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Total energy expenditure assessed by salivary doubly labelled water analysis and its relevance for short-term energy balance in humans

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RATIONALE: The doubly labelled water (DLW) method is a stable isotopic technique for measuring total energy expenditure (TEE). Saliva is the easiest sampling fluid for assessing isotopic enrichments, but blood is considered superior because of its rapid exchange with body water. Therefore, we compared a large range of isotopic enrichments in saliva and blood, and related TEE in subjects with their *ad libitum* total energy intake (TEI). The relevance of these parameters to body weight and fat change over an 8-day interval was also assessed.
METHODS: Thirty subjects underwent DLW analysis over either 8 or 14 days, during which time initial and final blood and saliva enrichments were compared. TEI was assessed by dieticians over the 8-day period only. Isotope ratio mass spectrometry was used for the measurement of δ^2H and $\delta^{18}O$ values.
RESULTS: No discrepancies were observed between sampling fluids over a wide range of enrichments. During the 8-day period, average TEI exceeded TEE by ~5% or less. Using saliva as sampling fluid, TEI and TEI-TEE, but not TEE, were positively correlated to body weight change. TEI-TEE and physical activity EE (AEE), but not TEI, correlated, respectively, positively and negatively to changes in fat mass.
CONCLUSIONS: The DLW method in humans can be reliably applied using saliva as sampling fluid. TEI-TEE as well as AEE contributes significantly to changes in fat mass over an 8-day period.

The doubly labelled water (DLW) method was proposed about half a century ago by Lifson and McClintock in their work on the fate of inspired oxygen, and is particularly useful for measuring total energy expenditure (TEE) under non-captive conditions in many species ranging from migratory birds to humans.[1–3] The method is based on the conjecture that hydrogen leaves the body primarily via water turnover, whereas oxygen leaves the body both via water turnover and via carbon dioxide as a product of fuel oxidation. Thus, enriching the body with tracer quantities of the rare stable isotopes of hydrogen (^2H) and oxygen (^{18}O), and subsequently subtracting elimination rates of ^2H (k_D) and ^{18}O (k_O), yield the carbon dioxide production, which can be converted into TEE.[4]

Because the DLW technique is harmless at low isotope concentrations, it can be safely applied for the study of TEE in humans.[5,6] Body fluids including blood, saliva and urine have been used for the measurement of isotopic enrichments, but the preferred fluid is blood since it is obviously in rapid exchange with the body water pool. Since blood may be more difficult to sample for practical reasons, saliva and urine have been used as alternatives.[7,8] Several (even large) cohorts of subjects show close agreement between urine and blood δ^2H and $\delta^{18}O$ values; however, this relationship depends strictly on the moment of void. Thus, depending on the moment of void and collection, urine may not be in complete equilibrium with the body water pool, and its isotopic enrichment may represent an integral over time.[9]

Sampling of saliva – at least in humans – is the easiest method, but its reliability has been suggested to suffer from isotopic fractionation and potential contamination by ingested material prior to sampling.[10] Studies comparing saliva and blood isotopic enrichment levels have only assessed a relatively narrow range of isotopic enrichment, without any attempt being made to actually calculate TEE from it.[7,8,11] Here we re-evaluated the use of saliva as a sampling fluid by comparing initial and final isotopic levels of ^2H and ^{18}O values in blood and saliva in 30 male human subjects over a wide range of enrichments, and subsequently calculated the TEE.

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A second aim was to relate the subjects' TEE assessed by DLW to total energy intake (TEI). If TEE measurements by DLW analysis and TEI assessment by dietary surveillance are valid, one would expect useful relationships between TEI and TEE and/or derived parameters of the latter – such as basal resting energy expenditure (REE) and physical activity EE (AEE) during the assessment of energy balance over an 8-day period. We furthermore hypothesized that precise and accurate assessment of the two would yield an energy budget (i.e., TEI-TEE) explaining potential differences in (components of) body weight over the course of measurement, and that this would predict weight change better than TEI or TEE alone.

