Two-day fasting affects kynurenine pathway with additional modulation of short-term whole-body cooling: a quasi-randomised crossover trial

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Abstract

Metabolites of the kynurenine (KYN) pathway of tryptophan (TRP) degradation have attracted interest as potential pathophysiological mediators and future diagnostic biomarkers. A greater knowledge of the pathological implications of the metabolites is associated with a need for a better understanding of how the normal behaviour and physiological activities impact their concentrations. This study aimed to investigate whether fasting (FAST) and whole-body cold-water immersion (CWI) affect KYN pathway metabolites. Thirteen young women were randomly assigned to receive the 2-d FAST with two 10-min CWI on separate days (FAST-CWI), 2-d FAST without CWI (FAST-CON), 2-d two CWI on separate days without FAST (CON-CWI) or the 2-d usual diet without CWI (CON-CON) in a randomised crossover fashion. Changes in plasma concentrations of TRP, kynurenic acid (KYNA), 3-hydroxy-kynurenine (3-HK), picolinic acid (PIC), quinolinic acid (QUIN) and nicotinamide (NAA) were determined with ultra-performance liquid chromatography-tandem mass spectrometer. FAST-CWI and FAST-CON lowered TRP concentration ($P < 0.05$, $\eta_p^2 = 0.24$), and increased concentrations of KYNA, 3-HK and PIC ($P < 0.05$, $\eta_p^2 = 0.21–0.71$) with no additional effects of CWI. The ratio of PIC/QUIN increased after FAST-CWI and FAST-CON trials ($P < 0.05$) but with a blunted effect in the FAST-CWI trial ($P < 0.05$) compared with the FAST-CON trials ($\eta_p^2 = 0.67$). Concentrations of QUIN and NAA were unaltered. This study demonstrated that fasting for 2 d considerably impacts the concentration of several metabolites in the KYN pathway. This should be considered when discussing the potential of KYN pathway metabolites as biomarkers.

Keywords: **3-Hydroxy-kynurenine: Energy deprivation: Kynurenic acid: Cold: Tryptophan**

In the last two decades, the pathogenesis and progression of various diseases and disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, Huntington's disease, cerebral ischaemia, AIDS dementia complex, dementia, malaria, cancer, diabetes, migraine, neuropathic pain, epilepsy, depression, schizophrenia and aortic stiffening, have been linked to kynurenine (KYN) pathway dysregulation[1–5]. Therefore, targeting the KYN pathway could be valuable not only for the treatment of existing conditions but also for chronic disease prevention. Furthermore, metabolites of the KYN pathway are small stable molecules and can be analysed in body tissues and fluids[6,7]. Some of the metabolites have been suggested as markers for the progression, severity and prognosis of the disease[8–11].

Evidence suggests that not only the pharmacological interventions or electroconvulsive therapy modulate the KYN pathway[3,4], but also healthy lifestyle changes such as exercise may influence the KYN pathway[2,12], which represents a potential link between a healthy lifestyle and disease prevention and treatment. There is evidence that interventions, including fasting and cold exposure, may lower the risk of various disorders and improve health status[13–15]. However, the mechanisms by which the interventions affect the KYN pathway remain unknown. The KYN pathway starts with the conversion of tryptophan (TRP) to KYN, which follows one of two possible branches from the pathway. KYN is metabolised either to the neuroprotective metabolite, kynurenic acid (KYNA) via four different kynurenine aminotransferase enzymes, or to the neurotoxic metabolite, quinolinic acid (QUIN) via the enzyme kynurenine 3-monooxygenase and others[6,16]. Studies in animal models have shown that fasting produces a robust increase of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α expression in the liver via glucagon and glucocorticoid signalling, which, in turn, stimulates hepatic gluconeogenesis and fatty acid oxidative metabolism[17,18]. Moreover, exposure to cold also

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Abbreviations: CWI, cold-water immersion; KYN, kynurenine; KYNA, kynurenic acid; PIC, picolinic acid; QUIN, quinolinic acid; TRP, tryptophan; 3-HK, 3-hydroxy-kynurenine.

