



Research article

Urinary DNA lesions as a biomarker for assessing male reproductive health

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Abstract: The study aimed to examine whether urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) could serve as a biomarker for assessing sperm DNA integrity. Urine and semen samples were collected from 124 coke-oven workers, who had chronically been exposed to polycyclic aromatic hydrocarbons (PAHs), from a steel plant in Kaohsiung, Taiwan. The coke-oven workers were divided into two subgroups: topside-oven workers and side-oven workers. Sperm DNA integrity was assessed in terms of DNA fragmentation and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo). Urine samples were used to detect 1-hydroxypyrene (1-OHP) and urinary 8-OHdG, which served to assess exposure to PAHs and the whole body oxidative stress, respectively. Urinary 1-OHP concentrations were significantly higher in the topside-oven workers than the side-oven workers ($p = 0.02$). Sperm 8-oxodGuo concentrations were correlated with percentages of sperm fragmentation ($p = 0.044$), but urinary 8-OHdG levels failed to correlate with sperm 8-oxodGuo levels and with percentages of fragmentation. In conclusion, exposure to PAHs was linked to increased oxidative stress on sperm. However, urinary 8-OHdG may not be a suitable biomarker for examining sperm DNA damage associated with oxidative stress.

Keywords: urinary 8-OHdG; 8-oxodGuo; sperm fragmentation; sperm denaturation; oxidative stress

Abbreviations:

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

PAHs: Polycyclic aromatic hydrocarbons

1-OHP: 1-hydroxypyrene

HPLC-EDC: high-performance liquid chromatography with electrochemical detection

8-OHdG: 8-hydroxy-2'-deoxyguanosine

SCSA: Sperm chromatin structure assay

8-oxodGuo: 8-oxo-7,8-dihydro-2'-deoxyguanosine

1. Introduction

PAH mixtures constitute a group of toxic and lipophilic chemicals that are widely distributed in the environment. Humans are constantly exposed to these complex mixtures through a variety of sources. These include airborne particulates, cooked foods, cigarette smoke, and emissions from industrial or contaminated waste sites [1]. PAHs can cause toxicity in several organs after metabolic activation to electrophilic intermediates [2,3]. These reactive intermediates are capable of covalent binding to DNA, potentially initiating a carcinogenic process [4]. Also, PAHs can form free-radical cations via the one-electron oxidation process, which yields the potency to cause oxidative stress to DNA [5,6,7]. Those intermediates are subject to biotransformation by glutathione S-transferase into metabolites (e.g. 1-hydroxypyrene (1-OHP)), that can be easily excreted from the body [8,9].

The most frequent member of these oxidized bases, 8-hydroxy-2'-deoxyguanosine (8-OHdG) [10], has been detected in DNA or in urine of subjects with occupational or environmental exposure [11,12]. 8-OHdG may originate from the hydrolysis of 8-oxo-7,9-dihydro-2'-deoxyguanosine 5'-triphosphate in the nucleotide pool 8-OHdG. This lesion is considered to be a highly mutagenic DNA lesion based on its propensity to form a stable base pair with adenine, resulting in G:C → T:A transversion mutations during DNA replication [13]. 8-OHdG in urine is considered an important biomarker of cellular oxidative stress for the whole biological system both in healthy subjects and in patients with cancers, diabetes, hypertension, stroke, and hematologic disorders [14,15,16]. The foregoing occurs because 1) the DNA lesion is stable in urine without being further metabolized, 2) urinary 8-OHdG has been found to be positively correlated with the oxidative damage levels in biological specimens and tissues (i.e. saliva, blood, and lung tissues from healthy subjects and patients) [5,11,15], and 3) the lesion has been studied widely as a noninvasive way to assess oxidative stress [14]. However, there is limited evidence about whether urinary 8-OHdG could serve as a molecular biomarker to assess oxidative damage in spermatozoa and/or in the reproductive system as a whole.

