Brief Communication: Oxygen Isotopes as a Biomarker for Sickle-Cell Disease? Results From Transgenic Mice Expressing Human Hemoglobin S Genes

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ABSTRACT The origins of sickle-cell disease (SCD) are well understood, as are its evolutionary pressures on humans and pathological presentation. However, because it has not been possible to identify SCD in archaeological contexts, its biocultural effects on past populations are unknown. Previous research investigating oxygen isotope fractionation during respiration among anemics suggests that oxygen isotopes in bone apatite may provide a biological marker for SCD in skeletal remains. This pilot study reports $\delta^{18}O$ ratios in bone apatite of transgenic laboratory mice expressing human SCD globins and compares them to healthy control mice. The $\delta^{18}O$ ratios of sick mice are significantly lower than those of healthy mice (−5.6‰ vs. −4.5‰; $P = 0.002$), and the sickest mice exhibit the lowest ratios of all (mean $\delta^{18}O = -5.8$‰). These preliminary results suggest that this method may be usefully applied to skeletal materials of past human populations whose diets and water sources do not differ substantially. Am J Phys Anthropol 145:495–498, 2011.

This preliminary investigation determines whether stable oxygen isotope ratios in bone apatite may provide a biomarker for sickle-cell disease (SCD) in mice transgenic for mutant hemoglobin S (HbS) genes. The etiology and origin of SCD are well documented. However, its presence in past human populations is difficult to assess, because it is not clearly visible in archaeological samples. Here, an isotopic method for identifying SCD in bones from transgenic laboratory mice is examined. This study is the first assessing use of stable oxygen isotope ratios to identify SCD in bone. When verified, this method may be useful for identifying SCD in human skeletal remains.

BACKGROUND

Sickle-cell disease

The sickle-cell trait is a widely cited example of environmental and cultural pressures altering human genomes. The sickle-cell allele originally was identified in West African populations, and several local variants have been identified in Asia, Africa, and the Middle East (Nagel and Fabry, 1985). Malaria is endemic in these tropical and subtropical regions of the Old World, where the parasite *P. falciparum* is transmitted by *Anopheles* mosquitoes.

Individuals homozygous for HbS typically have reduced life expectancy. Leading causes of death from sickle-cell disease (SCD) today are organ failure (especially the kidneys) and sickle crises involving congestion of irregularly shaped red blood cells in vessels, including stroke (Platt et al., 1994). Some sufferers are less symptomatic than others; the presence of circulating fetal hemoglobin is one ameliorating factor. Individuals who are heterozygous for the sickle-cell trait are resistant to malaria, do not suffer from SCD, but may have sickling crises.

Human modification of tropical landscapes by swidden agriculture over the past few thousand years encouraged expanding populations of malaria-transmitting mosquitoes. The clear-cutting of trees exposed pools of standing water in formerly forested areas to sunlight, providing an excellent breeding ground for mosquitoes. Although the sickle-cell allele reduces fertility, the perseverence of early agriculturalists using swidden agriculture provided a biocultural environment in which the sickle trait was slightly more advantageous due to the malaria resistance it conveyed (Wiesenfeld, 1967).

Apart from a reduction in fertility inferred from modern observations, other biocultural impacts of SCD in the past cannot be known without a direct way of identifying it archaeologically. In some cases, *P. falciparum* has been identified in preserved soft tissues (Bianucci et al., 2008). Cribra orbitalia and porotic hyperostosis are nonspecific indicators of anemias (Walker et al., 2009). Differential diagnosis among the different types of anemia (including sickle-cell) may be possible when postcranial skeletal remains are available (Hershkovitz et al., 1997). Because of SCD's impact on oxygen transport and multiple other pathophysiologies, it may be possible to identify the condition in skeletal remains using oxygen isotope ratios as a biomarker.