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increases PGC-1α expression in muscle and brown fat via the sympathetic nervous system β3-adrenergic receptor activation inducing mitochondrial biogenesis and adaptive thermogenesis[19,20]. PGC-1α1 enhances the expression of kynurenine aminotransferase, influencing TRP metabolism and enhancing the conversion of KYN into KYNA[21]. Thus, it can be hypothesised that also in humans, fasting would increase peripheral levels of KYNA, and this increase is strengthened by cold exposure.

Sex-specific differences in primary disease prevention are established, indicating that overall, women are more likely than men to engage in behaviours associated with health prevention[22]. Thus, the present study aimed to examine whether 2-d fasting (FAST) with two whole-body cold-water immersions (CWI) on separate days in a group of young healthy women affects the peripheral concentration of KYN pathway metabolites. This will allow not only to determine how these interventions affect the concentrations of KYN pathway metabolites but also allow for understanding whether food deprivation and cold exposure should be considered for the potential use of KYN pathway metabolites as biomarkers.

Materials and methods

Participants

Twenty-one women were assessed for eligibility. The inclusion criteria were (i) women; (ii) aged between 18 and 35 years; (iii) BMI from 19·5 to 30·0 kg/m^2; (iv) no blood/needle phobia and (v) no medications and/or dietary supplements that could affect experimental variables. Participants were excluded if they were smokers; involved in a weight reduction programme; involved in any regular physical activity programme (i.e. ≥3 times/week and ≤150 min of moderate-intensity or ≥75 min of vigorous-intensity activity/week) and/or in any temperature-manipulation programme or extreme temperature exposure for ≥3 months; history of alcohol, nicotine or drug abuse; neurological, cardiovascular, psychiatric and/or inflammatory diseases; or conditions that could be worsened by exposure to acute cold (14°C) water. In total, thirteen women (age: 25·8 (sd 4·7) years; weight: 68·9 (sd 12·7) kg; BMI: 23·4 (sd 2·9) kg/m^2) met the criteria and agreed to participate in this study (Fig. 1). All experiments were performed at the Institute of Sports Science and Innovations, Lithuanian Sports University, from September 2020 to May 2021.

Experimental protocol and study design

The experiments began at 08.00–09.00 hours when participants arrived at the laboratory after an overnight fast (9–11 h). The women were instructed to refrain from fatigue-related activities and abstain from ingesting alcoholic beverages, caffeine and medications for at least 72 h before each experimental assessment.

On arrival at the laboratory, an anthropometric measurement was performed, and the participant was asked to rest in a semi-recumbent position for 20 min in a quiet room at an ambient temperature of 24°C with 60 % relative humidity. Then, a venous blood sample was obtained.

The participants then received a break for 1 d before starting one of the prescribed interventions: the 2-d fasting with two whole-body CWI on separate days (FAST-CWI), the 2-d fasting without CWI (FAST-CON), the 2-d usual diet with two CWI on separate days (CON-CWI) or the 2-d usual diet without CWI (CON-CON) (Fig. 2). We used a crossover design, in which all women received all four interventions, at least 2 weeks apart. Allocation to a different sequence of trials was conducted quasi-randomly based on the order in which a participant was recruited for the experiment (Fig. 1). During CWI, the participant was immersed in a 14°C water bath in a semi-recumbent position up to the level of the manubrium for 10 min, as described previously[23]. Two CWI procedures were performed in the morning (08.00–10.00 hours) on separate days. During fasting, participants were instructed to follow a prescribed zero-energy diet with water provided *ad libitum* over a period of 2 d. During the usual diet, participants were instructed to maintain their previous eating habits. All interventions were followed by repeated blood collection as described above. During the study, no participant dropped out. Thus, a total of thirteen participants were included in the final analysis (Fig. 1). The menstrual cycle was not controlled for, but information about the cycle was collected. Distribution of women during follicular and luteal phases in CON-CWI (seven during follicular and six women during luteal phases), FAST-CWI (six during follicular and seven women during luteal phases) and FAST-CON (seven during follicular and six women during luteal phases) trials were similar and did not seem to affect current findings.