EXPERIMENTAL

Subjects and design

During two periods, 30 healthy male subjects were staying at the clinical research unit of PRA International (Zuidlaren, The Netherlands), as part of a randomized, double blind study in which 10 mg olanzapine was administered once daily over a 14-day period, with addition of topiramate (Days 1–6, 25 mg, and Days 7–14, 50 mg) or placebo twice daily. Drugs were offered in ingestible capsules, and checked for complete swallowing. This study was performed within the framework of the Dutch Top Institute Pharma project: T2-105. The latter project aimed at investigating drug-induced weight alterations to identify novel therapeutic strategies for the treatment of obesity, dylipidemia and diabetes. For Period 1, the subjects arrived at the clinic 3 days preceding the day of drug administration (assigned Day 1) and left on Day 16 (48 h after the last drug administration). For Period 2, the subjects arrived at the clinic for follow-up on Day 27 and on Day 28. The treatments were well tolerated and all subjects were discharged in good condition and recovery from olanzapine/topiramate effects was assessed in the clinic on Day 28 (after 14 days washout of all medication).

The subjects had normal hematology, chemistry and thyroid parameters during screening as well as at follow-up. They were non-smoking, were not allowed to drink alcoholic beverages during the study, and did not habitually perform heavy labour or athletic competition. The anthropometric parameters are shown in Table 1. The subjects had their own rooms with computer and multimedia, and could socialise as wished, but were asked to avoid strenuous exercise. *Ad libitum* standardised breakfasts, lunches and dinners were provided with known calorific content of which the amount and type of ingested food were analysed by documenting left-overs and converted into metabolisable TEI. The intake of freely available snacks and beverages was also recorded. The clinical study protocol was approved by the IEC of the 'Stichting Beoordeling Ethiek Bio-Medisch Onderzoek' (Assen, The Netherlands), and all subjects provided their written consent.

On Day 6, the subjects were sampled for baseline enrichments (i.e., the natural isotopic abundance in the subjects before dosing DLW) and dosed with DLW between 9:00 and 10:00 AM. Drews and Stein showed that salivary enrichment should be taken at least 1 h after drinking a water bolus. Thus (to be on the safe side), the subjects in this study were prior informed to avoid eating or drinking within 90 min before each sampling.[12] While the subjects were seated, a blood sample (5 mL) was taken from the right medial cubital vein and a saliva (~1 mL) sample was taken for the analysis of isotopic background levels. The subjects were then asked to drink the DLW mixture. After a 4-h equilibration period, 'initial' samples were taken of saliva and blood, and a second initial saliva sample (no blood) was taken at 6 h after enrichment. On Day 14 (i.e., 8 days after enrichment), subjects' saliva was sampled again ('final'). Immediately after collecting this 'final' sample, the subjects were dosed again with the DLW mixture (the final enrichment sample after the first dosing was used as background for the calculation of the body water pool for the second DLW experiment). Again, initial samples were collected after 4 (saliva and blood) and 6 h (saliva). Final blood and saliva samples were collected when the subjects were admitted again at PRA (Day 28), 14 days after enrichment. Aqueous fractions from saliva were obtained by centrifugation (1000 rpm, 10 min at 4°C). Whole blood and aqueous saliva fractions were readily flame-sealed in 25 µL glass capillaries (VWR, Roden, The Netherlands) using a propane torch.

Table 1. Characteristics of the subjects (n = 29) including total energy intake (TEI), total energy expenditure (TEE) and isotopic data for the DLW method

	Mean ± SE
Age (years)	36.1 ± 2.4
Initial weight (kg)	84.9 ± 2.3
Height (cm)	181.2 ± 1.5
Weight change (Δ kg)	0.73 ± 0.19
N_D (kg)	47.95 ± 1.08/48.48 ± 1.06
N_O (kg)	45.27 ± 1.04/45.40 ± 1.02
N_D/N_O[a]	1.059 ± 0.062/1.069 ± 0.074
K_O (ppm/day)	0.0048 ± 0.0001/0.0047 ± 0.0001
K_D (ppm/day)	0.0038 ± 0.0001/0.0037 ± 0.0001
K_O/K_D[a]	1.256 ± 0.101/1.271 ± 0.0107
rCO_2 (mol/h)	0.80 ± 0.04/0.77 ± 0.03
Food quotient	0.817 ± 0.0017
TEE (MJ/day)	10.60 ± 0.50/10.15 ± 0.43
TEI (MJ/day)	10.69 ± 0.37
REE (MJ/day)	7.14 ± 0.12/6.90 ± 0.11
PAL	1.48 ± 0.06/1.47 ± 0.06

Values are reported as mean ± SE unless otherwise specified, at the start or over the ensuing 8 days of assessment using either saliva or blood as sampling fluid (if appropriate respectively separated by '/').
[a]Mean ± 2 standard deviations.