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Oxygen isotopes and sickle-cell disease

Oxygen is incorporated into tissues of small mammals from drinking water (~55%), respired gas (~30%), and food (~15%) (Podlesak et al., 2008). Several metabolic processes cause fractionation of oxygen's stable isotopes, ^{16}O and ^{18}O, during uptake by the body. Fractionation occurs, because the bonds incorporating the lighter isotope ^{16}O are weaker than bonds incorporating the heavier isotope ^{18}O and break more readily during such events as diffusion and evaporation (Hoefs, 2004). The rate at which these events occur influences the degree of fractionation: slow reactions encourage fractionation, whereas rapid reactions inhibit fractionation (Hoefs, 2004). Because ^{16}O is preferentially taken up, the $\delta^{18}O$ value of oxygen used by the body is lower than that of the atmosphere (Epstein and Zeiri, 1988).

Known sources of oxygen isotope fractionation during the respiratory cycle include diffusion across pulmonary membranes and utilization of oxygen by cells. When the rate of these reactions is compromised, changes in isotope fractionation have been observed. For example, the inhibition of oxygen diffusion across calloused pulmonary membranes was observed to increase oxygen isotope fractionation in smokers (Epstein and Zeiri, 1988). Conversely, an increased rate of respiration caused by exercise has been linked to a decrease in overall oxygen isotope fractionation (Epstein and Zeiri, 1988; Zanconato et al., 1992; Widory, 2004).

Binding of oxygen to hemoglobin is also a fractionating process: O_2 bound to hemoglobin is 0.35% lighter than O_2 previously in a gaseous state (Pflug and Schuster, 1988). Previous research demonstrated that fractionation of oxygen isotopes is lower among anemics than among healthy individuals (Epstein and Zeiri, 1988; Heller et al., 1994). In both these cases, fractionation was measured in respired air and was attributed to low hemoglobin counts among anemics. It should be noted that, in these studies, a relationship was found between isotope ratios and other forms of anemia besides SCD; therefore, oxygen isotope variations should not be narrowly associated with a single disorder and may in fact apply to other anemias, including iron-deficiency anemia.

This study explores whether a relationship exists between sickle-cell anemia and $\delta^{18}O$ ratios of bone tissue, which are closely related to the $\delta^{18}O$ of respired air (Pflug et al., 1978; Luz, 1984). The hypothesis tested is that mice suffering from SCD exhibit higher $\delta^{18}O$ ratios in their bone apatite due to reduced fractionation during respiration. Bones from mice expressing human sickle-cell transgenes are used to provide a foundation for future research among archaeological human samples.

MATERIALS AND METHODS

Laboratory mice raised in a controlled environment were analyzed to validate the proposed method. Oxygen isotope signatures in human tissues are more difficult to interpret due to variable diets and high residential mobility. Transgenic mice expressing actual human HbS genes [originally developed by Pászty et al. (1997) for sickle-cell research] were used for this study. For details on the particular transgenic mouse lines examined, see Nagel and Fabry (2003).

Hindlimbs from 24 mice were donated by the Albert Einstein College of Medicine and prepared for oxygen isotope analysis. Mice are divided into three types for analysis. Eight mice were controls expressing healthy mouse globins (Type 1), and sixteen were transgenic mice expressing human HbS genes. Of the 16 unhealthy mice, 9 "less sick" mice (Type 2) exhibited 45–90% human HbS globins and 8–55% fetal hemoglobin (Hbγ), which has ameliorating effects on SCD (Fabry et al., 2001). Seven "very sick" mice (Type 3) exhibited almost 100% human HbS with fewer than 4% Hbγ. Data for all 24 mice are presented in Table 1.

