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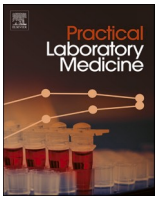

Temperature stability of urinary F_2-isoprostane and 8-hydroxy-2′-deoxyguanosine

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ABSTRACT

Background: Clinical and epidemiological studies employ long-term temperature storage but the effect of temperature on the stability of oxidative stress (OS) markers is unknown. We investigated the effects of storage at −20 °C and −80 °C over 4–9 months on F_2-isoprostanes (F_2-IsoP) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in urine of children, a population group among whom the measurement of these markers is still limited.
Methods: Paired spot urine samples from 87 children aged 8.9–16.9 years (52.9% boys) were analyzed. Samples were preserved with 0.005% (w/v) butylated hydroxytoluene, portioned and stored within 2.5 h (median) of collection. Samples were analyzed in duplicate or triplicate using commercial ELISA kits and their correlations were evaluated.
Results: F_2-IsoP and 8-OHdG showed high correlations (Spearman rho of 0.90 and 0.97, respectively; $P < 0.0001$) with storage at −20 °C and −80 °C. There was a strong agreement among categories of values for F_2-IsoP (Kappa = 0.76 ± 0.08, agreement = 83.9%, $P < 0.0001$) and 8-OHdG: (Kappa = 0.83 ± 0.08, agreement = 88.4%, $P < 0.0001$). The correlation between the temperatures for F_2-IsoP concentrations was also high when stored for <4 (0.93), 4 (0.93), and 5 months (0.88), all $P < 0.0001$. For 8-OHdG, Spearman correlations at <8, 8, and 9 months of storage at −20 °C and −80 °C were 0.95, 0.98, and 0.96 (all $P < 0.0001$), respectively.
Conclusions: Urine storage with BHT for up to nine months at a temperature of −20 °C to −80 °C yields highly comparable concentrations of F_2-IsoP and 8-OHdG.

1. Introduction

To assess oxidative stress (OS), urinary F_2-isoprostanes (F_2-IsoP, representing arachidonic acid and eicosanoid peroxidation) and 8-hydroxy-2′-deoxyguanosine (8-OHdG, representing damage in the DNA strand) are frequently used [1,2], albeit less so in children than adults. As these analyses are rarely performed in real-time, nearly all clinical and epidemiological studies employ long-term sample storage at temperatures ranging from −20 °C to −80 °C (for example [3,4]). Ultra-low temperature storage may be unavailable in resource-limited settings due to freezer cost or uncertain power supply. Some studies have investigated the stability of F_2-IsoP or 8-OHdG over the short-term, indicating that room temperature for one day, 4 °C for two days, −20 °C for 30 days, and up to three

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freeze-thaw cycles may have limited impact [5]. There is little evidence, however, to indicate how longer-term storage temperature may affect OS biomarker concentrations. Utilizing paired urine samples collected among school-age children and stored at −20 °C and −80 °C, we evaluated the effect of 4-9-month storage temperature on urinary F_2-IsoP and 8-OHdG concentrations. We hypothesized that storage temperature would have not effect on biomarker stability.

2. Materials and methods

2.1. Sample collection and preparation

Spot urine samples were collected March 1 to June 30, 2022 from children aged 8.9–16.9 years (52.9% boys) from Montevideo, Uruguay. Caregivers received 100-mL sterile, polypropylene sample cups and helped their children as needed to collect a first-morning sample, catching urine mid-stream. The samples were returned to the study offices at the Catholic University of Uruguay on the day of collection and processed immediately; >50% of samples were processed within 2–2.5 h of collection. A 10 mL aliquot of urine was vortexed for 10sec with 100 μ L butylated hydroxytoluene (BHT, 0.5% p/v), then the tube was centrifuged at 1500 g for 10min. Two 2 mL samples were portioned into cryovials and frozen, one at −20 °C and one at −80 °C. The samples were shipped on dry ice to the University at Buffalo and stored at their respective temperatures until analysis. The study was approved by institutional review boards of the University at Buffalo (STUDY00004845) and Catholic University of Uruguay (without number). Caregivers provided written consent for study participation.