Doubly labelled water and dosing

99.9% ^2H_2O was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and 10% $H_2{}^{18}O$ was purchased from ROTEM GmbH (Leipzig, Germany). All the DLW used here was gravimetrically mixed in two large 10-L sterile glass flasks. The mixture was micropore filtered (pore diameter: 0.2 µm) and sterilisation was verified by having no bacterial colony formation after application of the

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DLW on agar plates kept for several days at 37°C. The subjects drank DLW (between 125 and 212 mL) proportional to their weight in an amount of 0.183 g/kg ^{2}H 99.9% and 1.650 g/kg ^{18}O 10% from a cup. Dosing was provided to the nearest 0.05 g by weighing the cups holding the mixture before and after drinking without spillage. Right after finishing, another cup filled with 100 mL of tap water was drunk by the subjects to drain residual DLW from the oral cavity into the stomach.

Analysis of the DLW samples

Salivary and blood $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were determined by micro-distillation in a vacuum line, first heating the broken capillaries and then cryogenically trapping the emerging water vapour in a cooled (with liquid nitrogen) glass vial. Water samples from these glass vials were then automatically injected into a high-temperature pyrolysis unit (Hekatech, Wegberg, Germany), in which injected water reacted with glassy carbon at 1420°C.[13] The resultant H_2 and CO gases, emerging into a continuous helium flow through the system, were then led through a gas chromatography column to separate the two gases in time and finally fed into an isotope ratio mass spectrometer (IsoPrime, GV Instruments, Manchester, UK) for the measurement of $\delta^{18}O$ and $\delta^{2}H$ values. The measurements were corrected for memory effects using an algorithm; these and other procedures were all previously described in a methodological paper on DLW preparation, memory correction, calibration and quality assurances applied by our group.[14] These procedures are fully in line with the recommendations of the International Atomic Energy Agency.[15] For each analytical series at least three internal water references were chosen to cover the entire enrichment range of samples. Reference samples were prepared and analysed following the same methods. Based on the spread of these reference samples, and on our prior experience, we have determined combined uncertainties of our analysis to be the quadratic addition of 3‰ and 0.5% of the value for $\delta^{2}H$, and 0.4‰ and 0.2% of the value for $\delta^{18}O$ (this includes calibration uncertainties).[14]

Two-thirds of all randomly chosen samples were measured once. The remaining third was measured in duplicate and analysed in different batches to check the precision of the analysis. Allowed duplicate differences were maximally 5‰ and 2% of the quadratically added value for $\delta^{2}H$ values, and 0.8‰ and 1.5% of the value for $\delta^{18}O$ values.[14] If these differences were exceeded, we critically examined the data and omitted aberrant values, performed further calculations on a single replicate and/or analysed a third sample if available. Fortunately, such deviations only happened for 12 samples. The subjects' body water pool was determined by the average dilution space (i.e., to account for changes in the body water pools) using initial enrichments, by the intercept method.[16] Due to isotope exchange with tissue, the apparent body water pools for oxygen and hydrogen/deuterium, respectively N_O and N_D, are always slightly over-estimated relative to the real one (N). This effect is larger for ^{2}H than for ^{18}O, and is corrected on a group estimate rather than a population-derived estimate, such as done by Schoeller *et al.*:[4]

$$N = \frac{\left(N_o + \frac{N_d}{R_{dil}}\right)}{2} \qquad R_{dil} = \frac{N_d}{N_o}$$

The rate of CO_2 production (rCO_2, moles day^{-1}) for each individual was calculated as described by Speakman:[2]

