0568$, $P = 0.3739$). Lack of noticeable trends likely stems from preserved vascular volume (in which significant changes are likely only observable at severe levels of dehydration) and heterogenic amount of urine content between animals. Percent dehydration was calculated as [(non-fecal weight change)/(initial weight)] × 100. Error bars represent standard deviations from triplicate instrument measurements.

Dehydration validation

Weight change, urine, and plasma osmolality were used to assess animal dehydration. Weight loss increased proportionally to the amount of time spent in an elevated thermal environment. Quantified weight loss due to excrement was significantly less than the overall weight loss, which we attributed to water loss. Urine osmolality also increased proportionally as

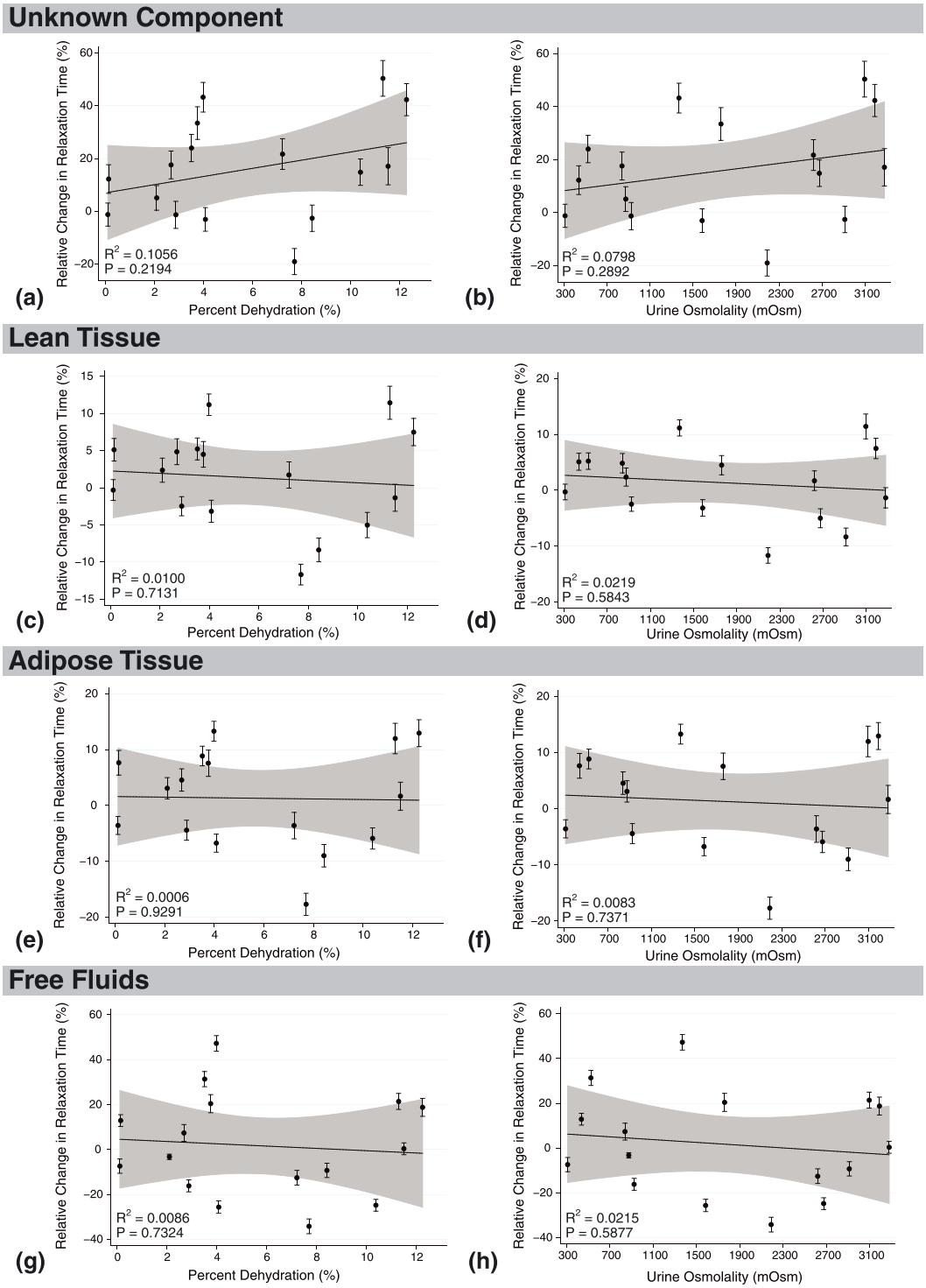

Figure 3. Multi-component NMR relaxation time changes as a function of percent dehydration and urine osmolality. Shaded grey areas indicate 95% confidence intervals of the linear fit. There is weak correlation between the unknown component ($R^2 = 0.1056$, $P = 0.2194$; $R^2 = 0.0798$, $P = 0.2892$), lean tissue ($R^2 = 0.0100$, $P = 0.7131$; $R^2 = 0.0219$, $P = 0.5843$), adipose tissue ($R^2 = 0.0006$, $P = 0.9291$; = 0.0083, $P = 0.7371$), and free fluids ($R^2 = 0.0086$, $P = 0.7324$; $R^2 = 0.0215$, $P = 0.5877$) and percent dehydration and urine osmolality respectively. Weak correlation indicates lack of intrinsic physical property changes due to dehydration. Percent dehydration was calculated as [(non-fecal weight change)/(initial weight)] × 100. Error bars represent standard deviations from triplicate instrument measurements.

dehydration ensued. As dehydration progresses, water stores, such as those in the bladder, become reabsorbed, leading to an increase in urine osmolality. Plasma osmolality, however, did not demonstrate any signs of correlation with dehydration levels. Plasma osmolality is a tightly regulated parameter, which homeostatic mechanisms attempt to keep within a narrow range (18,19). Shifts in plasma osmolality are likely only to be seen in severe cases of dehydration. Thus, it is not surprising to see a

weak correlation between plasma osmolality and dehydration level, at least within our experimental dehydration range.

Lean tissue

Lean tissue results only show changes in amplitude and not relaxation time. This indicates that water losses from lean tissue did not result in physical property changes, which would have led to shifts in relaxation time as well. Changes in lean tissue are expected. Water exodus from lean tissue inherently leads to less material being measured. This results in the observed drop in NMR signal amplitude after dehydration. Several early reports using non-NMR methods empirically demonstrated that lean tissue water content significantly contributes to overall water loss due to dehydration. Similar to the methods we described, Nose *et al*. used a thermal dehydration protocol to achieve roughly 10% dehydration measured by body weight change in rats (3). Established radiolabeling techniques and weight/dry weight measurements were used to assess dehydration associated water content change of various tissues. Significant ($P < 0.01$) changes in muscle tissue water content were observed between control animals and animals after 10% dehydration (3).

