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Original article 尿液中氮氧化物是自闭症谱系障碍的一种新的潜在生物标志物

Urinary NO_x, a novel potential biomarker for autism spectrum disorder

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一氧化氮（NO）参与了人类的许多生理和病理过程。尿液检查告诉我们很多关于健康的事情，这些都是方便和无害的。氧化还原压力，包括 活性氮物种及其代谢物NOx在自闭症谱系障碍（ASD）研究中受到越来越多的关注。然而，在ASD中尿亚硝酸盐和硝酸盐的浓度仍然不清楚。在本研究中，在44名ASD儿童、30名健康儿童（对照组）和28名健康成人的尿液标本中精确测量亚硝酸盐和硝酸盐。本文首次报道了ASD和健康儿童尿NOx的浓度。在ASD人群中亚硝酸盐含量高于对照组，浓度分别为0.8708±0.1121 μM（0.1556-3.0393 μM）和0.5938±0.07276 μM（0.1134-2.1004 μM）（p=0.0420）。成人组亚硝酸盐为0.5808±0.0985 μM（0.0808-1.9335 μM），与对照组相似。相反，ASD儿童尿硝酸盐浓度低于对照组，为2.875±0.2716mm（0.3264-7.1835mm），分别为4.558±0.5915mm（1.1860~15.8555mm）（p=0.0133）。成人硝酸盐也明显低于对照组，分别为2.799±0.3640mm（0.2507-8.6978mm）和4.558±0.5915mm（p 分别=0.0146）。ASD和对照组的亚硝酸盐/硝酸盐比值分别为0.3496±0.04382x10-3和0.1604±0.01862x10-3（p=0.0002），这再次表明NOx作为新的生物标记物。此外，没有发现NOx与性别之间的相关性，以及样本收集的时间。总之，NOx与ASD之间的关联是显著的。尿NO2-/NO3-可能作为ASD诊断的一种新的生物标志物，在追求无害、快速、方便的诊断方法的过程中。一氧化氮在ASD发病机制中的作用和代谢途径有待进一步研究 。

ABSTRACT

Nitric oxide (NO) participates in many physiological and pathological processes in human. Urine tests tell a lot about health, which are convenient and harmless. Redox stress, including imbalance of reactive nitrogen species and its metabolites NO_x, has been gaining increased attention in autism spectrum disorder (ASD) research. However, concentrations of urinary nitrite and nitrate among the ASD population stay unclear. In this study, nitrite and nitrate were precisely measured in urine specimens from 44 ASD children, 30 healthy children (the control group) and 28 healthy adults with an optimized and validated analytic method. For the first time, concentrations of urinary NO_x in ASD and healthy children were reported. Nitrite in the ASD population is higher than in the control group, with concentrations of 0.8708 ± 0.1121 μM (0.1556–3.0393 μM) and 0.5938 ± 0.07276 μM (0.1134–2.1004 μM) ($p = 0.0420$), respectively. Nitrite in the adult groups is 0.5808 ± 0.0985 μM (0.0808–1.9335 μM), which is similar to that in the control group. On the contrary, urinary nitrate concentration in ASD children is lower than that in the control group, which are 2.875 ± 0.2716 mM (0.3264–7.1835 mM) and 4.558 ± 0.5915 mM (1.1860–15.8555 mM) ($p = 0.0133$), respectively. Nitrate in adults is also significantly lower than that in the control, 2.799 ± 0.3640 mM (0.2507–8.6978 mM) and 4.558 ± 0.5915 mM ($p = 0.0146$), respectively. Nitrite/nitrate ratios for ASD and the control groups were 0.3496 ± 0.04382 x 10^{-3} and 0.1604 ± 0.01862 x 10^{-3} ($p = 0.0002$), which again indicated the probability of NO_x as a novel biomarker. Furthermore, no correlation between NO_x and gender, as well as sample collection timing was found. Taken together, the association between NO_x and ASD was significant. Urinary nitrite, nitrate and NO_2^-/NO_3^-, might serve as a new biomarker for ASD diagnosis during pursuit of harmless, fast, and convenient diagnostic method. Further studies are needed for the metabolic pathways of NO_x in ASD pathogenesis.

例如，美国约59名儿童中有1名儿童，中国约1000名儿童中有39名儿童被诊断为ASD[3]。由于ASD对个人和社会的巨大负面影响，很多人感兴趣 已被刺激到ASD的探索和鉴定生物标志物，以帮助早期诊断和治疗。如今，部分参与氧化还原平衡的生物标志物已陆续被报道，包括维生素 B12、血清素、催产素、谷胱甘肽等

1. Introduction 自闭症谱系障碍（ASD）是一种复杂的神经发育综合征，影响一个人如何感知和与他人交往。多发于儿童。

Autism spectrum disorder (ASD) is a complicated neurodevelopmental syndrome, influencing how a person perceives and socializes with other people [1,2]. With increasing recognition and more advanced diagnostic tools, more children have been found to suffer from ASD. For example, about 1 in 59 children in the United States and 39 in 1000 children in China are diagnosed with ASD [3]. Due to huge negative impact of ASD to individuals and the society, much interest has been spurred into exploration and identification biomarkers of ASD to aid early diagnosis and therapy. Nowadays, few biomarkers participated in redox balance have been reported, including vitamin B_{12}, serotonin, oxytocin, glutathione, etc [2]. As one of these reactive nitrogen species, nitric oxide (NO) is a potent signaling molecule involved in many physiological and pathological aspects of human cells. NO has dual roles and could be both neuroprotective and neurotoxic [4,5]. Recent reports about ASD found that NO could regulate the activity of polyunsaturated fatty acids [6] and the concentration of nitrite and nitrate in blood of ASD group were greatly higher than that of normal group [7–9], which is positively correlated with IFN-γ levels. However,