2.2. F_2-isoprostanes

For purification via solid phase extraction (SPE), samples were thawed at room temperature for 20min, then 1 mL was diluted with 2.5 mL acetate buffer (1 M, pH 4.0). SPE C18 (500 mg) affinity cartridges (Cayman Chemicals, MI, USA), attached to a vacuum manifold, were activated with 5 mL methanol, and rinsed with 5 mL ultrapure water. Diluted samples were run through the columns followed by 5 mL of ultrapure water. Columns were dried and F_2-IsoP was eluted with 2 mL methanol into glass vials. Vials were stored at −80 °C until ELISA analysis.

F_2-IsoP was quantified using a competitive ELISA assay (Cayman Chemicals, MI, USA) in August 2022. Methanolic SPE extracts were evaporated under a stream of nitrogen gas at 37 °C for ~40min and replaced with 1 mL ELISA buffer. A 32-fold sample dilution in ELISA buffer was prepared in 1.7 mL microcentrifuge tubes. The F_2-IsoP standard was also prepared in the ELISA buffer (ranging 500–0.8 pg/mL). The wells of mouse anti-rabbit IgG coated plate were filled with standards, samples, 8-isoprostane-acetylcholine esterase (AChE) conjugate (8-isoprostane tracer), and antiserum. Control wells contained non-specific binding ELISA buffer and tracer only; maximum binding wells contained ELISA buffer, tracer, and antiserum. Standard and samples were run in duplicate. The plate was sealed with plastic film and incubated at 4 °C for 18 h s. The next day, the plate was washed five times to remove unbound reagents. The plate was tapped onto a paper towel and 200 μL Ellman's reagent was added. The plate was sealed with plastic film, covered with aluminum foil, and placed on an orbital shaker set to 300 rpm for 90min at room temperature. A plate reader (BioTek Synergy HTX Multi-mode Plate Reader, Agilent Technology Inc., Winooski, Vermont) set to 412 nm and 25 °C was used to read the absorbance. The data were plotted and reduced using Cayman's Competitive ELISA Double Calculations Excel workbook. The concentration of the tracer is held constant; the intensity of the color is proportional to the amount of tracer and inversely proportional to the amount of F_2-IsoP.

2.3. 8-OHdG

The samples were shipped on dry ice to Creative Proteomics (Shirley, NY, USA) and analyzed in December 2022 with an in-house ELISA kit for the detection of 8-OHdG, utilizing a standard range of 1–320 pg/mL and Thermo Scientific™ Multiskan™ FC Microplate Photometer. Samples were analyzed in triplicate. The standard (50 μL each) was added to the designated well. Testing samples were diluted fivefold in the wells by adding 10 μL of sample followed by 40 μL sample dilution buffer. HRP-Conjugate reagent (100 μL) was added to all, except the blank well. The plate was sealed with plastic film and incubated for 60min at 37 °C. The wells were washed five times with the washing solution, dried by swing, and patted on paper towel. Color was developed in the dark for 15min at 37 °C by adding chromogen solutions A and B to each well. The reaction was stopped with 50 μL stop solution and absorbance was read at 450 nm within 15min. The blank was subtracted from the results. Each 8-OHdG oncentration was estimated from a XY-scatter plot and regression relating absorbance to the standard concentration.

2.4. Statistical analysis

The distribution of F_2-IsoP and 8-OHdG at each temperature was examined overall and according to the length (months) of storage. Considering −80 °C as the gold standard condition, concentrations at the two temperatures were graphed using scatter plots, and the relationship between the concentrations was obtained from a simple linear regression model. The marker distributions were divided into tertiles and the kappa statistic was used to determine the level of agreement for each marker at the two temperatures. Finally, Spearman rank correlations of each biomarker at the two temperatures were examined for the whole sample and according to the length of storage. The analyses were performed in Stata 14 (College Station, TX, USA).