Animal studies have revealed significant levels of PAH metabolites in the testis and epididymis of exposed rats [17,18]. Once PAHs and the metabolites pass through the blood-testis barrier, they could undergo metabolic activation to form DNA adducts [17,18,19]. Park et al. demonstrated that metabolic activation of PAHs by aldo-keto reductases leads to the formation of redox active o-quinones and reactive oxygen species [20], which forms 8-oxodG by oxidizing the C-8 position of the guanine base of DNA [21,22,23]. Similar to other tissues and cells, 8-oxodG, can lead to chromosomal aberrations in sperm and to the induction of mutations on GC to TA transversions [10]. Sperm DNA integrity is essential for the accurate transmission of genetic information. Damaged

genomic DNA of sperm has been implicated in many reproductive and fertility diseases, including impaired fertilization and disrupted preimplantation embryonic development [24,25,26]. Oxidative stress has been recognized as a biological mechanism associated with sperm DNA damage in that sperm are susceptible to oxidative stress, because of their deoxyribonucleic acid bases and phosphodiester backbones, and in that their cytoplasm contains low concentrations of scavenging enzymes [27]. Sensitivity to oxidative stress also poses a challenge in DNA isolation for oxidative DNA base detection. Recently, the European Committee for Standardization developed and recommended DNA isolation procedures that minimize DNA oxidation [28]. Also, a newly improved chromatographic method, such as high-performance liquid chromatography with electrochemical detection (HPLC-ECD) and an on-line solid-phase system, has been established for the analysis of oxidative DNA lesions in biological samples [29,30,31]. This method operates with high selectivity and sensitivity, and no derivatisation step is required. Such analytical development and advancements have increased the analytical capacity to detect oxidative DNA damage precisely [32,33].

Despite significant knowledge about a possible role of oxidative stress in the etiology of sperm function and improvements in analytical methods for oxidative DNA base measurements, limited data are available for evaluating the relationship between oxidative damage in the reproductive system and for selecting a less invasive, appropriate biomarker for determining oxidative damage in the reproductive system.

2. Materials and Methods

2.1. Human subjects and sample collection

One hundred and twenty-four coke-oven workers from a large steel company in Southern Taiwan were recruited for this study. Among them, 56 coke-oven workers operated at the topside oven, and thus, are called topside-oven workers; these workers may have been exposed to more PAHs than the 68 workers at the side-oven who are, thus, referred to as side-oven workers. The participants also had to meet the eligibility criteria including more than one-year of employment at the plant, no reproductive dysfunction that would preclude a sperm sample, and no pre-existing urological and/or andrological disorders, including infertility, testicular injury, and surgery. Participants agreed to abstain from ejaculation for at least three days prior to the biological sampling. After screening to determine eligibility of participants, a standardized questionnaire was administered to collect demographic information (e.g. age, body mass index, and educational level), occupational history, and covariance variables (e.g. alcohol consumption, smoking habits, and betel-quid chewing). A spot urinary sample and semen sample were collected from each participant. Semen quality, including sperm concentration, motility, and vitality, was analyzed according to the World Health Organization's (WHO) Standards Methods [34]. Urinary 1-OHP, a major metabolite of PAHs, was measured to assess PAH exposure to the workers. This study was approved by the Institutional of Review Board at Old Dominion University.

2.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The TUNEL assay was used to detect sperm DNA fragmentation [35]. A sperm pellet was

obtained after 200 μL of semen was centrifuged at $250 \times g$ for 5 min. The pellet was resuspended, washed with 1% human serum albumin in PBS, and spread onto slides. Then, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate at 4 °C for 2 min. A nucleotide labeling mixture, prepared according the Roche Diagnostic manufacturer's instructions, was deployed onto sperm cells. After the cells were incubated for one hour at 37 °C, the cells were washed twice with 1% HAS in PBS. Each test included both positive and negative controls. Cells in the positive control were treated with 50 μL of DNase solution, while cells in the negative control were not treated with the nucleotide labeling mixture. Fluorescence in sperm cells recorded as a positive for the TUNEL assay was assessed using an Olympus BX61 fluorescence microscope. At least 300 sperm cells from each sample were accounted for, and the percentage of TUNEL positive cells was calculated as the outcome of interest.