一氧化氮（NO）作为这些活性氮的一种，是一种强有力的信号分子，涉及人类细胞的许多生理和病理方面。NO有神经保护和神经毒性的双重作用。最近关于ASD的报道发现NO可以调节AS血液中多不饱和脂肪酸的活性以及ASD患者体内亚硝酸盐和硝酸盐的浓度明显高于正常组，与IFN-γ水平呈正相关。

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亚硝酸盐（NO2-）和硝酸盐（NO3-）又称NOx，是NO在血液和尿液中的两种主要稳定代谢产物。然而，只有少数研究报告了尿NO2-和NO3-与 生理学和发病机制。
1996年，Davidge等人发现子痫前期妇女尿液中NO2和NO3的浓度降低，但在血浆中无明显浓度差异。
Stichtenoth等人发现类风湿性关节炎患者尿硝酸盐的排泄量约为健康成年人的2.7倍。
Chao等人发现尿路感染（UTI）患者的亚硝酸盐浓度和亚硝酸盐/硝酸盐浓度比高于健康受试者。
感染者尿液在轻度酸化时产生大量NO，这可能解释了为什么尿酸化可以治愈UTI。
此外，一项针对大量人口的研究表明，尿中硝酸盐浓度与高血压和中风发病率以及心血管死亡风险有关。

urinary NO_x levels in ASD population haven't been declared yet.

Nitrite (NO_2^-) and nitrate (NO_3^-), also called NO_x, are the two major stable metabolites of NO in blood and urine. However, only a few studies reported the connection of urinary NO_2^- and NO_3^- with physiology and pathogenesis. In 1996, Davidge et al. found that the concentration of NO_2^- and NO_3^- in urine of women with preeclampsia decreased, and no significant concentration differences of these two anions were detected in plasma [10]. Stichtenoth et al. found that the excretion of urinary nitrate in patients with rheumatoid arthritis was about 2.7-fold greater than that of healthy adults [11]. Chao et al. found that the concentration of nitrite and the concentration ratio of nitrite/nitrate were higher in urinary tract infection (UTI) patients than that in healthy subjects [12]. Infected urine was found to produce large amount of NO upon mild acidification, which might explain why urinary acidification could cure UTI [13]. Lower blood pressure was associated with modest higher nitrate in adult urine, which was similar to 100 mmol reduction of sodium intake [14]. In addition, a study on large population had showed that urinary nitrate was associated with a lower prevalence of hypertension and stroke and with a lower risk of cardiovascular death [15].

到目前为止，ASD的尿NOx水平还没有报道。在本研究中，我们首先验证了亚硝酸根和硝酸盐在水溶液中的NOA分析方法。然后几种预处理方法 并行比较，优化尿液样品制备策略。然后对尿亚硝酸盐和硝酸盐进行精确定量。

To date, the level of urinary NO_x in ASD hasn't been reported. In this study, we first validated the NOA analytic method of nitrite and nitrate in aqueous solution. Then several pre-treatment methods were compared in parallel to optimize sample preparation strategy of urine. Precise quantitation of urinary nitrite and nitrate were then followed. The ranges of nitrite and nitrate in healthy and ASD children were reported for the first time, as well as in healthy adults who were between 18 and 25 years old. After that, the concentration of NO_x between ASD and control groups, adult and control groups were compared to suggest nitrite, nitrate and nitrite/nitrate ratio as a new potential biomarker for ASD diagnosis. Besides, sex and timing of sample collection was not a factor influencing concentrations of NO_x in urine.

2. Materials and methods

2.1. Subject recruitment

A total of 74 children and 28 adults were recruited at Hubei Maternity and Child Health Hospital (Wuhan City, Hubei Province, China). Among these children, 44 of them were diagnosed with ASD, and 30 of them were children with typical development, which was the control group.湖北妇幼保健院共招募74名儿童、28名成人。

对照组：30名健康儿童
实验组1：44位ASD儿童患者
实验组2：28名健康成人

2.2. Urine sampling

In order to monitor the concentration change of nitrite or nitrate with time, urine from adults was collected at 7:30 am, 9:00 am, 9:30 am, 10:30 am, 11:30 am, 1:00 pm, 1:30 pm, 2:30 pm, 5:30 pm, 7:00 pm, 7:30 pm, 8:30 pm in two consecutive day and analyzed immediately after sampling.

To analyze the difference of nitrite and nitrate between ASD patients and healthy people, spot urine specimens from control groups and adult groups were collected using propene polymer one-time urine cup at 10 am and then transferred into a 10 mL propene polymer tube for immediate analysis or storage for next-step management.