3. Results

The distributions and %CVs of the duplicate (F_2-IsoP, n = 87) or triplicate (8-OHdG, n = 86) measures of each sample are presented in Table 1. Scatterplots of observed concentrations at −20 °C and −80 °C storage are presented in Fig. 1. Urinary concentrations of F_2-IsoP (Spearman rho, p-value: 0.90, $p < 0.0001$) and 8-OHdG (0.97, $p < 0.0001$) at the two temperatures were highly correlated. The kappa statistics indicated strong agreement among categories of values for F_2-IsoP (Kappa = 0.76 ± 0.08, Z = 10.01, $p < 0.0001$, agreement = 83.9%) and 8-OHdG: (Kappa = 0.83 ± 0.08, Z = 10.83, $p < 0.0001$, agreement = 88.4%).

Urine samples for the measurement of F_2-IsoP were stored for <4 months (19.5% of samples), 4 months (36.8%) and 5 months (43.7%) prior to first thaw for solid phase extraction. Prior to 8-OHdG analysis, samples were stored for <8 (48.3%), 8 (27.6%), and 9 (24.1%) months. Biomarker concentrations by length of storage are provided in Table 1. Spearman correlations for F_2-IsoP concentrations at <4, 4, and 5 months of storage at −20 °C and −80 °C were 0.93 ($p < 0.0001$), 0.93 ($p < 0.0001$), and 0.88 (<0.0001), respectively. For 8-OHdG, Spearman correlations at <8, 8, and 9 months of storage at −20 °C and −80 °C were 0.95 ($p < 0.0001$), 0.98 ($p < 0.0001$), and 0.96 (<0.0001).

4. Discussion

Urinary F_2-IsoP and 8-OHdG are commonly measured and considered to be reliable markers of OS. Still, a lack of standard protocols for sample storage has been noted [6] and some studies do not specify storage conditions [7]. Ultra-low temperature storage likely predominates in well-resourced academic or biomedical settings [4,8]; it may, however, be limited in under-resourced laboratories and countries. The archiving of urine for the assessment of OS in epidemiological studies involving children is particularly valuable. To date, few studies have measured OS markers in this vulnerable population group.

Taking advantage of ~90 pairs of samples collected from children and stored at both −20 °C and −80 °C, we compared the concentrations of urinary F_2-IsoP and 8-OHdG. The mean biomarker concentrations were very similar at the two temperatures, as were the mean %CV of duplicate or triplicate runs. We also found high overall correlations of biomarker concentrations (Spearman's rho ≥0.90) at the two temperatures overall and across a range of storage time (Spearman's rho 0.88–0.98). The kappa statistics showed very good (F_2-IsoP) and excellent (8-OHdG) level of agreement, suggesting that up to 5–9 months, storage at −20 °C versus −80 °C has minimal effect on measured concentrations of the two biomarkers.

Some questions on the impact of storage conditions on the concentrations of urinary OS biomarkers remain. For example, we added BHT to the urine samples to a final concentration of 0.005% w/v prior to aliquoting and freezing. This preservation step was suggested by the Cayman Chemical F_2-IsoP kit to improve sample stability. It is unclear how lack of pre-treatment with BHT would affect temperature stability of the OS biomarkers. Second, storage time ranged 3–9 months, which is relatively short for epidemiological studies that may archive samples for many years. The effect of longer-term storage (1+ years) on biomarkers stability at different temperatures is unknown. Until further evidence is available, it is prudent to select storage at −80 °C for long-term archiving, laboratory conditions permitting. The relatively small sample size also prevented us from calculating kappa statistics for more narrow ranges of biomarker concentrations.

5. Conclusion

Sample storage with BHT for up to nine months at temperatures −20 °C to −80 °C has limited effect on the stability of urinary F_2-

Table 1
Distribution of F_2-isoprostanes (F_2-IsoP) and 8-hydroxy deoxy-2′-guanosine (8-OHdG) in children's urine, according to different storage temperatures and lengths.