$$R_{CO2} = \frac{N}{2}.078(k_O - R_{dil}k_D) - 0.0062 N R_{dil} k_D$$

where k_O and k_D are the respective fractional turnover rates of ^{18}O and ^{2}H, calculated against the individual-specific background concentrations measured before the first dose (for both measurement periods) and the individual-specific initial and final ^{18}O and ^{2}H concentrations. A two-point method was chosen over the multi-point method because the multi-point method does not appear to have a greater precision for estimation of TEE.[17,18] There is a slightly better association of fat-free mass to TEE with the multi-sample approach than with the two-sample approach, but we felt this advantage to be offset by economic and resource disadvantages.[17] We used the individual R_{dil} in the equation. Finally, the rate of CO_2 production, rCO_2, was converted into TEE using a rearrangement of the Weir equation.[5] The food quotient (FQ) was estimated from the dietary records according Black *et al.*[19] The fat-free mass (FFM) was calculated based on the assumption that the hydration factor for human subjects is 0.73.[20] The resting energy expenditure (REE) was calculated from FFM using the Mifflin-St. Jeor equation.[21] We used this equation since it was found to better predict REE in subjects taking olanzapine.[22] According to the World Health Organization (WHO), the level of TEE above REE was assigned as the activity energy expenditure (AEE), which includes all energy-consuming processes beyond REE, including diet-induced thermogenesis and muscular activity.[23] A Physical Activity Level (PAL) was calculated as TEE/REE.

Statistical analysis

The data are expressed as the mean and standard error of the mean (±SE) unless otherwise specified. The level of agreement between the δ values of blood and saliva, as well as between samples taken at 4 and 6 h, were analysed by plotting the difference between the two outcomes against one of them. The relationship between this difference and one of the two is then:

$$(X_1 - X_2) = \alpha X_1 + \beta$$

where X_1 and X_2 are the results from the two methods to be compared (i.e., blood compared with saliva enrichments, or enrichments at 4 h compared with those at 6 h), and α and β are linear least-squares fit coefficients. Potential slope deviations ($\alpha \neq 0$ and/or offsets ($\beta \neq 0$) are easily recognizable. The fit errors in the coefficients immediately show whether the found values are significant (a 95% confidence interval corresponds to two times the 1-σ error). By forcing $\alpha = 0$ in the fit we can investigate the significance of potential offsets (which corresponds to calculating the mean of the difference $X_1 - X_2$).

Data were analysed with Multiple Regression Analysis (SPSS18, Chicago, IL, USA). Specifically, TEI, TEE, REE, AEE, PAL, TEI-TEE, Δweight/day, ΔFAT/day, ΔFFM/day were included as factors.

RESULTS

Subject characteristics and isotopic data

One of the subjects was excluded from analysis due to an abnormal TEE (~25.07 MJ/day) over the two DLW intervals. The remaining 29 subjects had a mean age of 36.1 ± 2.4 years. The average daily TEE calculated by the DLW method using blood (10.15 ± 0.43 MJ/day) and saliva (10.60 ± 0.50 MJ/day) as sampling fluid over the first DLW period was not significantly different from the TEI (10.69 ± 0.37 MJ/day). We found an average (physical activity level) PAL score of 1.48 ± 0.06 (Mean ± SEM), indicating a sedentary lifestyle (1.40–1.69 PAL score).[23] These and other general characteristics of the study subjects and the isotopic data required for the calculation of TEE using the DLW method are shown in Table 1.

Comparison of saliva vs. blood isotopic enrichments

Figure 1 shows the difference between blood and saliva isotopic enrichment plotted against blood isotopic enrichment for both ^{2}H and ^{18}O (3 outliers were excluded). The slope of the regression line shows no significant difference from 0 for both isotopes. If we assume an offset only, we find that the mean difference between blood and saliva was –10 ± 4 for $\delta^{2}H$ values and –0.1 ± 0.4 for $\delta^{18}O$ values, thus a slight systematic bias between the two methods of sampling for ^{2}H, but not for ^{18}O.

The errors indicated in the plots are based on analytical procedures only (based on the numbers given above). In both plots, the spread of the differences increases with increasing enrichment, in line with the – also increasing – error bars. This indicates that individual variability plays an important role, causing the scattering of the points to be somewhat larger than one would expect from the analytical uncertainties alone. This is especially visible in the low enrichment part of the $\delta^{2}H$ values plot.