2.3. Pre-treatment of urinary samples

Urine specimens were prepared via five different methods at room temperature [16–19]: (1) omission of pre-treatment; (2) centrifugation: 2 mL of fresh urine was transfer to centrifuge tubes and centrifuged at 1000, 3000, 5000, 8000, 10000 rpm for 8 min, respectively. The supernatant was then filtered with 0.45 μM polyethersulfone filter; (3) protein precipitation with saturated zinc sulfate. 2 mL of freshly urine was transfer to centrifuge tubes. Then 0.1, 0.2, 0.5, 1, 2 mL of saturated zinc sulfate solution were added, respectively. After sealing, the tubes were treated in a water bath at 70 °C for 15 min. After cooling to room temperature, the supernatant was filtered through a 0.45 μM polyethersulfone filter; (4) decolorization with active carbon: 0.1, 0.2, 0.4, 0.5, 1g of activated carbon were added in 5 mL of fresh urine, respectively. After vortex for 30 s, samples were filtered with a 0.45 μM polyethersulfone filter; and (5) protein precipitation with ethanol: 0.1, 0.25, 0.4, 0.5, 1 mL of ethanol were added in 1 mL of fresh urine, respectively. And then the resulted urine was shaken and centrifuged at 5000 rpm for 8 min. Then the supernatant was filtered with 0.45 μM polyethersulfone filter. All the above obtained filtrate was analysis by Nitric Oxide Analyzer. When nitrate was analyzed, the obtained filtrate was diluted 1000 times with deionized water before measurement.

2.4. Measurement of urine pH at various time and temperature

The pH meter (Mettler Toledo, FiveEasy Plus FE28) was employed to measure the pH of urine. Before use, the pH meter was calibrated by standard calibration solutions (pH 4.01, 7.00 and 9.21). After storing at different temperatures at various length of time, samples were first warmed up or cooled down to room temperature before pH measurement.

2.5. Measurement of urinary nitrite and nitrate

Nitric Oxide Analyzer (NOA 280i, GE, US) was adopted to analyze the concentration of nitrite and nitrate with N_2 as carrier gas [20]. 0.011 g/mL iodine ion reagent was adopted for nitrite detection and 0.008 g/mL vanadium trichloride solution, which contained 1 M HCl, was loaded into the purge vessel for nitrate detection.

Duplicate injections of standard solutions into the NOA purge vessel was performed. Calibration curves were created according to literature [20]. The injection volume of urine specimens was 100 μL and all the urinary tests were repeated three times at room temperature.

2.6. Statistical analysis

Comparisons between two groups were performed using a two-tailed unpaired Student's t-test with Origin 8.0 (OriginLab) and Graph Pad Prism 7 (Graphpad). Values for all measurements were expressed as mean ± SD for parametric distributions. $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Subjects recruited for this study

Of recruited subjects, 44 children had a diagnosis of ASD (42 male and 2 females, 2–7 years old), 30 were classified as typically developing children (the control group, 18 male and 12 female, 3–7 years old), and 28 adults were healthy volunteers (14 male and 14 female, 18–25 years old). All ASD subjects received a diagnosis by two child development experts at Hubei Maternity and Child Health Hospital (Wuhan, Hubei Province, China), according to Autism Diagnostic Observation Schedule (ADOS) [21] and characteristics of onset pattern of ASD defined according to Ozonoff et al. [22]. All subjects recruited for this study have agreed with the research consent, who were not under medication.

3.2. Validation of Nitric Oxide Analyzer for analysis of nitrite and nitrate

Ozone-based chemiluminesence is a popular technique to measure NO_x, when appropriate reductive chemistry is applied. Nitrite or nitrate produces stoichiometric amount of NO with tri-iodide method [20] (Equation (1)) or Vanadium (III) trichloride (VCl_3) method [23] (Equation (2) and (3)). Nitrate is calculated after subtracting the amount of nitrite from the total quantity of NO, when the VCl_3 method

is used.

$$2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O \quad (1)$$

$$NO_3^- + 3V^{3+} + H_2O \rightarrow NO + 3VO^{2+} + 2H^+ \quad (2)$$

$$HNO_2 + V^{3+} \rightarrow NO + VO^{2+} + H^+ \quad (3)$$

To validate the relationship between the NOA chemiluminescence signal with nominal amount of NO, detections of nitrite and nitrate in standard solutions were performed and corresponding standard curves were plotted. Calibration curves were created based on area under curve (AUC) against nominal amount of nitrite or nitrate. Standard solutions of nitrite and nitrate were prepared in ultra-pure Milli-Q water with sodium nitrite and sodium nitrate as standard compounds. Nominal concentration was set at 0.04, 0.06, 0.08, 0.1, 0.2, 0.4 0.0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 8.0 and 10.0 μM for nitrite, and 1.0, 2.0, 3.0, 5.0, 8.0 and 10.0 μM for nitrate. The limits of detection (LODs) and the limits of quantification (LOQs) were calculated according to signal-to-noise ratio of 3:1 and 10:1, respectively. The intra-day and inter-day precisions and relative recovery were validated based on analysis of three-concentration standard solutions (0.04, 1.0 and 10 μM for nitrite; 1.0, 5.0 and 10 μM for nitrate). Duplicate injections of each sample (100 μL) were made into the NOA purge vessel. Raw data from the Sievers program were transferred into a second program, Origin 8.0 (OriginLab) for analysis. Due to existence of trace amount of nitrate in Milli-Q water, aqueous nitrate levels were normalized by subtracting nitrate in Milli-Q water.

The results showed that the chemiluminescent signal was linear with increasing concentrations of nitrite and nitrate, with slope equal to 0.1121 (units of area/pmol, $R^2 = 0.9992$) and 0.0920 (units of area/pmol, $R^2 = 0.9991$) for nitrite and nitrate, respectively (Fig. 1, Table 1). The slope of the line fell into the right range of the NOA design, which is 0.09–0.13 [20]. The LOD and LOQ of nitrite was 0.03 μM and 0.11 μM, and the LOD and LOQ of nitrate was 0.02 μM and 0.07 μM. The relative recoveries were in the range of 97.7–107.5% with relative standard deviation (RSD) no more than 3.5% and 3.8% for intra-day and inter-day experiment, respectively (Table 1, Supplementary Fig. S1). These results confirmed the reliability of this chemiluminescent method for urinary NO_x analysis.

