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Oral nitrate reduction is not impaired after training in chlorinated swimming pool water in elite swimmers

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Abstract
This study tested the hypothesis that exposure to chlorine-sterilised pool water would impair oral nitrate reduction (ONR). ONR was assessed in elite swimmers before and after morning and afternoon pool-based training. Non-swimmers were only assessed in the morning. ONR was similar in swimmers and non-swimmers (\( P = 1.000 \)) and unchanged pre to post morning and afternoon training (\( P \geq 0.341 \)). Therefore, exposure to chlorinated pool water does not interfere with ONR.

Key words: Nitrate, nitrite, oral nitrate-reducing capacity, swimming, chlorine
**Introduction**

Dietary nitrate (NO$_3^-$) supplementation has been shown to improve exercise performance in a variety of settings, with its ergogenic effect believed to be linked to the stepwise reduction of NO$_3^-$ to nitrite (NO$_2^-$) and NO$_2^-$ to nitric oxide (NO) (Jones et al. 2018). The increase in salivary and plasma [NO$_2^-$] after NO$_3^-$ ingestion is critically dependent on oral NO$_3^-$ reduction (ONR) catalysed by anaerobic bacteria residing in the oral cavity (Govoni et al. 2008). Indeed, numerous studies have demonstrated that removal of these bacteria with antibacterial mouthwash markedly blunts the increases in salivary and plasma [NO$_2^-$], and the associated NO-mediated biological effects, following inorganic NO$_3^-$ supplementation (Senkus and Crowe-White 2019). Moreover, it appears that the magnitude by which antibacterial mouthwashes blunt ONR and associated physiological responses after NO$_3^-$ supplementation is a function of their antibacterial potency (Woessner et al. 2016).

Chlorine confers potent antibacterial properties and is commonly used to disinfect swimming pool water. Accordingly, exposure to chlorine-sterilised pool water has the potential to impede NO$_3^-$ reducing oral microflora, and subsequently, the physiological and ergogenic benefits that attend NO$_3^-$ supplementation. Recent publications have not observed an ergogenic effect of NO$_3^-$ supplementation in trained swimmers (Lowings et al. 2017; Jonvik et al. 2018; Esen et al. 2019). Whilst this lack of an ergogenic effect following NO$_3^-$ supplementation could be a function of chlorine-mediated impairments in ONR, it has yet to be determined whether exposure to chlorine-sterilised pool water interferes with ONR. Therefore, the purpose of this study was to test the hypotheses that ONR would be: 1) lower in elite swimmers compared to non-swimmers; 2) lower after, compared to before, a pool-based training session in elite swimmers in the morning and afternoon, and lower following the afternoon compared to the morning session on the same day; and 3) negatively correlated with the pool-based training session duration.

**Methods**

**Participants**

Thirteen full-time elite swimmers (8 males, mean ± SD: age: 21 ± 2 years, height: 1.83 ± 0.09 m, body mass: 75.1 ± 10.4 kg) and fourteen non-swimmer controls (9 males, 25 ± 4 years, 1.77 ± 0.10 m, 70.1 ± 12.1 kg) volunteered to participate in this study.
The swimmers trained at Loughborough National Centre which is part of the British Swimming World Class Programme. On average the swimmers had 4.4 years of experience in national level swimming and above during the year of data collection. The swimmers completed between 10-13 exercise sessions per week (including up to 10 pool and 3 land sessions). The control participants were recreationally active and infrequent swimmers (using swimming pools less than once per month). Ethical approval for this study was granted by Loughborough University’s Ethics Advisory Committee. All participants provided written informed consent prior to participation in the study. Participants were given a list of nitrate-rich foods to avoid consuming 24 h before testing sessions and were asked to consume the same foods and drinks the evening prior to experimental visits. They were also told to avoid caffeine and alcohol ingestion 12 h and 24 h before trials, respectively. For the duration of the study, participants were instructed to abstain from using antibacterial mouthwash (Govoni et al. 2008) and to avoid brushing their teeth and tongue on the day of trials (Tribble et al. 2019).