Comparison of 4- to 6-h initial samples

Figure 2 shows the individual differences of isotopic enrichments for both isotopes between samples that were taken at 4 and 6 h after DLW dosing. For the $\delta^{2}H$ and $\delta^{18}O$ values, a linear fit with a slightly negative slope was found, indicating that differences between 4 and 6 h post-dosing is enrichment-dependent (which is logical). If we neglect the slopes, we find mean differences between isotopic enrichment at 4 and 6 h post-dosing of –53 ± 5‰ for $\delta^{2}H$ and –5.2 ± 0.4‰ for $\delta^{18}O$ values. At 6 h, ^{2}H and ^{18}O were already being eliminated from the body. Both the 4- and 6-h initial samples were used to calculate TEE, and they did not differ significantly (mean difference 0.38 ± 4%). Although the mean difference between TEE calculated from 4- and 6-h initial samples is quite small, the variation is relatively large, indicating that some individuals were still building up enrichments after dosing, while others were already at equilibrium before the 4-h time point. This effect is reflected by the scatter of data shown in Fig. 2, which is significantly larger than the analytical errors and is mostly due to individual variability among subjects.

How the analytical errors finally end up in the calculated TEE was provided in detail in Guidotti *et al*.[14] These errors range anywhere between 3 and 8%, which is well in line with the work of Schoeller.[24] Plots of TEE inferred from the 4-h initial versus the 6-h initial, as well as the plot of TEE inferred from blood vs saliva (with 4-h initials) show again that the individual variation is larger than the analytical uncertainties within samples (data on request). No significant offsets or

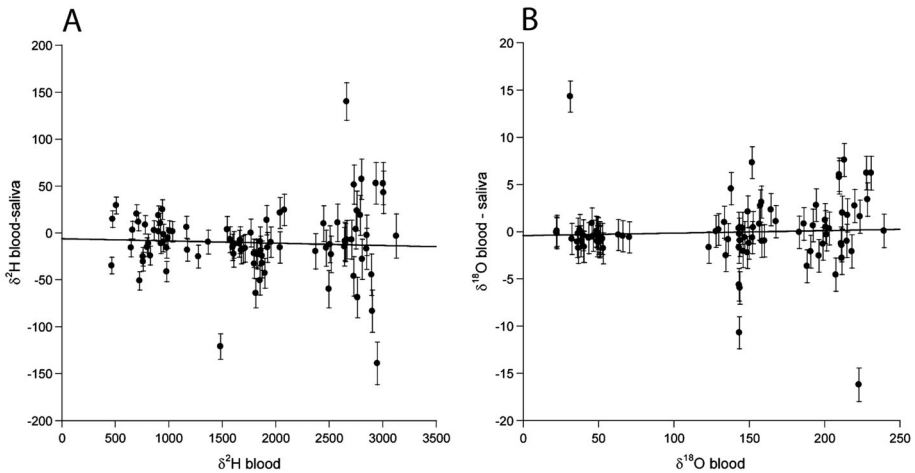

Figure 1. Individual differences between $\delta^{2}H$ (A) and $\delta^{18}O$ (B) isotopic ratio values measured in saliva and blood. For both $\delta^{2}H$ and $\delta^{18}O$ values a linear fit yields an insignificant slope. The offset of the $\delta^{2}H$ values between the two methods is significant but small: –10 ± 4‰ compared with values between 500 and 3000‰. For the $\delta^{18}O$ values the average offset between the two methods is zero within its error bars (of ±0.4‰). The errors indicated in the plot are based on the analytical procedures only; at higher enrichments, the individual scatter of the data points is larger than these error bars, indicating that individual variability is larger than the analytical scatter there.

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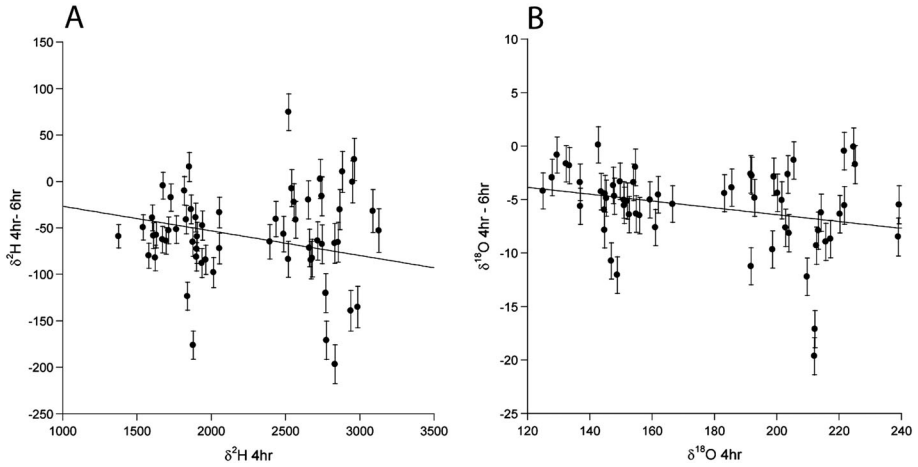