TABLE 1. Profile information and isotope data for all 24 mice

Sample ID	Mouse type	Age (months)	$\delta^{18}O_{VPDB}$ (‰)	%HbS	%Hbγ	Diet
41,527	1	7	−5.2	0	*n/d*	Control
39,592	1	8	−5.1	0	*n/d*	Control
42,128	1	4	−4.9	0	*n/d*	Control
42,067	1	4	−4.9	0	*n/d*	Control
42,069	1	4	−4.5	0	*n/d*	Control
39,593	1	8	−4.5	0	*n/d*	Control
42,127	1	4	−3.8	0	*n/d*	Control
42,031	1	5	−3.2	0	*n/d*	Control
39,122	2	10	−5.6	45.57	54.43	Sickle chow
39,746	2	10	−5.9	51.86	48.14	Sickle chow
40,252	2	3	−6.2	57.79	42.21	Sickle chow
40,210	2	4	−5.3	65.56	34.44	Sickle chow
36,798	2	5	−5.4	72.02	27.98	*n/d*
40,364	2	2	−4.4	75.32	24.68	Sickle chow
41,141	2	4	−5.5	82.09	8.24	*n/d*
41,035	2	3	−5.8	85.27	15.00	5% arginine
38,978	2	16	−5.0	90.16	13.62	Sickle chow
41,479	3	4	−4.6	96.38	3.62	5% arginine
39,525	3	12	−5.1	97.47	2.53	Sickle chow
40,119	3	10	−6.6	97.81	2.19	5% arginine
38,343	3	12	−6.5	99.00	<1.00	Sickle chow
39,617	3	6	−6.1	99.00	<1.00	Sickle chow
40,801	3	6	−6.0	99.00	<1.00	5% arginine
39,694	3	*n/d*	−5.9	99.00	<1.00	*n/d*

Water and diet influence oxygen isotope ratios in bone and other tissues. All experimental mice were raised in the same laboratory drinking from the same water source. However, diets of mice differed, because healthy and unhealthy mice have different nutritional needs. Healthy mice were fed a control diet, whereas affected mice were fed either sickle chow or a 5% arginine enriched diet (Table 1).

Samples were prepared according to the protocol of Garvie-Lok et al. (2004). Frozen hindlimbs were crushed to expose marrow cavities and soaked for 24 h in 2% sodium hypochlorite to remove soft tissues. After rinsing and drying, samples were powdered in a mortar and soaked for another 24 h in 2% sodium hypochlorite. Bone powders were again rinsed and dried and then soaked in 0.1 M acetic acid for 4 h. Halfway through acid treatment, sample tubes were exposed to vacuum to facilitate complete exposure of powders to the acid solution. After again rinsing and drying, samples were homogenized using an agate mortar and pestle. A subsample weighing between 1.2 and 1.3 mg was analyzed using an automated Carbonate Kiel device coupled to a Finnigan Delta IV Plus SIRMS in the Stable Isotope Biogeochemistry Laboratory at The Ohio State University. Samples were acidified under vacuum with 100% orthophosphoric acid, the resulting CO_2 was cryogenically purified and delivered to the mass spectrometer. Approximately 10% of all samples were run in duplicate. NBS-19 and UPS-1B standards were used. The standard deviation of repeated measurements of the internal standard UPS-1B was ±0.02‰ for

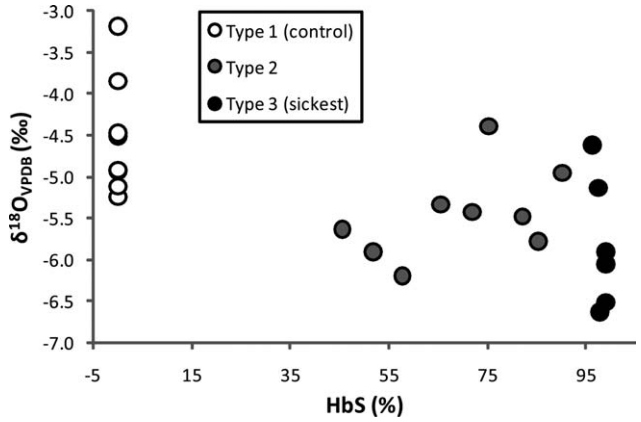

Fig. 1. Scatterplot of percent HbS hemoglobin measured in blood upon sacrifice, plotted against $\delta^{18}O$ ratios.