**Experimental design**

The elite swimmers completed 3 visits in total. During the first visit, swimmers were familiarised with the procedures involved in this study. In successive visits, as part of a randomised, double-blind crossover experimental design, ONR was assessed pre and post both a morning (~ 7.30 am; AM-Pre and AM-Post, respectively) and afternoon (~3.30 pm; PM-Pre and PM-Post, respectively) in-water training session in one of the two experimental conditions [nitrate-rich (NR) or nitrate-depleted (PL) mouth rinse]. Mouth rinse solutions were given on arrival at the pool before training and within 5 minutes of swimmers exiting the water after their session. The morning and afternoon sessions (91 ± 26 min and 103 ± 19 min, respectively) were separated by ~ 6 h. This was then repeated 2-14 days later with the alternative experimental treatment. Chlorine concentration (1.39 ± 0.10 mg/L) was adequately maintained within recommended guidelines of 1-3 mg/L throughout (WHO 2006). Non-swimmers attended the laboratory in the morning on one occasion for the assessment of ONR.

**Measurements**

*Oral nitrate-reducing capacity*
The mouth rinse solutions were prepared the day before administration and stored at 4°C. Prior to administration, all solutions were placed in a heated water bath at 36°C to match oral temperature (Sund-Levander et al. 2002). Participants were then asked to hold 10 mL of water (Buxton Mineral Water, UK) in their oral cavity for 3 min before expectorating into a 50 mL Falcon tube. Following 3 min rest, participants held either 10 mL of 1 mM KNO$_3$ solution (NR: Minerals-Water.ltd, Purfleet, UK) or a solution with a negligible NO$_3^-$ content (PL: Buxton Mineral Water alone) for 3 min before expectorating into a 50 mL Falcon tube. This protocol is similar to a previous protocol used to assess ONR (Doel et al. 2005). The expectorated samples were then frozen at -80°C for later analysis of ONR capacity through assessment of NO$_2^-$ production, having accounted for baseline oral [NO$_2^-$] in the initial water mouth rinse.

**Salivary [NO$_2^-$] determination**

After thawing at room temperature, samples were centrifuged for 10 min at 14,000 rpm and the supernatant was removed and diluted 100-fold with deionised water for subsequent analysis. The [NO$_2^-$] of the saliva samples was determined by its reduction to NO in the presence of glacial acetic acid and aqueous sodium iodide (4% w/v) via 50 μL injections into the septum of the air-tight purge vessel using a gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK).

**Statistical analysis**

Statistical analysis was performed using IBM SPSS Statistics version 25. Shapiro Wilk’s test was used to check data normality and, when violated, a non-parametric test was used. The Mann-Whitney U test was used to assess differences in ONR between swimmers and non-swimmers with effect size (ES) calculated as $Z / \sqrt{n}$. A two way (supplement × time) repeated-measures ANOVA was used to assess ONR in the swimmers with ES calculated using partial eta squared ($\eta_p^2$). Where the ANOVA revealed a significant effect, paired t-tests with LSD correction were utilised to determine the origin of any effects with ES calculated as Cohen’s $d_z$ ($t / \sqrt{n}$). Pearson’s product moment correlation coefficient was used to determine the relationship between swimming session duration and ONR. All parametric data are presented as mean ± SD and all non-parametric data are presented as median and inter-quartile range (25th and 75th percentiles) unless otherwise stated. Statistical significance was accepted at $P < 0.05$. 
Results
There was no difference in ONR between swimmers 99 (41, 143) nmol·min\(^{-1}\) and non-swimmers 56 (42, 165) nmol·min\(^{-1}\) (\(P = 1.000\); ES = 0.01; Figure 1). There was a main effect for supplement (\(P < 0.001\); ES = 0.71) and a supplement × time interaction effect for ONR in swimmers (\(P = 0.028\); ES = 0.22; Figure 1). The mean ONR across the AM-Pre, AM-Post, PM-Pre and PM-Post time points in the PL mouth rinse condition was 6 ± 25 nmol·min\(^{-1}\). ONR was higher at the AM-Pre, AM-Post, PM-Pre and PM-Post time points with the NR solution compared to all time points with the PL mouth rinse condition (all \(P \leq 0.002\)). ONR in NR was not different between AM-Pre (98 ± 67 nmol·min\(^{-1}\)) and AM-Post (124 ± 110 nmol·min\(^{-1}\); ES = 0.28; \(P = 0.341\)), or PM-Pre (156 ± 99 nmol·min\(^{-1}\)) and PM-Post (165 ± 148 nmol·min\(^{-1}\); ES = 0.09; \(P = 0.751\)). However, ONR in NR was 59.7% higher in PM-Pre compared to AM-Pre (\(P = 0.046\); ES = 0.62). There was no correlation between morning training session duration and ONR at the AM-Post, PM-Pre or PM-Post time points (\(r \leq 0.30, P \geq 0.329\)) or between afternoon session length and the PM-Post ONR (\(r = 0.19, P = 0.545\)).