3.3. Evaluation of pre-treatment methods

There exist complicated matrices (such as uric acid, protein, cell) in urine, which could interfere detection of analyte. An efficient pre-treatment method is required to reduce matrix interference and achieve high accuracy. At present, co-existence of multiple pre-treatment methods actually has caused confusion for NO_x quantitation in urine, for parallel comparison among them is lacking. In this study, four pre-treatment methods were adopted to select an efficient pretreatment method for analysis of urinary nitrite and nitrate [16–19]. And the results were shown in Fig. 2.

We carefully evaluated whether pre-treatment caused any artificial effects on urinary nitrite or nitrate. Nitrite level varied with centrifugation speeds, and nitrate level decreased with increased speeds in the range of 0–3000 rpm (Fig. 2A). Pre-treatment with 0.1–2 mL saturated $ZnSO_4$ lowered the nitrite level, without apparently influencing nitrate concentration in urine (Fig. 2B). Mixing with 0.1–1.0 g active carbon caused elevated levels of nitrite and varied levels of nitrate (Fig. 2C). Treatment with ethanol led to increased nitrite and varied levels of nitrate (Fig. 2D). Samples after pre-treatment were shown in Supplementary Fig. S2. To date, this is the first time that different pre-treatments were compared in parallel, which indicated that omission of treatment should be the best option for analysis of urinary NO_x. Therefore, urine specimens were directly injected into the purge vessel for nitrite and nitrate analysis.

3.4. Effect of storage temperature and time on urinary nitrite and nitrate

In order to find the balance between convenience of measurement and accuracy of NO_x in urine, we evaluated the influence of storage temperature and time on analytic results. Fresh urine samples were collected at 10 am from 11 ASD subjects, which were immediately

结果表明，随着亚硝酸盐和硝酸盐浓度的增加，化学发光信号呈线性关系，斜率分别为0.1121（面积/pmol单位，R2=0.9992）和0.0920（单位： 亚硝酸盐和硝酸盐的面积/p mol，R2=0.9991）

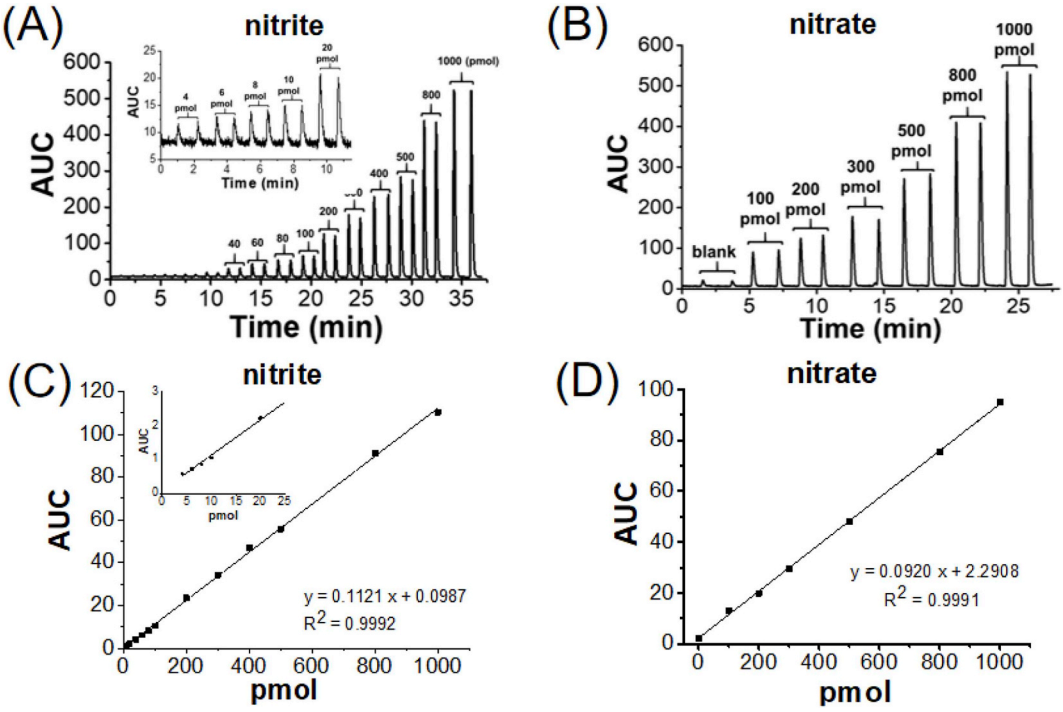


Fig. 1. Nitrite and nitrate standard curves. (A, C) nitrite standard curve in the tri-iodide method. Known amount of nitrite (0–1000 pmol) were injected in duplicate into the I_3^- solution. (B, D) nitrate standard curve in the vanadium trichloride method. Known amount of nitrate (0–1000 pmol) were injected in duplicate into the V^{3+} solution.

Table 1
Recovery of nitrite and nitrate from standard solutions.

