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H2OAthletes study protocol: effects of hydration changes on neuromuscular function in athletes

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Abstract

We aim to understand the effects of hydration changes on athletes' neuromuscular performance, on body water compartments, fat-free mass hydration and hydration biomarkers and to test the effects of the intervention on the response of acute dehydration in the hydration indexes. The H2OAthletes study (clinicaltrials.gov ID: NCT05380089) is a randomised controlled trial in thirty-eight national/international athletes of both sexes with low total water intake (WI) (i.e. < 35·0 ml/kg/d). In the intervention, participants will be randomly assigned to the control (CG, *n* 19) or experimental group (EG, *n* 19). During the 4-day intervention, WI will be maintained in the CG and increased in the EG (i.e. > 45·0 ml/kg/d). Exercise-induced dehydration protocols with thermal stress will be performed before and after the intervention. Neuromuscular performance (knee extension/flexion with electromyography and handgrip), hydration indexes (serum, urine and saliva osmolality), body water compartments and water flux (dilution techniques, body composition (four-compartment model) and biochemical parameters (vasopressin and Na) will be evaluated. This trial will provide novel evidence about the effects of hydration changes on neuromuscular function and hydration status in athletes with low WI, providing useful information for athletes and sports-related professionals aiming to improve athletic performance.

Keywords: **Electromyography: Hydration status: Muscle strength: Athletic performance: Dehydration**

There is scientific evidence showing the effects of hypohydration on performance even at small levels of body mass loss (BML). However, the literature is far from clear, with large variability, lacking detailed descriptions of the methods used to induce dehydration[1]. Regardless, it is accepted that dehydration may affect the electrolytes' intra- and extracellular concentrations, leading to changes in the membrane electrochemical potential[2]. As muscular strength development depends on a plethora of morphological and neural factors[3], these changes in neural factors may be one possible mechanism that explains the effects of dehydration. Although some studies aimed to test the effects of hydration changes on neuromuscular function using electromyography (EMG) analysis, there is no consensus among them[4–7]. While some authors showed parallel effects of dehydration on muscle endurance and EMG signal[5,7], namely reduced EMG median power frequency and increased rate of root-mean-square[7], others did not find any effects[4,6].

In normal circumstances, most individuals do not experience severe dehydration throughout their daily lives due to precise physiological and behavioural factors[8,9]. However, some individuals may achieve chronic dehydration without perceiving it or seeking water, potentially affecting both cells and renal

Abbreviations: AVP, arginine vasopressin; BIA, bioelectrical impedance analysis; BML, body mass loss; CG, control group; ECW, extracellular water; EG, experimental group; EMG, electromyography; FFM, fat-free mass; ICW, intracellular water; MVIC, maximum voluntary isometric contraction; RTD, rate of torque development; TBW, total body water; WI, water intake.

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function[10]. In this sense, evidence has identified people who are considered low drinkers (LOW; i.e. regular exposure to low water intake (WI)) and high drinkers (HIGH)[11]. The differences in WI may lead to different physiological responses such as serum arginine vasopressin (AVP)[12], urinary markers[13] and mood states[14]. For instance, in situations of water loss or restriction, the increased plasma osmolality stimulates osmoreceptors located in the hypothalamus, releasing AVP from the posterior pituitary gland[9]. Hence, low WI may increase AVP[11], interacting with the kidney and resulting in higher water reabsorption from the collecting ducts, restoring the plasma osmolality. Moreover, these different physiological responses between LOW and HIGH may disturb the body water compartments, as AVP is triggered by intracellular water (ICW) loss[9]. Nevertheless, while there is evidence regarding physiologic differences among the non-athletic population with different habitual WI, there is only a study involving young athletes[15]. Also, and despite the recognised effect of changes in total body water (TBW) and its compartments (ICW and extracellular water (ECW)) on sports performance[16–18], there are no studies connecting body water compartments with different levels of WI or changed WI.