Detection of tryptophan and kynurenine metabolites in human plasma

Venous blood samples for TRP and KYN metabolites analysis from the median antecubital vein were directly collected into 3-ml vacutainer tubes using EDTA with tri-potassium as an anticoagulant (K3EDTA tube; Fisher Scientific), mixed gently by inverting 8–10 times and kept at 2–8°C until centrifugation. The plasma was separated by centrifugation at 1200 × g for 15 min at 4°C within 30 min of blood collection. Plasma samples were stored in 0·5 ml aliquots at –80°C until analysis.

Blinded assessors used an ultra-performance liquid chromatography-tandem mass spectrometry system (UPLC–MS/MS) to measure plasma levels of TRP, KYNA, 3-hydroxy-kynurenine (3-HK), QUIN, nicotinamide and picolinic acid (PIC). The UPLC–MS/MS system used a Xevo TQ–XS triple quadrupole mass spectrometer (Waters) with a Z-spray electrospray interface, and the system was operated in electrospray positive multiple reaction monitoring mode.

The UPLC conditions were as follows. Separation was carried out using an Acquity UPLC® HSS T3 column (1·8 m, 2·1150 mm) from Waters (part number: 186003540) at 50°C with a guard column (Waters, Vanguard HSS T3 1·8 m, 2·150 mm column, part number: 186003976) to retain impurities from the mobile phase. The following were the components of the mobile phase: 0·6 % 30 formic acid in water (UPLC grade) and 0·6 % formic acid in methanol (B) (UPLC grade). The flow rate was 0·3 ml/min. While the MS was operating at a source temperature of 150°C,
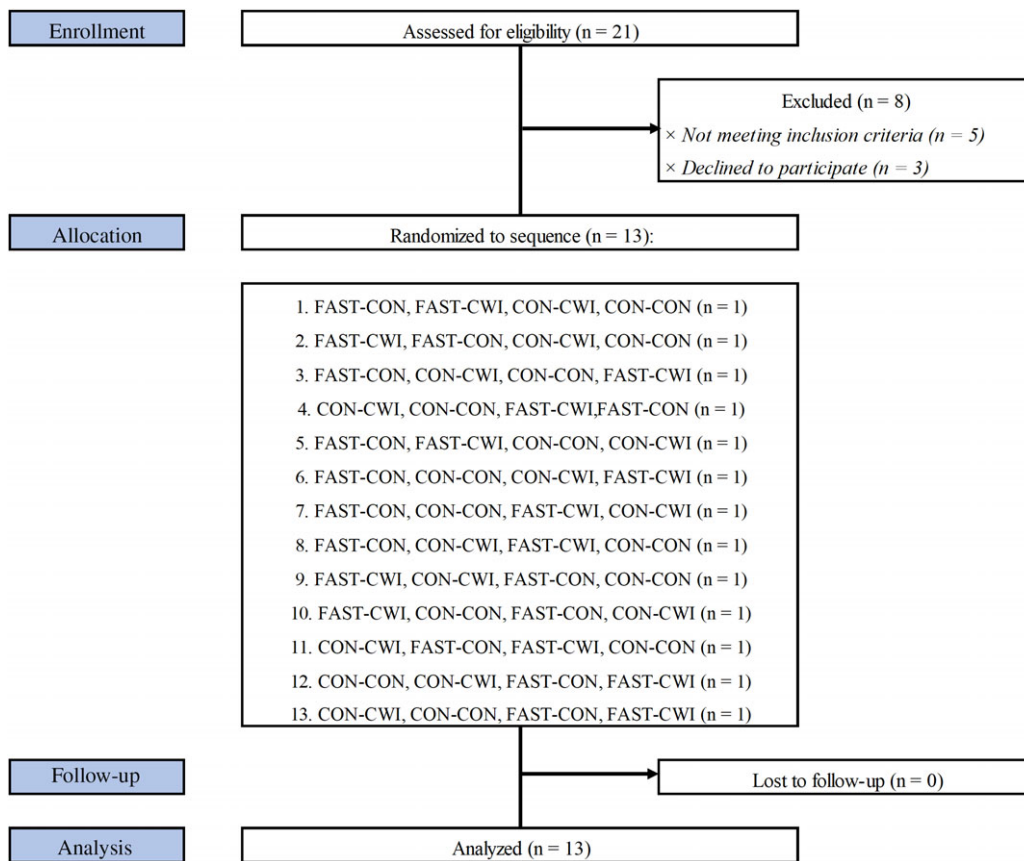
Fig. 1. CONSORT flow chart of the study. FAST-CWI, fasting with whole-body cold-water immersion; FAST-CON, fasting without whole-body cold-water immersion; CON-CWI, whole-body cold-water immersion without fasting; CON-CON, usual diet without whole-body cold-water immersion.
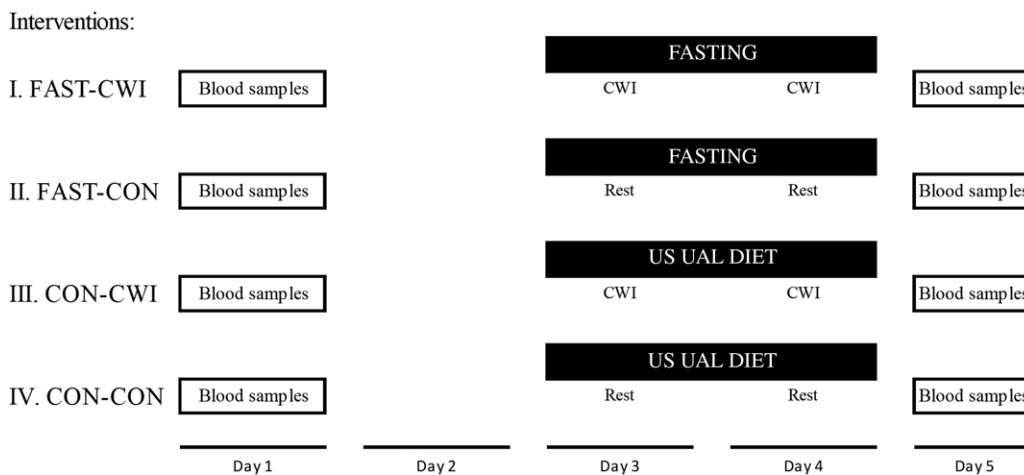