Analytes	Linear equation	Linear range (μM)	R^2	Added (μM)	Intra-day (n = 6)		Inter-day (n = 3)	
					Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Nitrite	y = 0.1121 x + 0.0987	0.04–10	0.9992	0.04	104.5	3.5	107.5	3.6
				1	99.2	2.5	103.3	3.8
				10	103.5	0.5	106.4	0.6
Nitrate	y = 0.0920 x + 2.2908	1–10	0.9991	1	104.2	2.1	104.3	2.5
				5	97.8	1.6	99.4	1.7
				10	97.7	0.4	99.9	0.5

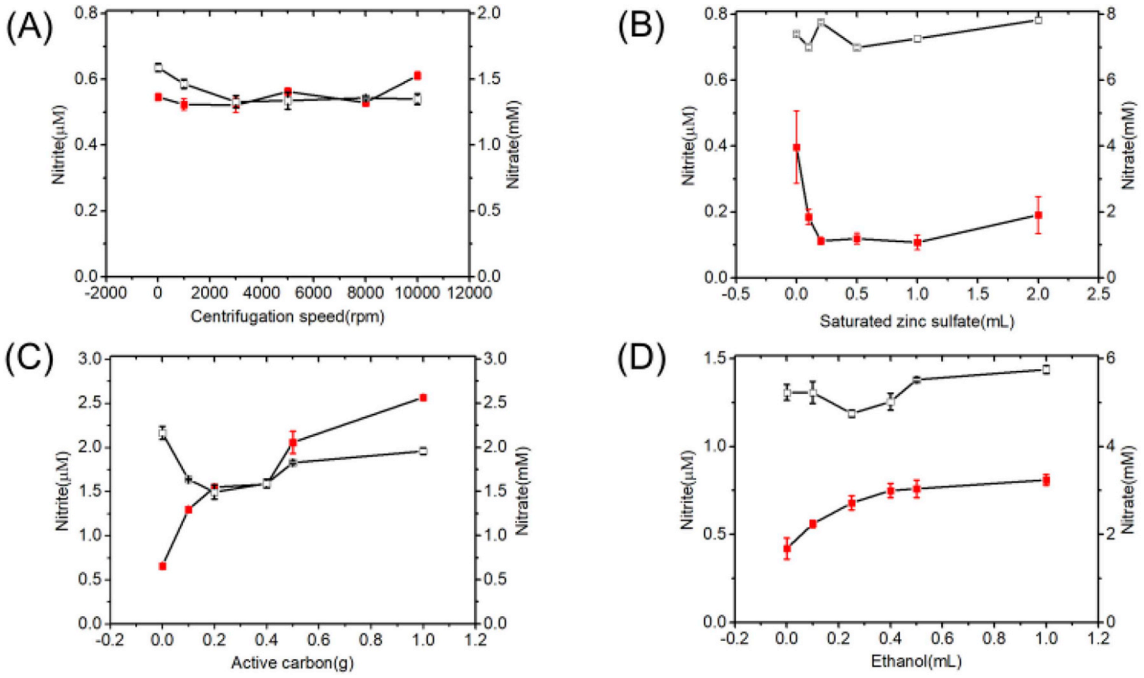


Fig. 2. Nitrite and nitrate measurements after various pre-treatments (nitrite was labelled as red solid square; nitrate was labelled as black empty square). (A) after centrifugation; (B) after treatment with saturated zinc sulfate; (C) after treatment with active carbon; (D) after treatment of ethanol. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analyzed. Then each sample was divided into aliquots for storage at different temperature with different length of time. After stored at -20 °C, 4 °C, 25 °C or 37 °C for 3, 6, 12, 24, 48, 72, 96 or 120 h, urinary nitrite and nitrate were quantitated again. Concentration of each sample was calculated and recorded based on average of three aliquots.

The result showed that nitrite increased with temperature and time during the five-day storage, but nitrite of certain samples increased first and then decreased afterwards. The maximum increase of nitrite in five days was around seven folds at -20 °C, eight folds at 4 °C, 1700 folds at 25 °C, and 2400 folds at 37 °C, respectively (Supplementary Figure S3 A-D). When nitrite levels were compared at different time points, significant elevation of nitrite levels were observed at 4 °C (3 h versus 24 h), 25 °C (3 h versus 24 h) and 37 °C (3 h versus 12 h) (Supplementary Figure S4 A-D). Apparently, higher temperature induced faster elevation of nitrite in urine.

On the other hand, an apparent drop of nitrate levels was observed from 3 h to 6 h after collection (Supplementary Figure S3 E-H). Afterwards, nitrate concentrations remained relatively stable, accompanying slight vibration at -20 °C, 4 °C and 25 °C and about three-fold decrease at 37 °C within 120 h. A closer look within 24 h indicated that urinary nitrate was significantly decreased after 6, 12, 24 h-storage, compared to 3 h-storage (Supplementary Figure S4 E-H).

Therefore, urine specimens need to be measured shortly after fresh collection, and measurement within 3 h could be a reasonable time frame considering the balance between convenience and accuracy. Decreased trend of nitrate was in accordance with increased trend of nitrite, which might be due to urinary bacteria-catalyzed nitrate decomposition and pH might participate in this process.

3.5. Effect of storage temperature and time on urine pH

Nitrogen variation of urine composition may be caused by genetic differences, physical activities, environmental conditions, as well as pH [24]. Urinary pH range of 29 ASD children was between 5.02 and 7.38 (6.23 ± 0.70). The urine pH of 30 healthy children was between 5.06 and 7.89 (6.11 ± 0.78), which was not significantly different from that in ASD children. In order to understand whether pH is correlated with temperature and time, we investigated the influence of different storage temperature and time on urinary pH in parallel.