Figure 2. Individual differences between δ^2H (A) and $\delta^{18}O$ (B) isotopic values measured in saliva at 4 and 6 h post-dosing. For both δ^2H and $\delta^{18}O$ values, a linear fit gives a negative (but not significant to the 95% level) slope. This implies that the difference between 4 and 6 h post-dosing is enrichment-dependent (which is logical). If we neglect the slopes, we find that the mean differences between isotopic values at 4 and 6 h post-dosing were –53 ± 5‰ for δ^2H and –5.2 ± 0.4‰ for $\delta^{18}O$ (the 6-h results being lower). The line represents the weighted mean for the δ^2H and $\delta^{18}O$ values.

deviations from 0 were found, indicating that there is no systematic bias for sampling time of the initial (i.e., 4 h vs 6 h) nor for the sampling fluid (i.e., blood vs saliva).

Matching total energy intake and total energy expenditure

As mentioned earlier, TEE was on average slightly lower than TEI over the 8-day interval. While these average levels are fairly comparable, there were relatively large inter-individual variations in TEI and TEE that were not correlated for both blood and saliva as sampling fluid, and differences between TEI and TEE (i.e., EI-TEE) resulting from variations in both TEI and TEE. While this variation is inter-dependent (i.e., the relations are mathematically equivalent), the negative correlation between TEE and TEI-TEE ($R^2 = 0.64$, $\alpha = -0.98 \pm 0.14$) was far stronger than the positive correlation between TEI and TEI-TEE ($R^2 = 0.32$, $\alpha = 0.96 \pm 0.26$), meaning that the variation in TEE could be a better predictor for variation in TEI-TEE than TEI. This was the case when TEE was derived from salivary enrichment and where the initial sample was taken at 4 h; a weaker relationship was observed using blood as the sampling fluid. One potential implication of the inter-individual variation in TEI-TEE is that the variation could explain the changes in body weight over the course of measurements. Indeed, plotting this relationship revealed a significant positive correlation in which 36% of the variation in body weight was explained (see Table 2) by TEI-TEE. Interestingly, TEI, but not TEE, revealed an even stronger positive correlation that explained 59% of the weight change (see Table 2).

Because the subjects were dosed again with DLW, this allowed us to dissociate changes in fat mass (ΔFAT) and fat-free mass (ΔFFM) underlying the weight change of subjects over the course of measurement. Although ΔFAT and ΔFFM alone were not correlated to weight change, TEI-TEE, but not TEI or TEE, was positively correlated to ΔFAT (see Table 2) when using saliva as the sampling fluid, but not when using blood as the sampling fluid. Regression

Table 2. Relationship between energy components such as total energy intake (TEI), total energy expenditure (TEE), TEI-TEE, resting energy expenditure (REE), physical activity energy expenditure (AEE) and physical activity levels (PAL) with short-term weight/body composition changes in 29 healthy subjects over an 8-day period using saliva as sampling fluid

	Δ weight		Δ FAT		Δ FFM	
	R^2	α	R^2	α	R^2	α
TEI	**0.59**	0.040 ± 0.06	0.02	NS*	0.01	NS
TEE	0.03	NS	0.13	NS	0.09	NS
TEI-TEE	**0.35**	0.019 ± 0.048	**0.15**	0.038 ± 0.179	0.04	NS
REE	0	NS	0.02	NS	0	NS
AEE	0.03	NS	**0.17**	–0.054 ± 0.229	0.12	NS
PAL	0.04	NS	**0.19**	–4.14 ± 1.64	**0.14**	3.58 ± 1.71

R^2 represents the square of a correlation coefficient, α values represent the slope of the regression line, and significant results ($p < 0.05$) are given in bold. NS: non-significant.

analysis revealed that AEE ($R^2 = 0.89$), but not REE, was strongly correlated to TEE (see Fig. 3) with saliva as the sampling fluid. The variation in TEE was also explained very well by the PAL score ($R^2 = 0.82$), which is a measure of physical activity.[25] Both AEE and PAL were significantly negatively correlated to ΔFAT.