2.3. Sperm chromatin structure assay (SCSA)

The SCSA was used to detect DNA denaturation as described by Evenson et al. [36]. Briefly, $1-2 \times 10^6$ sperm cells per mL were treated with a low pH 1.2 detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). After 30 s, the cells were stained by adding 1.2 mL acridine orange stain in a phosphate-citrate buffer, pH 6.0, for 3 min. Then, the cell suspensions were analyzed using a flow cytometer. Sperm chromatin damage can be quantified by cytometric measurements of the metachromatic shift from green (native double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA). Flow cytometry measurement of 5000 sperm per sample was used to determine the ratio of red to green sperm and the extent of the DNA denaturation.

2.4. Sperm DNA isolation

Sperm DNA were isolated according to the procedure recommended by the European Standard Committee on Oxidative DNA Damage (ESCODD) [28], with modifications to minimize DNA oxidization during DNA isolation procedures. Briefly, sperm samples ($15-30 \times 10^6$ sperm⁻¹) were washed with 1% HSA in PBS and centrifuged at $3000 \times g$ for 5 min. The resulting pellet was added to 600 μL of ice-cold extraction buffer (10% (w/v) SDS and 1M DTT), 30 μL of proteinase K, 30 μL of RNase A (1×10^{-2} mg/L) and 8 μL of RNase T1 (1 U/ μL). The mixture was incubated at 37 °C for 1 h and then cooled to 4 °C for 5 min. Subsequently, 1.2 mL of NaI solution and 2 mL of 2-propanol were added. After centrifugation at 5000 g for 5 min, the DNA pellet was washed with 1 mL of ice-cold 40% (v/v) 2-propanol, collected by centrifugation and dissolved in 200 μL of 0.1 mM DFO overnight. DNA concentration was measured by absorbance at 260 nm. Protein contamination was checked using the absorbance ratio A260/A280. An absorbance ratio over 1.6 was acceptable.

2.5. Detection of 8-oxo-dGuo adducts in sperm

DNA hydrolysis was performed as described by Gedik et al. [37] with some modifications. Briefly, sperm DNA samples (10–20 μg) were spiked with 2.82 pmol of [¹⁵N₅]-8-oxodGuo and 84.3 pmol of [¹⁵N₅]-dG. Then, 5 μL of 0.2 U/ μL nuclease P1 (in 300 mM sodium acetate and 1 mM ZnSO₄, pH 5.3) were added to the DNA solutions and the DNA was incubated at 37 °C for 2 h.

Thereafter, 10 μL of 10 \times alkaline phosphatase buffer (500 mM Tris/HCl, pH 8, 1 mM EDTA) together with 4 μL of alkaline phosphatase was added and the incubation was continued at 37 $^{\circ}\text{C}$ for 2 h. Subsequently, 10 μL of 0.1 M HCl was added to neutralize the solution and the neutralized DNA hydrolysates were ready for 8-oxo-dGuo analysis.

8-oxodGuo adduct in sperm DNA was determined by a LC-MS/MS with an on-line solid-phase extraction procedure as reported in recent studies [22,38]. After automatic sample cleanup, DNA samples were injected into an Agilent 1100 series HPLC system interfaced with a PE-SCIEX API 3000 triple quadrupole mass spectrometer with an electrospray ion source. Detection was performed in the positive ion multiple reaction monitoring mode for simultaneous quantification of 8-oxo-dGuo and dG. Transitions of the precursors to the product ions were as follows: 8-oxo-dGuo (m/z 284 \rightarrow 168), [$^{15}\text{N}_5$]-8-oxo-dGuo (m/z 289 \rightarrow 173), dG (m/z 268 \rightarrow 152), and [$^{15}\text{N}_5$]-dG (m/z 273 \rightarrow 157). With the use of isotopic internal standards and on-line SPE, this method exhibited a low detection limit of 1.8 fmol for 8-oxo-dGuo, which corresponds to 0.13 adducts 10^6 dG $^{-1}$ when using 20 μg of DNA per analysis.