Costill *et al*. implemented human exercise protocols at an elevated temperature (39.5 °C) to induce dehydration in study participants. Overall dehydration was calculated based on study participant weight change. Wet/dry weight and chemical electrolyte extraction methods were employed on muscle biopsy samples to calculate tissue dehydration level and water distribution changes in interstitial and intracellular compartments. A correlation between overall dehydration and muscle water loss, from both interstitial and intracellular compartments, was demonstrated (4).

It is also noted that baseline ($t = 0$) signal amplitude values are actually positive and not zero. This can potentially be explained by the increase in blood flow to lean tissue caused by a fight or flight response. This response is typical during animal handling and introduction to unknown environments.

Adipose tissue

Amplitude and relaxation time NMR measurements of adipose tissue did not demonstrate a strong correlation with animal dehydration. Relaxation times showed much weaker correlation than signal amplitude. As mentioned earlier, adipose is significantly less hydrated than lean tissue with an order of magnitude less water (0.35 mL versus 3.0 mL of water per gram of dry tissue) (3). Adipose tissue has also demonstrated non-significant mass changes in control versus dehydrated rats (3). The weak correlation between dehydration level and signal changes in adipose tissue is not surprising.

Free fluids

Amplitude and relaxation time NMR measurements of free fluids did not demonstrate significant correlations with dehydration. Free fluids encompass an array of bodily fluids ranging from vasculature to interstitial fluids to urine being held in the bladder. These fluidic compartments also have a wide range of relaxation times, ranging from approximately 200 ms (oxygenated blood) to 2000 ms (urine) (20). The aggregate relaxation time is a combination of these different components. Variations in one physiological compartment, such as urine amount, may have a great effect on the overall aggregate amplitude and relaxation time and increase heterogeneity when comparing values between animals. The lack of correlation is also expected from a vascular/plasma volume perspective. Plasma osmolality is a tightly regulated parameter, which homeostatic mechanisms attempt to keep within a narrow range (10). The body aims to preserve vascular volume to maintain adequate cardiac output and blood delivery to vital organs. This results in minimal changes in vascular volume as long as sufficient tissue water stores, such as lean tissue, are available. This may also be a corollary to the consistent values of plasma osmolality observed across all dehydration levels. Shifts in plasma osmolality are likely only to be seen in severe cases of dehydration. We likely did not achieve a severe enough level of dehydration to demonstrate significant, measureable vascular changes.