DISCUSSION

The first aim of this study was to evaluate the reliability of saliva as sampling fluid for DLW isotopic enrichment and to assess its applicability in studies on human energetics. This is of particular interest because, due to its fast equilibration with body water pools, blood or plasma would clearly be the optimal sampling fluid for DLW analysis. Although agreement between saliva and blood has been shown for a relatively small range of enrichments, more extensive verification is required before saliva can be considered as a suitable sampling fluid for DLW analysis.[7,8] In the comparison between saliva and blood isotopic enrichments in a broad spectrum of isotopic enrichment, ranging from 500 to 3000 for δ^2H values, and from 20 to 250 for $\delta^{18}O$ values, we found no systematic bias and overall an excellent level of agreement between the two fluids. Thus, we conclude that saliva, following the design of our experiment, can be reliably used as a sampling fluid for human DLW studies. An important consideration about the reliability of saliva is that it could suffer from contamination by recently ingested foods and fluids and thus supervision of the study participants is required for the ingestion of the water. We avoided this issue by carefully instructing the participants not to drink or eat within 90 min before dosing or sampling.[12] Another issue raised by Schoeller is that saliva may suffer from isotopic fractionation (i.e., heavy isotopes evaporate more slowly than lighter ones) from the mouth.[10] We found a limited bias of about 10‰ between blood and saliva for δ^2H values (saliva being higher), and no bias at all for $\delta^{18}O$ values. Thus, although oral evaporation does take place, there is probably ample mixing of saliva with the body water pool such that isotope fractionation in the mouth plays no significant role in the study outcome.[7]

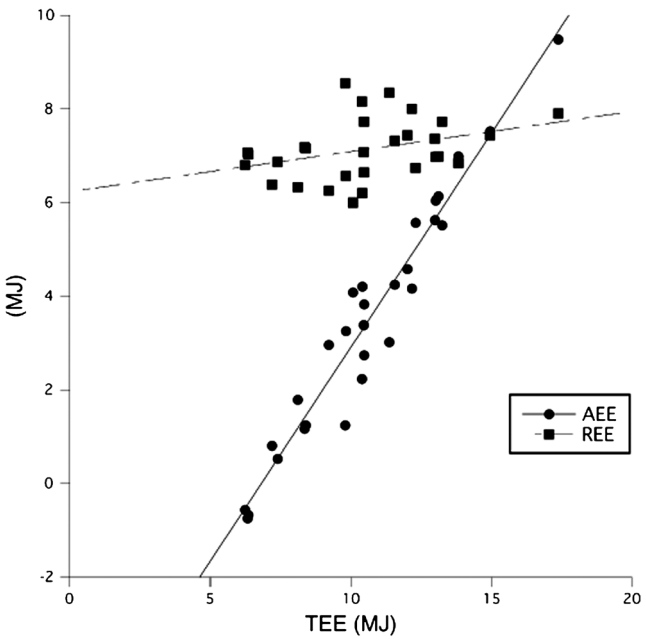

Figure 3. Relationship between TEE and its components: REE (squares) and AEE (circles). AEE explains ($R^2 = 89\%$) more of the variation in TEE than REE ($R^2 = 13\%$).

A second issue in using saliva as the sampling fluid is that fluids in general reach their 'plateau' or isotopic equilibrium with the body water pool at different time points. Urine, for example, would be expected to have a large delay in reaching equilibrium.[8,11] The 6-h initial samples showed slightly lower enrichment levels than the 4-h initial samples, indicating that the equilibration process was, for all practical purposes, complete after 6 h. The rate of elimination by then had surpassed the rate of appearance of the isotopes in the body water pool, which is consistent with the data of Wong *et al.*[8]

A third issue is that the individual variability between enrichments in saliva and blood are larger than the analytical uncertainties alone. In a comparison of isotopic enrichments in urine versus blood or saliva, this could be explained, for instance, by differences in post-void residual urine. In the present study, however, such an explanation would not suffice for the differences between blood and saliva. Saliva has some characteristics overlapping with blood, including the presence of electrolytes and some enzymes (such as carbonic anhydrase), but the total protein concentration in saliva compared with that in blood is very little (less than 1%). Proteins can bind water differently, but this would not matter with respect to enrichment levels, unless there is some mechanism that discriminates isotopes. Recently, Kreuzer and colleagues found evidence that mammalian fibroblast as well as muscular tissue maintains a large isotopic gradient across their membranes after exposure to DLW.[26] They estimated the proportion of intracellular H and O atoms to be isotopically distinct from extracellular water by at least 12%. We suspect that the processes underlying these differences introduce additional variable pools, which may underlie subtle differences between enrichments in saliva and blood.