Lastly, the assessment of the hydration state has been considered a controversial topic[19–21], with no clear protocol regarding the best practice for assessing hydration status in athletes despite an existing substantial body of research[22]. Additionally, a large variability of the fat-free mass (FFM) hydration has been observed in athletes using reference methods[23]. New methods that provide hydration status safely, accurately, reliably and feasibly are also needed. In this sense, bioelectrical impedance analysis (BIA) is a technique for this specific context that offers both whole-body and segmental analysis[24]. This method utilises the components of impedance (resistance (R) and reactance (Xc)), also providing phase angle (PhA), a relevant indicator of cellular health and muscle functionality[25–27]. Thus, changes in BIA variables have been suggested to be related to hydration changes, namely R with fluid changes or Xc and PhA with cell structure integrity[28].

Our primary aim is to understand the effects of hydration changes on muscle strength (with EMG analysis for the neuromuscular response) in an athletic population. Secondary aims include (i) to analyse the effects of the 4 d WI intervention on TBW, ECW, ICW, FFM hydration, hydration indexes (serum, saliva and urine osmolality), biochemical markers (AVP and Na serum concentrations) and raw BIA-derived parameters (R, Xc, impedance (Z) and PhA); (ii) to explore the effects of acute dehydration on hydration indexes, biochemical markers and raw BIA-derived parameters and (iii) to test the effects of increasing WI during the 4 d intervention on the response of acute dehydration in the hydration related parameters. We hypothesise that EMG changes are associated with muscular fatigue after dehydration. After the 4 d of intervention, we hypothesise an increase in TBW, ICW, ECW and FFM hydration. Also, considering that it is expected a higher concentration of body fluids after dehydration, we anticipate that all hydration biomarkers (serum, saliva and urine osmolality, AVP and Na serum concentrations) and thirst feelings will significantly increase after exercise and heat-induced dehydration and will be reduced after the 4 d WI intervention. We hypothesise that raw BIA are influenced by changes in hydration status after the dehydration protocols and the intervention.

Materials and methods

Study design

H_2OAthletes is a randomised controlled intervention designed to increase WI among national/international[29] athletes (n 38) from both sexes with habitual low total WI (i.e. < 35 ml/kg/d)[30].

The study was approved by the Ethics Committee of the Faculty of Human Kinetics, University of Lisbon (FMH-UL) (CEIFMH: 12/2021) and will be conducted in accordance with the declaration of Helsinki for human studies from the World Medical Association. This study was registered at (clinicaltrials.gov ID: NCT05380089).

All measurements and interventions will take place at the Exercise and Health Laboratory and Neuromuscular Research Lab in the FMH-UL starting in 2022 and finishing in 2023 as shown in Fig. 1. A schematic representation of the study's phases is shown in Fig. 2.

Before enrollment in the study, athletes will be contacted by a telephone call, and if they are interested they will perform a screening visit to confirm eligibility. Athletes who meet the criteria will be randomly assigned to the control (CG, n 19) or experimental group (EG, n 19). All participants will perform baseline assessments in three sessions (over 48 h, including a cardiorespiratory fitness test, hydration status, body composition and BIA) followed by an exercise-induced dehydration protocol. Next, athletes will either increase WI through fluid intake (EG) or maintain WI (CG) over the course of four days. After this, participants will be assessed, similar to the baseline. Lastly, participants will perform a second exercise-induced dehydration protocol. A schematic description of the visits and measurements is presented in Fig. 3, but a more detailed description of each stage is provided below.

Sample recruitment and selection

Participants will be recruited through federations, sports clubs and high-performance sports centers, specifically those close to FMH-UL. We will recruit national/International[29] athletes (> 6 h/week of training with an emphasis on improving performance and participating in official competitions), aged between 18–39 years, considered LOW (i.e. ≤ 35 ml/kg/d[30]). We will exclude athletes with a clinical history compatible with exertional heat illness, taking medication known to alter the normal fluid-electrolyte balance or the chronotropic response to exercise, injuries that would limit exercise performance and pregnancy/having been pregnant within the past 6 months, or breast-feeding.

As it is not possible to evaluate many athletes simultaneously, recruitment will be maintained during the study (2022–2023).

