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Coffee Drinking Increases Levels of Urinary Hydrogen Peroxide Detected in Healthy Human Volunteers

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Freshly-voided human urine contains significant concentrations of hydrogen peroxide (H_2O_2). This H_2O_2 appears to arise in whole or in part by superoxide-dependent autoxidation of urinary biomolecules. Since instant coffee also contains high levels of H_2O_2, we examined the effect of coffee drinking on urinary levels of H_2O_2. Studies on healthy human volunteers showed that coffee drinking is rapidly and reproducibly followed by increased levels of H_2O_2 detectable in the urine for up to 2 h after drinking the coffee. The levels of H_2O_2 detected in urine suggest that exposure of human tissues to H_2O_2 may be greater than is commonly supposed. It is possible that H_2O_2 in urine could act as an antibacterial agent, and that H_2O_2 is involved in the regulation of glomerular function.

Keywords: Coffee, hydrogen peroxide, urine, antioxidant, redox regulation, kidney, glomerulus

INTRODUCTION

Hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are usually regarded as toxic agents whose levels must be minimized by the action of antioxidant defence enzymes (reviewed in [1]). Enzymes capable of scavenging H_2O_2 include catalases, selenoprotein glutathione peroxidases and thioredoxin-dependent systems.[1,2] However, there is increasing evidence that H_2O_2 plays a role in redox regulation of gene transcription,[3,4] which implies that it is not completely destroyed *in vivo*. Indeed, H_2O_2 can be detected in expired human breath[5,6] and in human urine.[7–10] Three different assay methods have been used to show that freshly-voided human urine can sometimes contain H_2O_2 at levels $>100\,\mu M$ (e.g. spot samples in the UK gave a range 11–173 μM; mean ± SD of 10 samples $38.5 \pm 46.3\,\mu M$; 17 subjects in Singapore gave a range of 5.8–109.6 μM; mean ± SD $32.1 \pm 39.7\,\mu M$).[10] Levels of H_2O_2 slowly increase on allowing urine samples to stand in air (e.g. the percentage increases ranged from 17% to 139% after 3.5 h)[10] suggesting continuing generation of H_2O_2. However the significant levels found in

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freshly-voided immediately-assayed samples strongly suggest that some H_2O_2 could be generated within the bladder.[10] Centrifuging the urine had no effect on levels of H_2O_2 detected.[10]

What is the origin of this H_2O_2? It could arise from excretion of H_2O_2 produced during aerobic metabolism and/or from $O_2^{\bullet -}$-dependent oxidation of certain urinary constituents both *in vivo* and after voiding.[10] However, an origin from diet must also be considered, since many alleged "biomarkers" of oxidative damage (if indeed urinary H_2O_2 can be described as such) can be confounded by diet (reviewed in [11]). Several hot beverages commonly drunk by humans contain high levels of H_2O_2, especially instant coffee.[12–16] Indeed, it has been shown that some of this H_2O_2 can generate hydroxyl radical ($OH^{\bullet}$), within the coffee, leading to hydroxylation of caffeine.[17] We therefore wondered if drinking instant coffee could lead to increased urinary excretion of H_2O_2, or whether the H_2O_2 present in coffee is destroyed by antioxidant defence systems, e.g. catalases and peroxidases in the cells lining the gastrointestinal tract.[1] In the present paper, we have therefore investigated the effects of coffee drinking on urinary H_2O_2 levels in healthy human volunteers.

MATERIALS AND METHODS

Reagents

All reagents (except for methanol from Fluka, Switzerland) were of the highest quality available from Sigma Chemical Corp.: catalase was C40 from bovine liver, specific activity 25,000 units/mg protein.

Volunteers

Ten healthy human volunteers participated in the experiments: all had BMI within the normal range. They were asked to provide spot urine samples several times during the day, but at least 2 h after eating or drinking. Four of the subjects then drank one cup of instant coffee within 10 min. Only one of the volunteers was a regular coffee drinker (4–6 cups/day), and abstained from coffee for 10 h before the experiment. One volunteer regularly consumed a multivitamin supplement containing low levels of vitamins C and E (60 mg ascorbate and 10 mg α-tocopherol respectively) but did not consume this for 48 h prior to the experiment. Otherwise subjects undertook no special preparations for the experiment. This study did not require ethical permission, as advised by the review board of the National University Hospital of Singapore (NUH).