$\delta^{13}C$ and $\pm 0.07‰$ for $\delta^{18}O$. Nonparametric statistical tests were conducted using SYSTAT 12 software.

RESULTS

Isotope results are presented in Table 1. Oxygen isotope ratios are plotted against percent HbS hemoglobin in Figure 1. When considered as a group, "sick" mice (Types 2 and 3) exhibit lower $\delta^{18}O$ ratios than do healthy Type 1 mice (see Fig. 2). The eight Type 1 control mice exhibit a mean $\delta^{18}O$ ratio of $-4.5 \pm 0.7‰$, whereas the 16 noncontrol mice average $-5.6 \pm 0.6‰$, a difference of 1.1‰ which is statistically significant (Mann–Whitney U test, $P = 0.002$).

Sick mice were then divided into their respective hemoglobin (%HbS) groups, with Type 2 having a lower percentage of unhealthy HbS hemoglobin and Type 3 having the highest percentage. Thus divided, a clear trend emerges: Types 1, 2, and 3 mice exhibit progressively lower mean $\delta^{18}O$ ratios of $-4.5 \pm 0.7‰$, $-5.5 \pm 0.5‰$, and $-5.8 \pm 0.7‰$, respectively (see Fig. 2). A Kruskal–Wallace analysis of variance confirms significant $\delta^{18}O$ differences between the three groups ($P = 0.005$), although a Conover–Inman pair-wise comparison of Types 2 and 3 showed this difference to be statistically nonsignificant ($P = 0.229$). A Spearman's correlation analysis showed that despite significant differences in $\delta^{18}O$ ratios between groups, there was no consistent correlation between $\delta^{18}O$ values and %HbS in the 16 unhealthy mice ($r_s = -0.336$).

To investigate the relationship between diet and oxygen isotope ratios, $\delta^{18}O$ and $\delta^{13}C$ ratios were compared. Because carbon isotope ratios in bone reflect the $\delta^{13}C$ ratios of foods consumed during life, they were considered a reliable indicator of differences in diet among the sample. The $\delta^{13}C$ ratios of food were determined by analyzing desiccated powdered samples of the laboratory mouse chows. The control, sickle chow, and 5% arginine had $\delta^{13}C$ ratios of -19.4, -19.8, and $-19.9‰$, respectively. A Spearman's correlation analysis confirmed the absence of a statistically significant relationship between $\delta^{18}O$ and $\delta^{13}C$ ratios throughout the sample ($r_s = -0.261$). A Mann–Whitney test to compare the effect of different diets (5% arg and sickle chow) on $\delta^{18}O$ ratios within 13 sick showed no significant relationship between diets and $\delta^{18}O$ ratios (Mann–Whitney U test, $P = 0.643$). Because all control mice were raised on the control diet, they were excluded from this test. There was no relationship between mouse age in months and $\delta^{18}O$ ratios ($r_s = -0.194$) or between age and $\delta^{13}C$ ratios ($r_s = -0.058$).

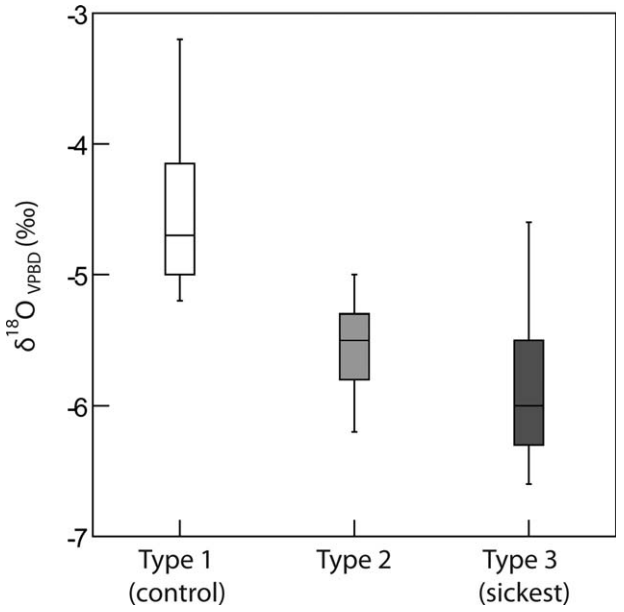

Fig. 2. Box plot of mice grouped into three-type categories. Boxes represent the interquartile range, and bars represent all data points within 1.5 × the interquartile range from the median.