Fig. 2. Schematic representation of the experimental protocols. FAST-CWI, fasting (0 kcal/d) with whole-body cold-water immersion (10 min at 14°C); FAST-CON, fasting (0 kcal/d) without whole-body cold-water immersion; CON-CWI, whole-body cold-water immersion (10 min at 14°) without fasting; CON-CON, usual diet without whole-body cold-water immersion. All participants (*n* 13) received all four interventions in a randomised order, at least 2 weeks apart.

the capillary voltage was set to $+3{\cdot}0$ kV. The cone gas flow was 150 l/h and the desolvation gas flow rate was 1000 l/h, while the desolvation temperature was 650°C. The autosampler was set at 5°C and each sample took $13{\cdot}0$ min to run. Data processing and acquisition were performed using the software package MassLynx v 4.1 SCN943 SCN979 (© 2016 Waters Inc.). The detailed description of the method has been previously published[6].
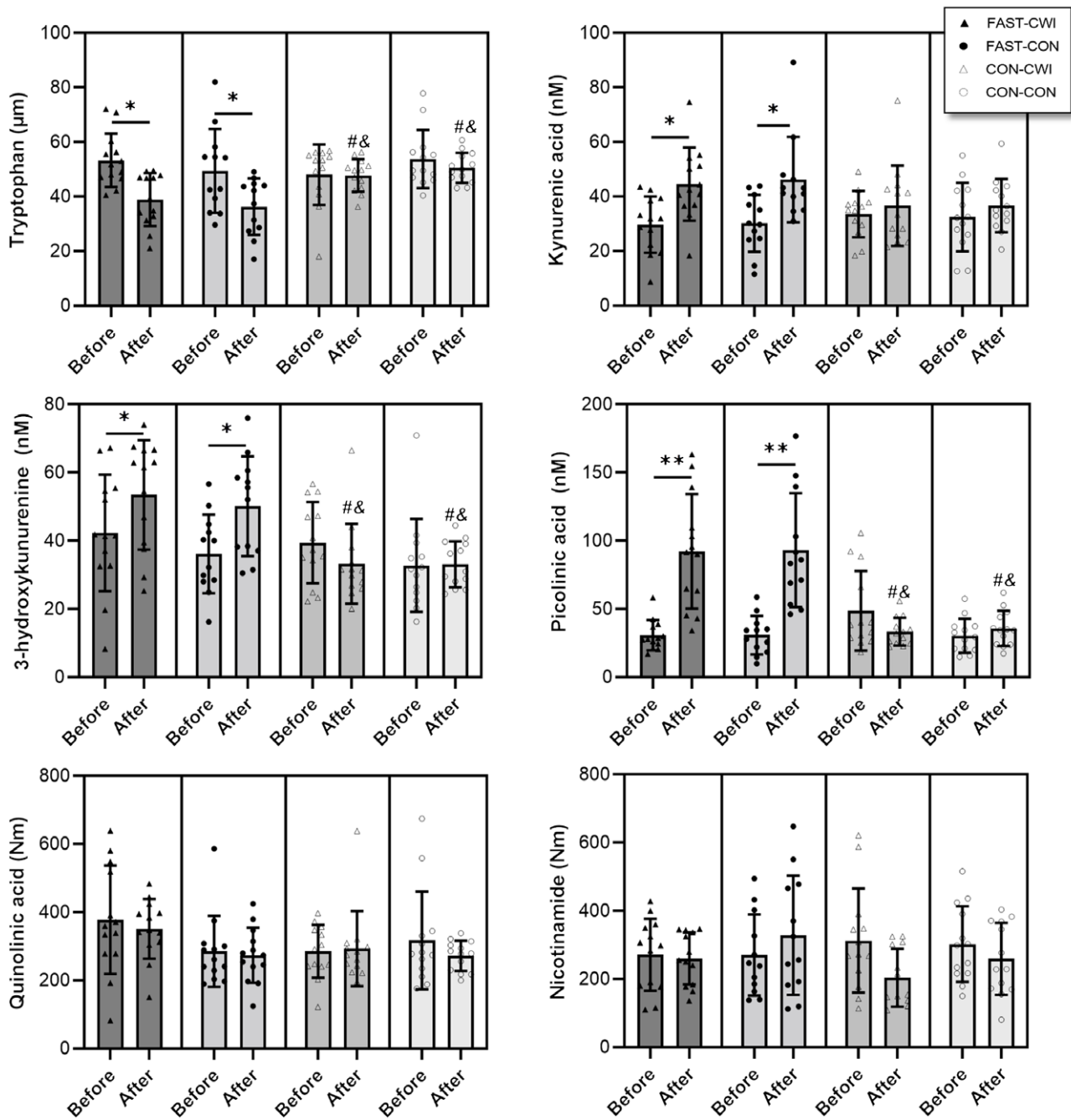
Fig. 3. Plasma concentrations of kynurenine metabolites before and after the 2-d fasting with two whole-body cold-water immersion (FAST-CWI), 2-d FAST without CWI (FAST-CON), 2-d two CWI without FAST (CON-CWI) and after the 2-d usual diet without CWI (CON-CON). Data are presented as mean values and standard deviations. *$P < 0.05$, **$P < 0.001$, compared with baseline values; #$P < 0.05$, compared with FAST-CWI; &$P < 0.05$, compared with FAST-CON.