Metabolic considerations

The overwhelming majority of observed weight loss is believed to be associated with dehydration related water loss and not metabolic or catabolic processes, since the dehydration process was relatively acute. Weight loss contribution from excrement never exceeded 1% of total weight loss, and such percentages were only seen in animals with longer heat exposure times. Qualitatively, it was observed that mice decreased their activity levels in the elevated thermal environment. The elevated temperature reduces the gradient between the body and environment through which an animal can cool off.

Additionally, an animal's metabolic rate should be held at a minimum. A recent report demonstrated a "thermoneutral" range of 28 °C–38 °C over which a mouse's oxygen consumption rate, associated with metabolic rate, was held at a minimum (21). This, in conjunction with the decreased metabolic demand from diminished movement, should keep metabolic weight losses to a minimum. There should also be zero to minimal catabolic events that would result in weight loss given the animal's decreased metabolic rate. The animals did not undergo a fasting period and had *ad libitum* access to food and water just prior to the initial baseline measurement.

Model validation

The close agreement between relaxation times of discrete tissue samples and values derived from multi-exponential, whole body analysis gives us confidence that any post-mortem change in tissue properties was insignificant. Multi-exponential fitted values of lean tissue and adipose fall very well within the range of *ex vivo* measurements. Free fluid values are reasonable given the broad spectrum of relaxation times encompassed by this category. NMR measurements were made within a half hour of animal euthanasia in order to minimize processes such as tissue degradation and/or dehydration. It was noted that *ex vivo* muscle was measured at a slightly higher relaxation time than the four-exponential model value. Small adipose deposits within the muscle that were not completely removed during tissue excision may account for this observation.

It should be noted that we were not able to empirically identify the "unknown" component that exhibited the fastest relaxation time. It is well known that solid materials, macromolecules (i.e. proteins), water bound to such macromolecules, and generally materials with slower molecular tumbling rates exhibit fast relaxation rates at this order of magnitude (22,23). This may manifest itself in the form of fur, cortical bone, or, as mentioned, macromolecules. If this were truly the case, such tissues would likely

not exhibit much change in relation to dehydration owing to their minimally hydrated states (3,24). We did not explore this component in depth due to the lack NMR signal correlation with clinical dehydration metrics.

We further observed good correlation between relative tissue percentages calculated from our multi-exponential fit as compared with empirical data from literature (25). We were unfortunately unable to evaluate statistical significance with full rigor due to the absence of error bounds from the literature. Our calculated values, however, demonstrate the correct order of magnitude, with values agreeing quite closely with the empirical data. We note that calculated blood volumes are a little over twice what we calculate from our multi-exponential fitting. This likely arises from the well-known influence of the paramagnetic effect of blood oxygenation levels on relaxation times (26,27). It has been shown that higher blood oxygen contents contribute to longer relaxation times and vice versa for less deoxygenated blood (26,27). This fact is relevant because roughly 60–70% of bodily blood in mammals is deoxygenated while the remainder is oxygenated (28). It is conceivable that the longer relaxation blood detected in our multi-exponential fit represents oxygenated blood while the shorter relaxation deoxygenated blood may be lumped in with adipose tissue during multi-exponential fitting. This may explain the slight amplitude overestimation of adipose tissue. This segregation between oxygenated bloods likely explains the underestimation by our multi-exponential fitting amplitude assessment since we are only assessing oxygenated blood and not the entire blood pool.

High resolution T_2 decays allowed us to reliably extrapolate contributing physiological components. There are numerous methods through which physical systems composed of multi-exponential components can be analyzed (29). More indiscriminate methods exist, but the four-exponential model worked well for our purposes with coefficient of determination values (R^2) of over 0.99 (example goodness of fit, Supplementary Fig. 1). These indiscriminate methods do not rely on *a priori* assumptions of the number of components. They are, however, more analytically challenging. We attempted a non-negative least squares optimization method; the method resolved at least four components but was inconsistent across measurements and animals.