DISCUSSION AND FUTURE DIRECTIONS

Mice expressing the human HbS gene and healthy control mice exhibit significantly different $\delta^{18}O$ ratios. However, contrary to our original hypothesis, the $\delta^{18}O$ ratios of sickle-cell mouse bones are lower, not higher, than those of healthy mice. When divided into three groups that vary in %HbS, a trend emerges with mice expressing the highest percentage of HbS hemoglobin also exhibiting the lightest $\delta^{18}O$ ratios.

One possible explanation for this observation concerns activity levels of the mice. Zanconato et al. (1992) and Widory (2004) demonstrated that fractionation decreases with increased activity level. Increases in oxygen diffusion across capillary membranes, heart rate, and water vapor loss contribute to this effect (Kohn, 1996; Widory, 2004). If the sickest mice were the least active, it could explain a higher degree of fractionation and concomitant depletion of ^{18}O in bone tissue in comparison with healthy, more active mice.

Fractionation of oxygen isotopes during respiration increases with increased hemoglobin count (Epstein and Zeiri, 1988). Among sickle-cell transgenic mice, hemoglobin becomes "trapped" in lungs and kidneys due to vasoocclusion of sickled cells. Fabry et al. (1992) observed that lungs of transgenic mice contained approximately six times more hemoglobin than did lungs of control mice. This may account for why sickle-cell mice in the present study exhibit a high degree of fractionation, whereas human anemics in previous studies exhibit a low degree. If this interpretation is correct, SCD is not directly comparable to other anemias in terms of its isotopic effects.

Although this study identified statistically significant differences between overall Types 1, 2, and 3 mice (see

Fig. 2), a scatterplot of individual sick mice did not reveal a linear relationship between $\delta^{18}O$ ratios and %HbS (see Fig. 1). This suggests that hemoglobin-specific fractionation is not the only source of differences between the groups and that the complex etiology of SCD should be considered in greater detail. Pathophysiologies such as urine concentrating ability, blood pressure, oxygen dissociation of sickle cells, and other pleiotropic or epistatic effects of the sickle-cell mutation (Becklake et al., 1955; Harrington et al., 1977; Nagel and Fabry, 1985; Fabry and Costantini, 1992; Romero et al., 1997) could all cause isotopic variations between healthy and sick individuals. If so, anemia may be the ultimate, though not the proximate, cause of $\delta^{18}O$ variations in bone. Importantly, other anemias and conditions impairing oxygen uptake (e.g., altitude sickness, tuberculosis, mining, and smoking) may also cause variations in $\delta^{18}O$ ratios of bone.

This pilot study demonstrates a relationship between the oxygen isotope ratios of bone apatite and HbS expression among mice. Further research is necessary to identify the source of this relationship. The hypothesis of this study was based on previously published observations of $\delta^{18}O$ fractionation during respiration and reactions of oxygen with hemoglobin in alveoli. In future studies, more consideration should be given to oxygen's pathway through the digestive tract, through which the majority of oxygen in bioapatites is routed.

Although these results are the opposite of what was expected based on previous research involving human respiration, pathology-dependent fractionation of oxygen isotopes does occur with SCD in mice. Humans and small mammals differ in ways that affect oxygen isotope ratios in tissues, including metabolism, water economy, body size, and thermoregulatory mechanisms (Bryant and Froelich, 1995; Kohn, 1996; Gautier, 2000; Scantlebury et al., 2010). However, considering these results alongside respiration studies reporting isotopic differences in humans with anemia, we conclude that this technique may be usefully applied to human skeletal materials where diets and water sources do not differ substantially between individuals.

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