Plasma preparation

Thirty microlitre of human plasma sample, quality control or standard mix, was mixed with 30 μl of internal standard 0·5 μM in 10 % ammonia for 15 s. Next, 60 μl of 200 nM ZnSO4 (5°C) was added and mixed for 15 s. This was followed by adding 30 μl of methanol (5°C) and mixed for 15 s. In addition, the mixture was centrifuged for 10 min at 2841 × g at room temperature. 30 μl of the supernatant obtained after the centrifugation was mixed with 30 μl of formic acid 5 % in LC-MS

Certified Clear Glass 12 × 32-mm vials (Waters, product no. 186005662CV). Samples were transferred to an autosampler that was set at 5°C. The volume injected into the UPLC–MS/MS system was 1·5 μl.

All metabolites measured in plasma samples were detected in higher concentrations than the lowest level of quantification (TRY, 6 nM; KYNA, 6 nM; 3-HK, 10 nM; PIC, 10 nM; QUIN, 10 nM; and nicotinamide, 10 nM). The CV for quality controls within a run (intra-assay, during 15 h) was less than 6 % and between run (inter-assay) less than 7 % for all metabolites measured.

Ethical approval

This study was conducted according to the Declaration of Helsinki guidelines and all procedures involving human subjects/patients were approved by the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-23). A written informed consent was obtained from all subjects.

Statistical analysis

The number of participants was selected based on the calculated sample effect size, following the use of the data involving the first four subjects who completed the study. At an α value of 0·05 and β (power) value of 80 %, our power analysis indicated that twelve participants in a within-condition comparison would be required to detect a large effect for hypothesised parameters.

Statistical analyses were performed using IBM SPSS Statistics for Windows (version 26.0; IBM SPSS). Data were tested for normality using the Shapiro–Wilk test before statistical analyses. All data were found to be normally distributed. The changes in plasma concentration of KYN metabolites were assessed using two-way repeated-measures ANOVA with a within-subjects factor of time (before, after) and group (FAST-CWI, FAST-CON, CON-CWI, CON-CON). The effect size of the evoked significant changes was determined by calculating partial eta squared (η_p^2). If a significant interaction of time × group was observed, paired t tests were used to determine the changes evoked within and between each group. Data were presented as means and standard deviations, and $P < 0.05$ was considered statistically significant.

Results

To investigate whether fasting and cold affect the KYN pathway, concentrations of plasma TRP, KYNA, 3-HK, QUIN, nicotinamide and PIC were measured before and after each trial (Fig. 3). No differences in baseline levels of any metabolite measured were observed among trials ($P > 0.05$). A two-way repeated-measures ANOVA revealed significant effects of time for concentrations of TRP ($P = 0.002$, $\eta_p^2 = 0.60$), KYNA ($P = 0.001$, $\eta_p^2 = 0.67$) and PIC ($P = 0.002$, $\eta_p^2 = 0.62$), and group for concentrations of 3-HK ($P = 0.001$, $\eta_p^2 = 0.4$) and PIC ($P < 0.001$, $\eta_p^2 = 0.42$), and a significant time × group interactions for concentrations of TRP ($P = 0.030$, $\eta_p^2 = 0.24$), KYNA ($P = 0.045$, $\eta_p^2 = 0.21$), 3-HK ($P < 0.001$, $\eta_p^2 = 0.51$) and PIC ($P < 0.001$, $\eta_p^2 = 0.71$). Subsequent analysis showed that the FAST-CWI and FAST-CON trials decreased plasma levels of TRP ($P < 0.02$) and increased KYNA ($P < 0.01$), 3-HK ($P < 0.02$) and PIC ($P < 0.001$) concentrations, whereas CON-CWI tended to decrease plasma concentrations of 3-HK ($P = 0.104$) and PIC ($P = 0.060$). The mean plasma concentration of TRP ($P < 0.02$) was lower and the mean concentrations of 3-HK and PIC levels ($P < 0.003$) were higher in both FAST-CWI and FAST-CON trials compared with their respective control trials (CON-CWI and CON-CON). The mean plasma concentration of KYNA ($P = 0.03$) was higher after FAST-CON trial than in the CON-CWI trial. Meanwhile, two-way ANOVA revealed no significant effect of time or the time × group interaction on plasma levels of QUIN or nicotinamide.
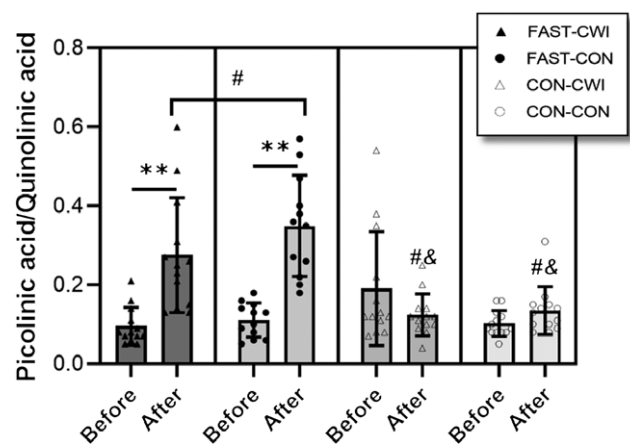