2.6. Detection of urinary 8-OHdG

8-OHdG concentrations in urine were also measured using a validated method of LC-MS/MS with on-line SPE as previously reported [22,38]. Twenty microliter of urine was diluted 10-fold with 5% methanol containing 0.1% formic acid. After the addition of 40 μL of [$^{15}\text{N}_5$]-8-OHdG solution (20 $\mu\text{g}/\text{L}$ in 5% methanol/0.1% formic acid) as an internal standard, 100 μL of prepared urine sample was directly injected into the same on-line SPE LC-MS/MS as described above. The precision of the present method was determined by performing replicate determinations of 8-OHdG in three different urine samples. The intra- and inter-day CV were 2–3% and 4–5%, respectively [22]. With the use of isotopic internal standards and on-line solid-phase extraction, this method had a limit of detection of 5.7 ng/L (2.0 fmol) for 8-OHdG. To control for variation in urinary output, the concentration of urinary 8-OHdG was adjusted to the urinary creatinine (ng 8-OHdG/mg creatinine).

2.7. Statistical analysis

Data analysis was performed using a SAS version 9.1 (SAS Institute Inc., Cary, NC) with the results expressed as the mean \pm SEM. The distributions of semen quality parameters were examined for normality. Measures were log-transformed if their skewness indicated they were not normally distributed. For each response variable, means of semen quality parameters, sperm DNA integrity, and urinary 8-OHdG measurements were compared using Bonferroni adjusted t -tests. Multivariate regression analysis was conducted to determine the correlation between urinary 8-OHdG and sperm DNA integrity parameters, while controlling for confounding factors. All hypothesis testing was two-sided with a probability value of 0.05 or less being considered significant.

3. Results and Discussion

Table 1 provides the demographic characteristics of study subjects. Topside-oven workers and side-oven workers were similar in age, BMI, smoking habits, and alcohol consumption. The mean age of the workers was 42 years old and their mean BMI was 25. Fewer than 16% of workers

reported drinking regularly and < 45% of workers in either group were smokers. Both smoking and alcohol consumption did not significantly associate with sperm quality and DNA integrity markers. Semen parameters for the topside-oven workers were comparable with those of the side-oven workers, except for sperm vitality. The topside-oven workers had a numerically lower percentage of vitality (69.5%) as compared with the side-oven workers (75.2%). However, these percentages were not statistically different. PAH exposure did not significantly change sperm quality.

Table 1. Characteristics of subjects and sperm quality.

| | Topside oven workers <i>n</i> = 56 | Side oven workers <i>n</i> = 68 | General population ^a |
|---|---------------------------------------|------------------------------------|---------------------------------|
| Age (years, mean ± SD) | 43 | 41 | |
| BMI (kg/m ² , mean ± SD) | 25.4 ± 3.81 | 24.4 ± 3.89 | |
| Drinking status [n(%)] | 58.6 | 51.4 | |
| Smoking [n(%)] | 44.5 | 40.5 | |
| Semen quality | | | |
| Total count (mL) | 261.8 ± 108.6 | 232.7 ± 92.3 | |
| Concentration (10 ⁶ /mL) | 119.4 ± 100.2 | 110.8 ± 97 | 78.5–113.6 |
| Motility (%) | 53.9 ± 18.9 | 57.8 ± 22.2 | 55.0–66.3 |
| Morphology (%) [*] | 24.7 ± 4.3 | 20.9 ± 3.6 | 7.9–70 |
| Vitality (%) | 69.5 ± 17.7 | 75.3 ± 21.1 | 78.8–93.3 |
| Sperm concentration < 15 × 10 ⁶ /mL (%) ^b | 0 | 1.0 | |
| Motility < 40% (%) ^b | 20 | 9 | |
| Vitality < 58% (%) ^b | 14 | 12 | |
| Norm forms < 4% (%) ^b | 20 | 19 | |
| 1-OHP concentration (µg/g creatinine) | 12.8 ± 15.0 | 4.4 ± 10.9 | |

* normal form

^a General population: Rubes et al.2005; Zribi et al., 2011; Singh et al., 2011 [39,40,41]

^b Percentages of participants who have semen parameters below cut-off values recommended by the WHO (WHO 2010)