Temperature	Full sample	By length of storage		
	Mean ± SD	**Mean ± SD**		
	%CV (Mean ± SD)	**%CV (Mean ± SD)**		
F_2-IsoP (pg/mL)	N = 87	<4 months	4 months	5 months
		N = 17	N = 32	N = 38
−20 °C	2332.0 ± 1692.6[a]	2501.2 ± 2673.4	2313.5 ± 1348.6	2271.9 ± 1423.9
	11.9 ± 13.2[b]	10.3 ± 14.1	13.4 ± 10.1	11.4 ± 15.1
−80 °C	2384.0 ± 1664.5	2605.7 ± 2472.2	2595.4 ± 1564.3	2106.9 ± 1267.3
	12.7 ± 9.3[b]	18.5 ± 8.0	12.5 ± 9.8	10.4 ± 8.4
8-OHdG (pg/mL)	N = 86	<8 months	8 months	9 months
		N = 41	N = 24	N = 21
−20 °C	233.3 ± 68.6	224.4 ± 66.3	235.8 ± 70.1	247.8 ± 71.8
	4.5 ± 2.5[c]	4.7 ± 2.6	5.1 ± 1.5	3.5 ± 2.2
−80 °C	234.5 ± 66.8	225.5 ± 65.5	239.9 ± 67.5	245.8 ± 69.5
	4.3 ± 2.3[c]	4.5 ± 2.2	4.2 ± 3.0	4.0 ± 1.7

[a] Values given as mean ± SD.
[b] %CV based on duplicate measures of a single sample.
[c] %CV based on triplicate measures of a single sample.

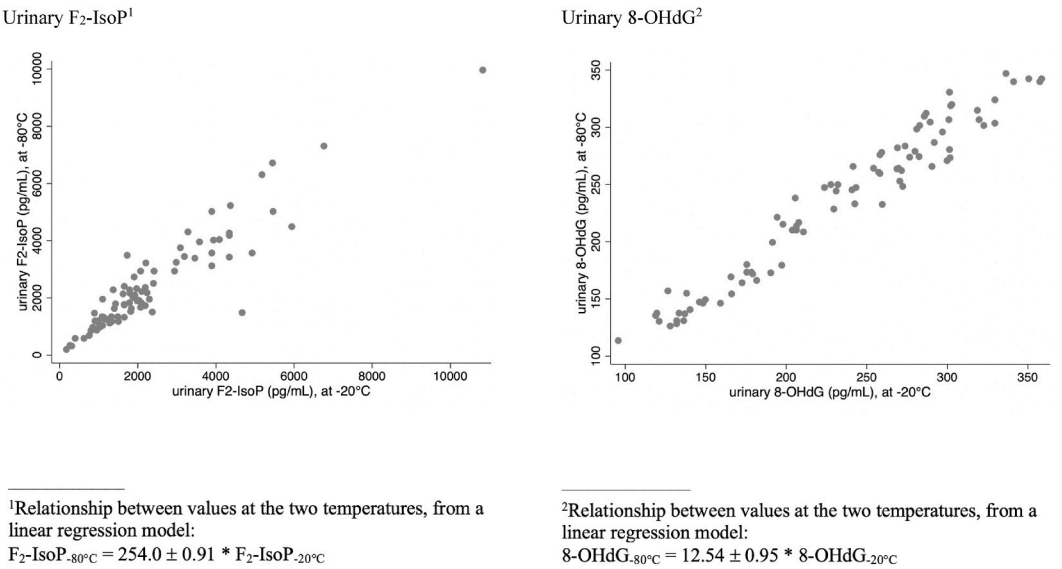

Fig. 1. Scatterplots of urinary concentrations of two oxidative stress markers upon storage at −20 °C and −80 °C.

IsoP and 8-OHdG, and yields highly comparable biomarker concentrations.

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CRediT authorship contribution statement

Katarzyna Kordas: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Conceptualization. **Diala Ghazal:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Elena I. Queirolo:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **James R. Olson:** Writing – review & editing, Funding acquisition. **María Inés Beledo:** Writing – review & editing, Resources, Project administration, Methodology. **Richard W. Browne:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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