The hypothesis is that pH increases with storage time and higher temperature [25]. Immediately after fresh collection from four ASD children, the original pH was measured. Afterwards, pH was monitored after stored for 3, 6, 12, 24, 48, 72, 96, or 120 h at each temperature (-20 °C, 4 °C, 25 °C or 37 °C) (Supplementary Fig. S5). Triplicate measurements were carried out, averaged and recorded. Within five days, the maximum increase of pH (ΔpH) was 0.54 at -20 °C, 0.38 at 4 °C, 0.61 at 25 °C, and 2.82 at 37 °C. Apparently, the greatest change of urine pH occurred at 37 °C. Though both NO_x and pH changed with

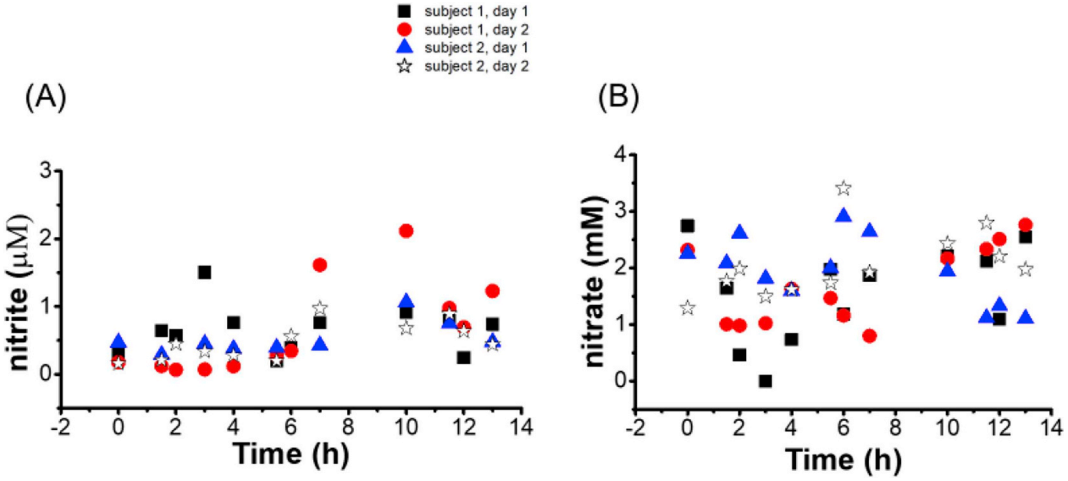


Fig. 3. Measurement of urinary nitrite and nitrate were performed at 12 time points per day for two consecutive days. (A) nitrite analysis of two subjects from 7:30am (set as 0 h of x axis) to 8:30pm (set as 13 h of x axis). (B) nitrate analysis of two subjects from 7:30am (set as 0 h of x axis) to 8:30pm (set as 13 h of x axis). Samples were collected at 7:30am, 9:00am, 9:30am, 10:30am, 11:30am, 1:00pm, 1:30pm, 2:30pm, 5:30pm, 7:00pm, 7:30pm, and 8:30pm. Breakfast, lunch and dinner were taken at 8:30 am, 12:30 pm and 6:30 pm. Nitrite in urine samples was measured by NOA without any pretreatment, and nitrate in urine samples was measured by NOA after 1000-fold dilution.

temperature and time, the association between urine pH and NO_x was equivocal at this stage.

3.6. Effect of sampling time on urine nitrite and nitrate

The first urine in the morning was usually used for NO_x analysis, but whether sampling time affects the concentrations is unclear. The hypothesis is that NO_x doesn't follow certain pattern during one individual's daily routine. Due to the difficulty obtaining multiple samples from children, we monitored the concentration change of nitrite and nitrate with time in two adults.

After 12 collections of urine at different time of a day, fresh samples were immediately measured in the lab after collection (Fig. 3). By tracking NO_x for two consecutive days, no regular pattern was found for nitrite or nitrate. Food intake didn't give rise to NO_x in urine, either. Therefore, in order to coordinate all volunteers for sampling, urine was collected at 10 am for all three groups, i.e. ASD children, healthy children and adults.

3.7. Quantitation of urinary nitrite and nitrate in ASD children, the control group and adults

Spot urine specimens were collected at 10 am, which were analyzed within 3 h. Among 44 ASD children (42 male and 2 female), nitrite level was 0.8708 ± 0.1121 μM, in the range of 0.1556–3.0393 μM (Fig. 4, Table 2). Among 30 healthy children (18 male and 12 female), nitrite level was 0.5938 ± 0.07276 μM, in the range of 0.1134–2.1004 μM. The difference of nitrite concentrations between ASD and healthy groups was significant ($p = 0.0420$) (Fig. 4A). Higher urinary NO_2^- concentration in ASD than that in the control group was in accordance with what was reported in blood samples [7–9]. In addition, urinary nitrite in adults (14 male and 14 female) was also measured, which was 0.5808 ± 0.0985 μM and fell in a range of 0.0808–1.9335 μM (Fig. 4B, Table 2). No significant difference of nitrite level was observed for adults and the healthy children. Association between nitrite and gender was not found, which might be due to small pool of samples.