The concentrations of urinary 1-OHP from PAHs in the topside-oven workers were significantly higher than those in the side-oven workers ($p = 0.02$) (Table 1). These results suggested that the topside-oven workers had been exposed to higher PAH concentrations than the side-oven workers [34]. The topside-oven workers had increased levels of urinary 8-OHdG levels and sperm 8oxodGuo as compared with those of the side-oven workers (Table 2). Such results suggest that PAH exposure could lead to the formation of oxidative adducts, which increases oxidative stress on the reproductive system. In accordance with WHO guidelines, it was determined whether subjects had asthenozoospermia and/or oligozoospermia. In accordance with the WHO guidelines in 2010, levels for urinary 1-OHP and DNA integrity parameters were determined for those with sperm concentrations < 15 × 10⁶/mL, motility < 40%, and vitality < 58%. The levels of urinary 1-OHP and DNA integrity parameters of participants with sperm concentrations < 15 × 10⁶/mL were comparable

with those of all of the participants (Table 2). Participants with motility < 40% and vitality < 58% had experienced increased levels of DNA integrity parameters as compared to all of the participants, whereas urinary 1-OHP seemingly remained stable between the two groups. Urinary 8-OHdG levels were not correlated significantly with sperm motility and concentration. Other studies have also reported similar results and suggest that semen quality parameters may be independent of oxidative damage to the reproductive system.

Table 2. Concentrations of sperm DNA integrity and urinary oxidative stress status among the study groups*.

| | Urine | Sperm | | |
|--|---------------------------------|--------------|-------------|---|
| | 8-OHdG (ng/mg creatinine) | TUNEL (%) | SCSA (%) | 8-oxodGuo (/10 ⁶ dG) ^a |
| Topside-oven workers (<i>n</i> = 88) | 2.95 ± 1.29 | 39.7 ± 24.8 | 14.5 ± 8.2 | 30.2 ± 30.7 |
| Side-oven workers (<i>n</i> = 98) | 2.79 ± 0.89 | 32.9 ± 20.2 | 12.3 ± 6.3 | 18.3 ± 19.1 |
| Sperm concentration < 15 × 10 ⁶ (<i>n</i> = 18) | 2.99 ± 1.25 | 40.5 ± 21.7 | 14.7 ± 7.6 | 26.3 ± 25.7 |
| Motility < 40% (<i>n</i> = 66) | 3.13 ± 1.42 | 46.7 ± 16.3 | 14.8 ± 8.2 | 27.8 ± 24.2 |
| Vitality < 58% (<i>n</i> = 62) | 3.01 ± 1.46 | 48.9 ± 15.3 | 15.2 ± 8.9 | 28.1 ± 25.2 |

* values are mean ± SD; different; SCSA indicates sperm chromatin structure assay; TUNEL, TdT-mediated-dUTP nick and labeling

^a The 8-oxodGuo level for the topside-oven workers was significantly higher than the concentration for the side-oven workers (*p* = 0.027).

Sperm 8-oxodGuo was positively correlated with DNA fragmentation and denaturation measured by the TUNEL assay and SCSA, respectively (Table 3). The TUNEL assay allows the detection of single- and double-stranded DNA fragmentation [15,38]. These results are consistent with findings from other studies [38,42] regarding oxidative stress being a biological mechanism contributing to DNA damage to sperm. A study suggested that the generation of TUNEL-reactive 3'-OH termini in sperm DNA is a perimortem change that takes place sometime after the initial oxidative insult, whereas the DNA denaturation, a single-strand break, is detected in the early stage of spermiogenesis [43]. The positive correlation between DNA denaturation and fragmentation may suggest the occurrence of oxidative DNA damage in the spermiogenesis stage (Table 2). Oxidative stress induced PAHs could affect early stages of DNA packing and lead to losses of DNA integrity, which may reduce the efficiency of DNA compaction during the terminal stages of spermiogenesis [43,44,45]. If the lesions are repaired by the base excision repair (BER) pathway, sperm could increase protective potency against oxidative insult after the final stage of chromatin organization in which protamine cross-linking occurs by disulphide bond formation for chromatin compaction [43].