Fig. 4. Ratio of picolinic acid to quinolinic acid before and after the 2-d fasting with two whole-body cold-water immersion (FAST-CWI), 2-d FAST without CWI (FAST-C ON), 2-d two CWI without FAST (CON-CWI) and the 2-d usual diet without CWI (CON-CON). Data are presented as mean values and standard deviations. **$P < 0.001$, compared with baseline values; #$P < 0.05$, compared with FAST-CWI; &$P < 0.05$, compared with FAST-CON.

A two-way repeated-measures ANOVA also revealed significant effects of time ($P < 0.001$, $\eta_p^2 = 0.66$) and group ($P = 0.001$, $\eta_p^2 = 0.37$), and a significant time × group interactions ($P < 0.001$, $\eta_p^2 = 0.67$) for the plasma ratio of PIC/QUIN (Fig. 4). Subsequent analysis showed that the FAST-CWI and FAST-CON trials increased the ratio of PIC/QUIN ($P < 0.001$), and a higher ratio was found when comparing FAST-CWI and FAST-CON trials with the CON-CWI and CON-CON trials ($P < 0.002$), respectively. Furthermore, the ratio of PIC/QUIN concentrations was higher after the FAST-CON trial than in the FAST-CWI trial ($P = 0.005$). Noteworthy, a trend towards decreased PIC/QUIN ratio ($P = 0.065$) was detected in the CON-CWI trial.

Discussion

In the present study, we investigated whether fasting and short-term whole-body immersion (for 10 min) in cold water (14°C) would affect the plasma concentration of KYN pathway metabolites in young women. Our results show that 2-d fasting decreased TRP and increased plasma levels of KYNA, 3-HK, PIC and PIC/QUIN ratio. Furthermore, two short durations of cold exposures tended to affect metabolites in the kynurenine 3-monooxygenase branch of the KYN pathway with a trend towards decreased plasma levels of 3-HK and PIC and a significantly suppressed PIC/QUIN ratio induced by fasting.

Predominantly, TDO controls TRP degradation via the KYN pathway in the periphery[24]. Although TDO activity is generally stable and mainly controlled by the availability of TRP level itself, the activity of TDO can be regulated by increases in hormones, such as cortisol, insulin or epinephrine[25,26]. There is evidence of reduced insulin, and reduced or unchanged norepinephrine and epinephrine following 36–48 h fasting[27,28]. Meanwhile, short-term fasting results in greater hypothalamic-pituitary-adrenal axis activity[29] and shifts the diurnal cortisol profile towards higher levels during the day and a flatter profile with a slower

cortisol decline[30], indicating decreased cortisol elimination, which may occur due to protracted occupancy time and increased activation of glucocorticoid receptors[31]. In this regard, fasting-induced release of cortisol may be responsible for the induction of the KYN pathway and TRP degradation seen in the present study. By contrast, TRP degradation was not affected after treatment with cold exposure. In a recent study by Eimonte *et al.*[23], it was established that whole-body immersion in cold water (14°C) for 10 min led to residual changes in epinephrine and cortisol levels in men. However, in our previous study, we have shown that second exposure to cold evokes lower perceptual and cardiorespiratory strain (i.e. lower heart rate, ventilation and shivering, and discomfort level)[32]. Such effect may in turn blunt the expected TRP response. Despite the similar experience of cold strain in men and women, the immediate neuroendocrine response with elevated concentrations of epinephrine and cortisol was only detected in men[33]. If a similar residual pattern remains, then TRP degradation after cold-water exposure was not affected in women and must be further investigated.