Preparation of Coffee

One sachet of instant coffee mix (OWL International Pte Ltd., Singapore) containing 2.5 g of instant coffee, 9 g of sugar and 6.5 g of non-dairy creamer was added to hot water and made up to 200 ml. The sugar and creamer in solution do not affect H_2O_2 production by coffee or generate H_2O_2 themselves (data not shown).

Assay of Creatinine

This was performed using the creatinine assay kit (A-555) from Sigma Diagnostics, which is based on the method of Slot.[18] Essentially, the formation of an acid-sensitive chromogen after reduction of the sample with picrate is measured at 500 nm.

Assay of H_2O_2

This was performed by the ferrous ion oxidation-xylenol orange (FOX) assay based on an absorbance change at 560 nm as described previously.[10] Usually 90 µl of urine was analysed: it was not centrifuged before use and no samples were cloudy. A control with 10^3 units of added catalase was included in all experiments[10] to check that absorbance changes were due to H_2O_2

TABLE I Urinary hydrogen peroxide in healthy human subjects

Subject	Age	Gender	$[H_2O_2]$ μM		Creatinine (mg/dl)		$[H_2O_2]$ μM Creatinine corrected	
			Pre-coffee	60 min after coffee	Pre-coffee	60 min after coffee	Pre-coffee	60 min afer coffee
A	36	F	2.1	2.6	86.0	21.9	2.1	10.2
B	49	M	28.8	85.0	218.0	228.4	28.8	81.1
C	26	M	3.8	5.1	23.4	14.2	3.8	8.4
D*	32	M	8.4	7.4	165.1	146.1	8.4	8.4

*In subject D the rise in H_2O_2 occurred after 60 min (Figure 1).

and not to any other oxidising agents that could conceivably be present in urine. This small amount of catalase (0.04 mg) did not interfere with the FOX assay, as tested using cumene hydroperoxide.[12] Some samples were analysed using a Hansatech O_2 electrode as described in;[10] briefly 1.0 ml of urine plus 0.4 ml of phosphate-buffered saline, pH 7.4, was added to the reaction chamber, the O_2 concentration allowed to stabilize and 10^3 units of catalase in 100 μl of phosphate-buffered saline injected through the cap. The "burst" of O_2 release was used to calculate the amount of H_2O_2 present.

RESULTS

It had already been observed that "spot" urine samples taken from healthy human subjects at various times during the day, but at least 2 h after eating or drinking, were approximately constant, varying by $\leq 20\%$ over 24 h (10 subjects). There was more variability (up to 50%) in samples taken in different months: e.g. subject B gave a value of 28.8 μM on May 20 1999 (Table I) and 15.2 μM on October 6 1999 (Table II). Thus it was possible to examine the short-term effects of coffee drinking on urinary H_2O_2 levels. To confirm the lack of variation in "basal" H_2O_2 levels in short-term experiments, control studies were also performed in which subjects drank an equal volume of the water used to make the coffee.

Four healthy human subjects provided urine samples at least 2 h after eating or drinking. As observed previously,[7–10] significant and easily-measurable levels of H_2O_2 were present, although there was considerable inter-individual variation (Table I). Each subject then drank one cup (200 ml) of instant coffee and provided urine samples at intervals thereafter. Table I (data before coffee, and 60 min after coffee) and Figure 1 (data at a range of time-points) shows that in all cases coffee drinking was followed by a rise in detectable urinary H_2O_2 levels before 100 min, although the time-course differed between subjects. This rise was detected by both the FOX assay and the O_2 electrode method. Because drinking fluids alters water balance, the levels of H_2O_2 in samples taken after coffee drinking were corrected on the basis of changes in urinary creatinine content (Figure 1). The coffee used in the experiments in

TABLE II The effect of coffee drinking on urinary H_2O_2 levels in a healthy human subject: a balance sheet

Time after starting to drink coffee (min)	$[H_2O_2]$	$[H_2O_2]$ corrected for creatinine	Fold increase
Pre-coffee*	15.2	15.2	—
26	41.1	58.7	3.86
56	18.4	25.6	1.68
86	11.1	15.8	1.04
116	11.2	14.8	0.97
161	11.0	15.9	1.05
221*	7.6	9.7	
28	22.9	30.1	3.10
58	24.0	29.2	3.01
88	20.1	24.0	2.47
118	15.3	17.6	1.81
178	11.8	11.3	1.16

In the first cycle 4.6% of the H_2O_2 in the coffee appeared to be recovered in the urine; in the second cycle 5.8%.
*Immediately after these samples were taken coffee drinking was started, and completed within 10 min.