Table 3. Correlations between urinary 8-OHdG, sperm quality, and DNA integrity from all subjects

| | Urinary 8-OHdG R (<i>p</i>) | Sperm 8-oxodGuo R (<i>p</i>) |
|--|----------------------------------|-----------------------------------|
| Sperm DNA integrity | | |
| 8-oxodGuo | 0.67 (0.09) | – |
| TUNEL | 0.56 (0.32) | 0.32 (0.044)* |
| SCSA | 0.12 (0.42) | 0.24 (0.032)* |
| Semen quality | | |
| Concentration | –0.40 (0.46) | –0.081 (0.28) |
| Motility | | |
| Progressive | –0.32 (0.21) | –0.039 (0.83) |
| Non-linear | –0.04 (0.67) | –0.045 (0.36) |
| Non-progressive | –0.11 (0.83) | –0.021 (0.56) |
| Morphology | | |
| Normal form | –0.03 (0.23) | –0.089 (0.45) |
| Head defects | –0.06 (0.48) | –0.092 (0.54) |
| Vitality | –0.32 (0.65) | –0.056 (0.32) |
| Sperm Concentration < 15 × 10 ⁶ /mL | –0.12 (0.34) | –0.08 (0.11) |
| Motility < 40% | –0.09 (0.11) | –0.22 (0.09) |
| Vitality < 58% | –0.06 (0.18) | –0.13 (0.06) |

* *p* values less than 0.05

Our analysis indicated that 8-oxodG levels in sperm DNA were less than that in urine. These results differed from Guz et al.'s finding that the level of 8-oxodG in sperm DNA was about 25% higher than in leukocytes [44]. The possible explanation for the different observations could come from differences in subjects, who may have different exposure levels and reproductive conditions. Guz et al. studied infertile patients who may have experienced a higher degree of oxidative DNA damage in the male germ line than did our participants who were exposed to PAHs. The protective mechanism of the testis-blood barrier could lead to less PAHs entering the testis compared to the whole biological system.

We did not observe that urinary 8-OHdG correlated with sperm 8-oxodGuo or with DNA fragmentation (Table 3), suggesting no relationship between the whole body oxidative stress and that of sperm. The insignificant correlation may be due to 1) the origination of lesions from different pathways and repair mechanisms involved; 2) repair timing, and 3) differences in levels of antioxidants. Urinary 8-oxodGuo concentrations, which reflect the overall oxidative DNA damage in the biological system, were released initially into the bloodstream and then excreted into the urine. Urinary 8-OHdG reflected the results of a cellular DNA excision repair process including nucleotide excision repair (NER) and the base excision repair (BER) in the whole body, rather than the result of changes within specific tissues or cell types [43,45].

For the sperm, the repair pathway mainly involved the NER. The second factor for the difference could be the stage of DNA repair for sperm. Although oxidative DNA adducts in most germ cell stages could be repairable early during spermatogenesis (spermatocytes and early spermatids), DNA repair does not occur in condensed spermatids and sperm in epididymis where protamine has replaced

somatic histone, rendering the DNA transcriptionally inert [46]. In addition, NER is limited to testicular cells, spermatocytes, and round spermatids [47]. Finally, activity of enzymes involved in DNA repair could be different in the reproductive system than the whole biological system. Guz et al's study showed that the degree of change to antioxidant capacity due to environmental exposure differs between the whole biological system and the reproductive system [15]. A significant decrease in ascorbic acid concentration in smokers' blood existed, while the exceptionally high level of ascorbic acid in sperm plasma had been detected and constitutes a good buffer to protect sperm DNA against ROS attack [48]. The study showed that the reproductive system likely compensates for reduced antioxidant capacity due to the limited volume of sperm cytoplasm via a high concentration of low molecular weight antioxidants, such as ascorbic acid [48,49].

4. Conclusion

The study demonstrated that PAH exposure did not significantly change sperm quality, however, such exposure did induce oxidative damage to sperm DNA. No relationship between the whole body oxidative stress and that of sperm plasma was found, which suggests that the oxidative stress status of semen may be rather independent of this characteristic for other human tissues. A lack of correlation between urinary 8-OHdG levels and sperm quality and oxidative damage markers suggested that urinary 8-OHdG may not be a sufficient molecular biomarker for the assessment of oxidative damage to sperm and the reproductive system. However, additional studies are needed to verify the outcome of this study.

Conflict of interest

The authors have no potential conflict of interests on financial or commercial matters associated with the study.

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