TRP degradation via the KYN pathway might also be linked to the activity of the inflammation-induced enzyme indoleamine 2,3-dioxygenase[34]. Different dietary interventions such as energy restriction, time-restricted feeding and fasting potentially manipulate immune functions followed by changes in cytokines (see Okawa *et al.*[35]). Interestingly, experimental studies in rats show that fasting enhances the secretion of the pro-inflammatory cytokine interferon-γ[36]. Interferon-γ is further known to induce the expression of indoleamine 2,3-dioxygenase, kynurenine 3-monooxygenase and kynurenine aminotransferase leading to increased production of KYN and its metabolites[37–44].

In the present study, fasting increased plasma levels of 3-HK and PIC but did not affect plasma levels of QUIN. Interestingly, fasting induces a robust increase in PGC-1α expression, a transcription coactivator recently shown to induce the expression of aminocarboxymuconate-semialdehyde decarboxylase[45]. Increased plasma concentration of PIC following fasting might therefore be related to PGC-1α-induced expression of aminocarboxymuconate-semialdehyde decarboxylase. This hypothesis is in agreement with previous studies suggesting that fasting induces aminocarboxymuconate-semialdehyde decarboxylase expression[46,47]. The expression and/or activity of aminocarboxymuconate-semialdehyde decarboxylase is further shown to balance the synthesis between QUIN and PIC and suggested to be the master regulator at the intersection of acetyl-CoA and NAD+ metabolism from TRP in the KYN pathway[48].

3-HK is a controversial KYN metabolite as it is shown to possess both pro-oxidants and antioxidant properties, and the behaviour of 3-HK depends on the redox status of the cell[49]. A recent study by Wilhelmi de Toledo *et al.*[50] reported that fasting improves redox status in the blood. In this context, it can be proposed that increased plasma concentration of 3-HK may account for the improved antioxidant activity. In contrast, cold exposure tended to decrease concentrations of 3-HK and consequently PIC, leading to suppressed PIC/QUIN ratios after FAST-CWI trial as compared with FAST-CON trial. There is evidence that kynurenine 3-monooxygenase activity can be inhibited by anti-inflammatory cytokines (IL-4 and IL-10, etc.)[51]. In a recent study, it was demonstrated that short-duration CWI has residual effects on pro-inflammatory cytokines[23] and it was assumed that anti-inflammatory cytokines are released in response to increased secretion of pro-inflammatory cytokines[52].

Our study is not without limitations. First, we only measured TRP and KYN metabolites in plasma and not in cerebrospinal fluid. UPLC–MS/MS is a robust, accurate and precise method to quantify KYN metabolites in both cerebrospinal fluid [7] and plasma[6]. However, the values and evoked effect size differ between cerebrospinal fluid and plasma and therefore the use of results in the diagnostic process remains unknown. Second, short-term fasting has significant effects on metabolites. However, limitations exist in determining if the similar changes remain after prolonged fasting (> 48 h) or repeated bouts of fasting (intermittent fasting). Third, our study was performed on healthy young women. Thus, it would be interesting to include men as well, and individuals with different diseases and disorders to explore the therapeutic potential of this mechanism.

In conclusion, our study demonstrated that 2-d fasting affects the concentration of metabolites of the KYN pathway in women with additional modulation of cold exposure. Thus, food deprivation with or without cold exposure and food intake should be taken into consideration before the utilisation of KYN pathway metabolites as potential biomarkers.

Acknowledgements

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