The nitrate levels among ASD, control and adult groups were measured with the reductive VCl_3 method, which were 2.875 ± 0.2716 mM (in the range of 0.3264–7.1835 mM), 4.558 ± 0.5915 mM (in the range of 1.1860–15.8555 mM) and 2.799 ± 0.3640 mM (in the range of 0.2507–8.6978 mM), respectively (Fig. 4C–D, Table 2). The difference of nitrate concentrations between ASD and control groups was significant ($p = 0.0133$) (Fig. 4C), as well as between the adults and control groups ($p = 0.0146$) (Fig. 4D). Furthermore, the concentration ratio of nitrite versus nitrate was compared between the ASD and the control groups, which were $0.3496 \pm 0.04382 \times 10^{-3}$ and $0.1604 \pm 0.01862 \times 10^{-3}$, respectively and p was 0.0002 (Fig. 4E). The ratio of nitrite versus nitrate was not significantly different between the adult ($0.2838 \pm 0.07372 \times 10^{-3}$) and the control, with $p = 0.0999$ (Fig. 4F). Similarly, association between nitrate and gender was not found.

Taken together, urinary nitrite in ASD children was dramatically higher than that in the control, and nitrate in ASD children urine was lower than that in the control group, which made the ratio NO_2^-/NO_3^- a potential biomarker for fast and harmless diagnosis of ASD.

综上所述，ASD儿童尿中亚硝酸盐含量显著高于对照组，而ASD儿童尿中硝酸盐含量低于对照组，这使得NO2-/NO3-比值是快速、无害诊断ASD的潜在生物标志物。

4. Discussion

In the past decades, different analytic methods have been used for detection of NO_x, along with various preparation methods of urinary samples. Pre-treatment with centrifugation, saturated zinc sulfate, active carbon or ethanol has been reported [16–19], though parallel comparisons among them were missing. We compared levels of standard nitrite and nitrate with all known pre-treatments, which caused artificial effect and got measured values off nominal concentrations. Centrifugation at various speeds didn't make any difference, which made centrifugation unnecessary. Both active carbon and ethanol led to higher nitrite concentration, which might be due to water extraction from urine and led to NO_x enrichment in urine. Saturated zinc sulfate made nitrite concentration artificially lowered, without affecting urinary nitrate. Therefore, pre-treatment is inappropriate for nitrite and nitrate analysis in urinary sample. Direct injection of urine specimens into the NOA vessel is the best method so far.

For ASD children, collecting the first urine in the morning may be difficult due to parents' concern. Whether urinary NO_x follows a daily pattern is unknown. Urinary nitrate was reported to reaches a maximum concentration 4–6 h after nitrate challenge and returned to baseline within 24 h [30]. Nitrate challenge had been designed as nitrate-free diet for three days that was followed by an oral dose of KNO_3, which is distinctive from nitrate intake from our regular daily routine [31]. In our study, urine samples from two adults were collected and analyzed at 12 times points, from the first morning urine to the sample obtained at 8:30 pm. No regular pattern of NO_x concentrations was found. This is also the first time to search for a pattern of NO_x by

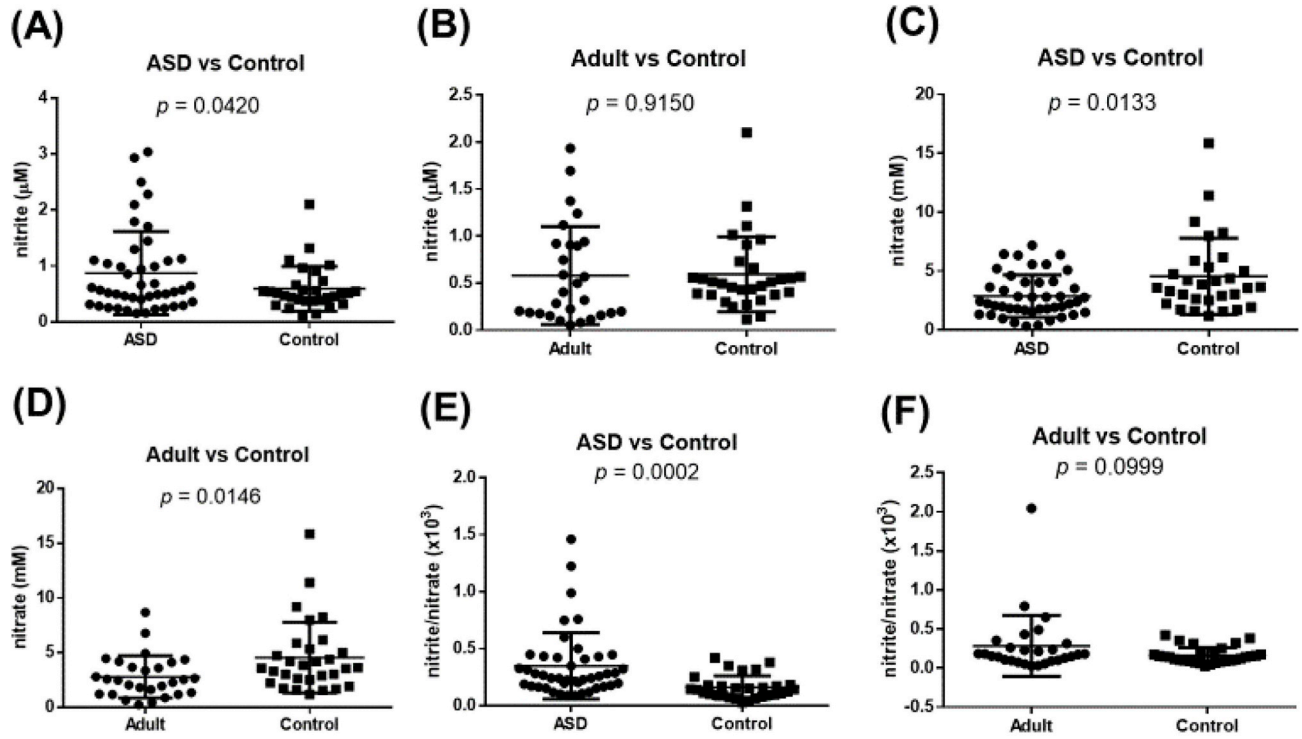


Fig. 4. Concentrations of urinary nitrite and nitrate from the ASD, control and adult groups. (A) nitrite in the ASD and control groups (p = 0.0420), (B) nitrite in the adult and control groups (p = 0.9150), (C) nitrate in the ASD and control groups (p = 0.0133), (D) nitrate in the adult and control groups (p = 0.0146), (E) nitrite/nitrate ratios in the ASD and control groups (p = 0.0002), (F) nitrite/nitrate ratios in the adult and control groups (p = 0.0999).