Discussion
The novel findings from this study in elite swimmers were: 1) ONR did not differ from that observed in non-swimmers; 2) ONR was not attenuated following a single or multiple pool sessions, but was enhanced in the afternoon compared with the morning, on the same day; and 3) ONR was not correlated with the duration of the pool-based training sessions. Collectively, these observations suggest that exposure to chlorinated pool water does not interfere with ONR in elite swimmers.

Several previous studies have reported a detrimental effect of antibacterial chlorohexidine mouthwash on ONR capacity (Kapil et al. 2013, 2018; Bondonno et al. 2015; McDonagh et al. 2015; Sundqvist et al. 2016; Woessner et al. 2016; Ashworth et al. 2019; Bescos et al. 2020). However, despite the established antimicrobial properties of chlorine, and contrary to our hypothesis, there was no difference between the abilities of swimmers and non-swimmers to form NO\(_2^–\) in the oral cavity when administered a NR mouth rinse. Furthermore, there was no impairment in ONR
following morning or afternoon in-water training sessions on the same day, nor was the session duration correlated with the post session ONR in the current study. Our findings suggest that frequent exposure to chlorinated water through regular pool-based training does not influence ONR in elite swimmers compared to non-swimmers. Therefore, the lack of an ergogenic effect of NO$_3^-$ supplementation previously reported in trained swimmers (Lowings et al. 2017; Jonvik et al. 2018; Esen et al. 2019) is unlikely to be a function of impaired ONR. However, additional research is required to elucidate whether the oral microbiome of frequent swimmers develops a tolerance to chlorine-sterilised pool water and whether infrequent pool-users would experience acute reductions in ONR upon exposure to chlorinated pool water. We also cannot exclude the possibility that maintaining the pool chlorine concentration closer to the upper limit of the recommended guidelines of 1-3 mg/L (WHO 2006) could impair ONR in swimmers. We did not directly assess the aerobic fitness status of our non-swimming controls and acknowledge this as a study limitation since ONR has been shown to correlate positively with aerobic fitness (Thomas et al. 2019).

Although ONR was not impaired after, compared to before, pool-based training, ONR was higher prior to the afternoon training session compared to the morning. It is well documented that the oral microflora catalyse the reduction of NO$_3^-$ to NO$_2^-$, and several important NO$_3^-$ reducing bacterial genera have been identified including: *Veillonella*, *Neisseria*, *Prevotella*, *Actinomyces*, *Rothia*, *Granulicatella*, *Staphylococcus*, *Propionibacterium* and *Haemophilus* (Doel et al. 2005; Hyde et al. 2014). Indeed, the abundance of these genera of oral NO$_3^-$ reducers has been shown to impact the magnitude of salivary and plasma [NO$_2^-$] following NO$_3^-$ ingestion (Burleigh et al. 2018; Vanhatalo et al. 2018). It appears that the oral microbiome exhibits circadian oscillation patterns, with proliferation of *Prevotella* in the morning and *Neisseria* and *Rothia* genera in the afternoon (Takayasu et al. 2017). Since Vanhatalo et al. (2018) indicated that high abundances of *Rothia* and *Neisseria* and low abundances of *Prevotella* were conducive to greater increases in NO$_2^-$ synthesis following dietary NO$_3^-$ supplementation, this might have accounted for the greater ONR observed in the current study in the afternoon compared to the morning.

In conclusion, our findings indicate that exposure to chlorinated pool water does not interfere with the capacity of elite swimmers to reduce NO$_3^-$ in the oral cavity.
Conflict of interest

The authors declare no conflict of interest.

Reference list


Takayasu, L., Suda, W., Takanashi, K., lioka, E., Kurokawa, R., Shindo, C., Hattori,


**Figure legend**

**Figure 1.** Morning oral nitrite production in non-swimmers and swimmers. Data are presented as group median (solid line) and individual responses (circles). No differences were observed between groups ($P = 1.000$). Oral nitrite production in elite swimmers pre and post morning (AM-Pre and AM-Post) and afternoon (PM-Pre and PM-Post) pool-based training sessions in nitrate-rich (NR, squares) and nitrate-depleted (PL, circles) conditions. Data are presented as group mean ± SEM. * indicates oral nitrite production was higher in the NR condition compared to all time points in the PL condition ($P \leq 0.002$). # indicates oral nitrite production was higher PM-Pre compared to AM-Pre in the NR condition ($P = 0.046$).