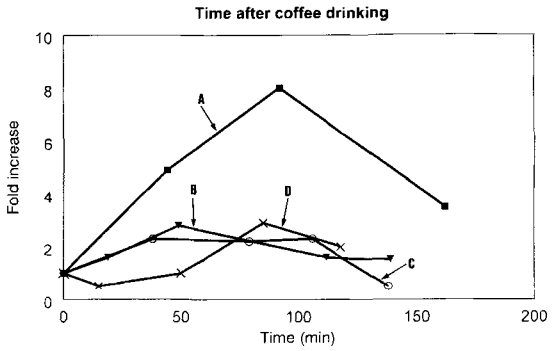

FIGURE 1 Increases in urinary H_2O_2 detected in healthy human subjects after coffee drinking. Because of the wide variation in initial H_2O_2 concentrations (Table I), data are expressed as a fold increase over the value before coffee drinking and are corrected for creatinine levels. Table I shows some of the raw data on which the calculations are based. Times shown are the times from which coffee drinking began.

Figure 1/Table I contained 23.7 μM H_2O_2. Drinking the same volume of the hot water used to make the coffee did not increase creatinine-standardized urinary H_2O_2 levels in any subject.

One volunteer (B) was subjected to further experiments to check the reproducibility of the phenomenon and to calculate the urinary recovery of the H_2O_2 in the coffee, by measuring the volumes of each urine sample taken (Table II). The rise in H_2O_2 after coffee drinking could be reproduced on 5 different days (maximum fold increase in H_2O_2 3.2 ± 0.6, $n = 5$). It could also be reproduced twice on the same day, leaving a 3 h gap between the two drinkings of coffee (Table II). The percentage of the H_2O_2 in the coffee drank that could be accounted for in the urine was 4.6–5.8% (Table II). In two other volunteers, the phenomenon could be repeated with a fold increase similar within 15% on two different days, and the percentage recovery of the H_2O_2 was 8.2–8.6%.

DISCUSSION

Instant coffee,[12–15] like many other beverages,[10] contains H_2O_2, although the levels present are often greater in instant coffee than in the other beverages examined.[12] Our data show that the drinking of instant coffee leads to a rise in urinary H_2O_2 levels within 2 h. The variability in initial levels of H_2O_2 has been described previously,[10] and could conceivably be related to differences in the diets of the subjects as well as to endogenous rates of H_2O_2 generation and H_2O_2 catabolism. Our subjects fasted for at least 2 h before providing samples and were not consuming antioxidants, but otherwise made no special preparations for the experiment.

One possible explanation of our data is that some of the H_2O_2 in the instant coffee is excreted unchanged. For example, 200 ml of coffee with 23.7 μM H_2O_2 concentration contains approximately 5 μmol of H_2O_2. If this amount were excreted in, say 100 ml of urine, it would increase the urinary level by about 50 μM. Balance sheet studies showed that the rise in urinary H_2O_2 accounted for less than 10% of the H_2O_2 in the coffee consumed, so that most of the H_2O_2 appears to be destroyed *in vivo*. It must also not be assumed that the H_2O_2 in urine arises directly from the H_2O_2 in the coffee, since there is evidence[10,19] that at least some urinary H_2O_2 is generated within the urine itself by autoxidation reactions involving $O_2^{\bullet -}$. Coffee is rich in polyphenols such as chlorogenic acid which can easily oxidize, especially if transition metal ions are present.[20,21] Another agent involved in generating H_2O_2 in coffee appears to be hydroxyhydroquinone.[16] In other words, it could be that these products are excreted in urine and then oxidize to make H_2O_2 in the bladder after voiding. Despite this, there is no clear evidence that coffee consumption is harmful to humans.[22]

Further work is needed to investigate the nature of the chemicals that lead to urinary H_2O_2 production, to extend the work to other beverages, and to assess the biological significance of H_2O_2 generation. H_2O_2 has an antibacterial effect and it may be that its production at high levels in urine could be advantageous in diminishing infections of the bladder and urinary tract. On the other hand, the impact of H_2O_2

generation *in vivo* upon the cells of the kidney, ureters, bladder and urinary tract must be considered. Indeed, there are several suggestions that reactive oxygen species are involved in the regulation of renal function.[23,24]

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