monitoring urinary NO_x for 13 h per day. Apparently, first urine should not be the only option for NO_x analysis. Collection at 10 am actually provided convenience for sampling and made urine tests more flexible.

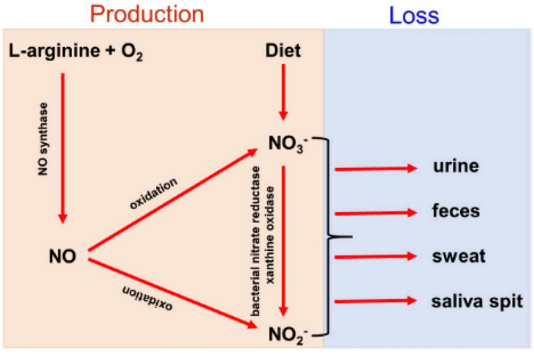
Urinary NO_x levels have been reported before in adults, with ranges of 0.02–1 μM and 20–2000 μM for nitrite and nitrate, respectively (Table 2) [25–29]. Our studies on 30 healthy adults between 18 and 25 years old have provided similar concentrations of these two anions in urine, which actually provided a wider range than previously reported. For the first time, we did unpaired comparisons on urinary nitrite and nitrate among ASD children, the control group (children without ASD) and adults, which may help future clinical diagnosis of ASD via urinary analysis.

NO_x is known to play an important role in the cardiovascular [32], immune [33], and neurological systems [34,35], while the precise metabolic regulation of nitrate in urine is unclear. Urinary nitrate is the net results of intake from diet, endogenous synthesis and metabolic loss. ^{15}N-labelling studies showed that endogenous nitrate biosynthesis was independent of ingestion and comparable to diet intake [36]. Endogenous L-arginine was reported to contributed to urinary nitrate in murine models [37]. However, another study suggested that urinary nitrate mainly came from undetected dietary nitrate, instead of endogenous synthesis [31]. On the other hand, endogenous nitrite can be generated from NO oxidation or nitrate reduction. Therefore, elucidation of the source of nitrate and nitrite in urine would help answer the endogenous nitrogen cycle in ASD children, helping understand whether and how oxidative stress, diet or arginine pathways affects NO_x in metabolism (Scheme 1). Actually, NO interacts with quite a few potential biomarkers of ASD, including folic acid, vitamin B_{12}, vitamin D_3, melatonin, glutathione, dopamine and serotonin. Some are antioxidants and others add oxidative stress. Understanding the relationship of NO with these molecules would further clarify if nitrite/nitrate may be a new biomarker of ASD diagnosis in children.

Table 2
Summary of concentrations of urinary nitrite and nitrate.

subjects	number of subjects	analytical method	nitrite (μM)	nitrate (μM)	reference
ASD children	44	Chemiluminescence	0.16–3.04	326–7183	this work
healthy children	30	Chemiluminescence	0.11–2.10	1186–15856	this work
adults	28	Chemiluminescence	0.08–1.93	251–8698	this work
human	–	HPLC	–	250–2000	[25]
adults	22	HPLC	319–441	1075–1615	[26]
adults	8	GC/MS	–	20–170	[27]
human	10	Griess reaction	–	188–1098	[16]
human	12	Capillary electrophoresis	0.02–0.54	118–1254	[28]
human	–	Chemiluminescence	0.1–0.5	–	[19]
human	10	Spectrophotometry	0.19–1	–	[17]
human	10	FASS–OT-CEC	–	118.56–620	[29]

已知NOx在心血管[32]、免疫[33]和神经系统[34，35]中起着重要作用，而尿液中硝酸盐的精确代谢调节尚不清楚。尿硝酸盐 是饮食摄入、内源性合成和代谢损失的净结果。15N标记研究表明内源性硝酸盐生物合成与摄入无关，与饮食相当 摄入量[36]。内源性L-精氨酸在小鼠模型中被报道为尿硝酸盐的贡献[37]..然而，另一项研究表明，尿硝酸盐主要来自未被发现的饮食中的硝酸盐 而不是内源性合成[31]。另一方面，内源亚硝酸盐可由NO氧化或硝酸盐还原产生。因此，阐明硝酸盐和亚硝酸盐的来源 尿液将有助于回答ASD儿童的内源性氮循环，帮助了解氧化应激、饮食或精氨酸途径是否和如何影响代谢中的NOx（方案1）。实际上，N 氧与ASD的一些潜在生物标志物相互作用，包括叶酸、维生素B12、维生素D3、褪黑素、谷胱甘肽、多巴胺和5-羟色胺。有的是抗氧化剂，有的加入氧化 压力。了解NO与这些分子的关系将进一步阐明亚硝酸盐/硝酸盐是否可能是儿童ASD诊断的新生物标志物。



Scheme 1. Production and loss of nitrite and nitrate.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.11.001.

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