The next aim of this study was to relate TEI and TEE over an 8-day measurement. Over the period in which TEI and TEE were compared, the subjects were staying at the clinical research facility of PRA International, where all the subjects' ingested food items (which were freely available on request) and leftovers were recorded and calculated into TEI by dieticians. Thus, carefully assessing TEI was an important feature in this study. Within the group as a whole (excluding the one with an unexplainable high MR of 25 MJ/day), the average TEI (10.69 ± 0.37 MJ/day) exceeded TEE using saliva (10.60 ± 0.50 MJ/day) as sampling fluid by only 0.09 MJ/day. This backs up the notion that DLW analysis can be used to measure TEI in free living human subjects, provided that these individuals are relatively weight stable.[27,28]

The individual variation in TEI and TEE, however, was quite large among subjects, and these were not strongly inter-related. In an attempt to reconcile the variability in the imbalance between TEI and TEE, we calculated the subjects' energy budgets by subtracting TEE derived from either salivary and blood enrichments from TEI, and investigated components that contributed to the variation in TEI-TEE. We found that variation in TEE correlated better with TEI-TEE than TEI did with TEI-TEE. TEE assessed by DLW

analysis can be broken down into REE and factors beyond REE, collectively termed physical activity EE (AEE). Although the subjects were housed under sedentary conditions and were asked to refrain from strenuous exercise, differences in AEE, but not REE, using saliva as sampling fluid largely underlay the differences in TEE. AEE (R^2 = 0.65) and PAL (R^2 = 0.64) correlated even better to TEI-TEE than TEE alone, suggesting that physical activity played an important role in determining the variation in TEI-TEE. We do not know to what extent this effect was due to differences in voluntary activity (also termed non-exercise physical activity, NEAT[29]) or planned activities, such as walking, stair climbing, etc. Remarkably, these and other correlations were weaker with blood as sampling fluid than with saliva. This seems to indicate that the DLW characteristics inferred from salivary enrichment, at least in our setting and approach, are more accurate than when using blood. Further studies need to be performed to reinforce and closer inspect these findings.

When using saliva, but not blood, as the sampling fluid, regression analysis revealed that the variation in TEI-TEE over the course of the 8-day period explained ~36% of the weight change of the subjects (see Fig. 3). The positive slope of this linear fit suggests that adding 1 kg of body weight requires 15.4 MJ excess energy. This is far less energy than has been suggested for weight loss.[30,31] In the present study, however, 15 of the 29 subjects gained weight, which obviously requires other physiological/metabolic mechanisms than weight loss. Performing this study in a larger group of subjects might reveal different slopes for weight loss and weight gain in the interaction with EI-TEE.

To our surprise, we observed that TEI explained differences in weight gain better than TEI-TEE; however, TEI was not correlated to individual components underlying weight gain (i.e., ΔFAT, ΔFFM, a change in body water content). Instead, variation in TEI-TEE correlated significantly in a positive direction to ΔFAT, as would be expected. Variation in AEE and PAL also contributed to ΔFAT, but in a negative direction, which is consistent with studies pointing out this inverse relationship between PAL and %body fat.[32] The importance of our study, however, is that we actually assessed changes in fat mass over a relatively short period, perhaps as a consequence of physical activity, rather than comparing snapshots of physical activity levels and %body fat at different time intervals.[33,34]

CONCLUSIONS

Our study extends the findings by Wong *et al*.[8] and Schoeller *et al*.[9] that saliva is a reliable sampling fluid for DLW studies, and that there are no strong differences between initial samples taken 4–6 h post-dosing of DLW. While TEE assessed by the DLW method is well in line with TEI in the group of subjects in the present study, the variation in the imbalance is primarily caused by differences in TEE, and particularly AEE, and to a lesser extent in TEI. The imbalance between TEI and TEE explains weight change consistently and, although TEI is a better predictor for weight change, TEI-TEE as well as AEE/PAL, but not TEI, are predictors for a short-term change in body fat mass.

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