Practical considerations

This study has demonstrated that an NMR approximation of lean tissue exhibits NMR detectable changes that correlate with dehydration. We performed whole body animal measurements with a focus on extracting relevant tissue level information. It is entirely conceivable, however, that only specific regions of physiological and NMR relevancy need to be probed in the clinic. Locations enriched for lean tissue, such as the arms and legs, may be prime targets for dehydration. There furthermore exist commercial and experimental NMR sensors that do not rely on imaging and provide analytical utility through relaxometry measurements. The NMR-MOUSE (NMR Mobile Universal Surface Explorer; Magritek, Wellington, New Zealand) is a single-sided NMR system that has been successfully used on humans (30–32). More experimental methods from the Blümler group are developing MANDHALAS (magnetic arrangement for novel discrete Halbach layout) instruments with a central bore through which one can envision placing a digit or appendage to perform a measurement (33–35). Both modalities are packaged in a portable and practical manner.

One can envision the great utility such devices may bring to the clinic. There are, however, several limitations that should be addressed. We presently discuss use of relative amplitude assessment, which require a baseline measurement. Such baselines are not always available in the clinic. Further studies will need to be performed to establish a means of making absolute measurements, perhaps through population normalization and/or use of internal standards. Hardware also needs to be taken into consideration. Obtaining good signal to noise ratio in an unobtrusive clinical instrument is non-trivial. A balance between instrument size, field strength, and sample size must be found. Effective temperature control must also be implemented to ensure that static field strength and coil frequency remain properly tuned. While these challenges exist, we do not believe them to be insurmountable. These instruments have the potential to preclude impractical whole body MRI measurements for routine hydration monitoring.

Clinical comparators

Our results revealed trends in highly relevant lean tissue (but not adipose and free fluids) that appear to correlate with an animal's state of dehydration as measured by body weight and urine osmolality. Body weight and urine osmolality are clinical tools that are valid in a controlled setting, such as the experiments herein performed. Their real world applications, however, are limited due to persistent confounding factors such as food and fluid intake. One cannot interpret results at face value without knowledge of intake. These factors may alter body weight and osmolality measurements without actually causing a change in hydration state. We believe that NMR methods are of greater utility because they are not as susceptible to such confounding factors.

Comparison with previous methods

This is one of the first reports that indicate NMR as a non-invasive tool for monitoring dehydration state in a live animal without the use of imaging and with proper clinical correlates. The work herein described differs from our previously published work in several key ways. The acute nature of the thermal dehydration ensured that physiological changes were predominantly associated with dehydration and not metabolic and catabolic processes that may have affected our previous long-term (up to 72 h) study (15). The analytical methods are also more robust in this present study. Our previous work used a single-exponential fit when assessing whole body measurements. We contrast this with a four-exponential fit in the present work, with an R^2 greater than 0.99. Whole body measurements are best represented as multi-exponential, and it is not possible to tightly fit a multi-exponential curve with a single-exponential regression. The previously reported fittings are thus statistically inferior compared with this study. Our earlier study also demonstrated whole body changes in relaxation time, whereas we show changes in signal amplitude in this present study. The fact that we previously saw relaxation time changes and now amplitude changes in our current study may be resultant from differences in analytical methods (single- versus four-exponential fits).

The previous study also directly looked at excised, post-mortem muscle tissue. What we believed to be the interstitial fluid compartment in excised muscle tissue demonstrated signal amplitude decrements that appeared to correlate with levels of dehydration. We did not use such methods in this most recent study because we were only interested in live measurements. It

is unclear what potentially confounding factors come into play post mortem. Excised tissues were also observed to rapidly dehydrate once exposed to air in our previous study. We did not want to introduce such an enormous confounder in our current studies. Another drawback of *ex vivo* analysis is that measurements on discrete tissue eliminate the complex dynamics that occurs in a living system. Even with distinct experimental differences, the general downward trends between previous muscle interstitial fluids and extracted lean tissue values from this most recent study appear to be similar, with lower amplitudes at greater levels of dehydration.

CONCLUSION

The power of the NMR methods herein described lies in the ability to probe the water content of various physiological compartments of interest. Our method, combined with advances in portable spectrometers and magnets, could offer rapid, onsite hydration state assessment to guide fluid replacement therapy in the field and the clinic. These NMR methods may also be applicable as a diagnostic for congestive heart failure and/or renal failure patients where excessive fluid accumulation occurs. Hydration status is a clearly critical clinical parameter. Validation and success of practical NMR based methods to quantitate fluid status has the potential to greatly improve quality of life and reduce healthcare burden.

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