Screening process

In the first phase, participants will be contacted through telephone as the primary mode of communication. The purpose of this initial contact is to establish communication and to

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	STUDY PERIOD									
	Recruitment	Allocation	Data collection							Close-out
2022	*March*	April	*Sept*	*Oct*	*Nov*	*Dec*	*Jan*	*Feb*	*March*	---
2023	*March*	April	*Sept*	*Oct*	*Nov*	*Dec*	*Jan*	*Feb*	*March*	---
2024	---	---	---	---	---	---	---	---	---	*March 2024*
ENROLMENT:										
Eligibility screen	X									
Informed consent	X									
Randomisation		X								
INTERVENTIONS:			X	X	X	X	X	X	X	X
ASSESSMENTS:			X	X	X	X	X	X	X	
Dehydration protocol			X	X	X	X	X	X	X	
Graded exercise test			X	X	X	X	X	X	X	
Food records			X	X	X	X	X	X	X	
Hydration indexes			X	X	X	X	X	X	X	
Dilution techniques			X	X	X	X	X	X	X	
Bioelectrical impedance analysis			X	X	X	X	X	X	X	
Body composition			X	X	X	X	X	X	X	
Neuromuscular function			X	X	X	X	X	X	X	
Profile mood of states			X	X	X	X	X	X	X	
DATA ANALYSIS			X	X	X	X	X	X	X	X

Fig. 1. Study timeline.

explore their potential interest in participating in the study. This preliminary contact will serve as an opportunity to provide participants with detailed information about the study, address any queries or concerns they may have and to assess their eligibility for further involvement. Second, participants will be scheduled for an orientation/screening visit where participants' availability will be confirmed, including: (i) debriefing session with detailed information about the study (e.g. the number and type of assessments, the intervention, the time commitment required) and, if interested, participants will have an informed written consent form to read and sign and (ii) assessment of habitual WI through three non-consecutive day food records. After the athletes complete their food records, a nutritionist on the research team will analyse them. The athlete will only continue the study if they are considered a low drinker (i.e. < 35 ml/kg/d). Also, based on previous studies,[12,13,31,32] we expect some physiological differences between LOW and HIGH conditions. For example, it has been reported that LOW has a lower daily water intake (identified by food records), higher AVP, higher urine osmolality and urine specific gravity. Thus, we will exclude athletes with measurements compatible with the HIGH condition.

To decrease the training effect, familiarisation with the neuromuscular tests will be performed during the screening visit, as described below in the 'Neuromuscular function tests' section.

Randomisation

Eligible participants will be randomly assigned to two arms of the study: CG or EP. Randomisation will be performed according to an automated computer-generated scheme controlled by a team member not involved in data collection and the intervention process. The software that will be used for the randomisation process is the R-studio version 1.4.1717 (R Core Team, 2013) with

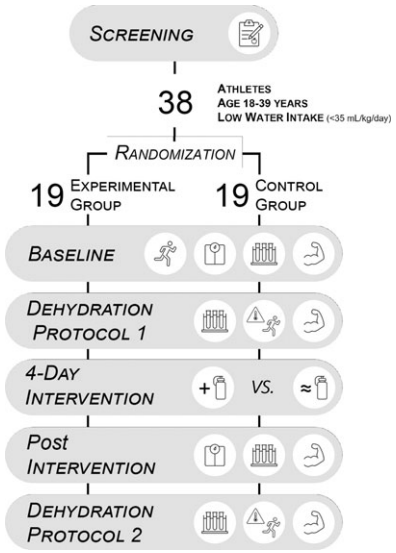

Fig. 2. Schematic description of the study design.

lmtest package. Athletes will be randomised using a balanced (1:1 ratio) design of permuted blocks stratified by sex. Given the nature of the study, it is not possible to blind either participants or research team members regarding the allocated groups.

Dehydration protocol

Before and after the intervention, participants will be submitted to an exercise-induced dehydration protocol on a treadmill in an environment with heat stress. Before starting, samples of urine, saliva and blood will be collected, and participants will be weighed wearing only underwear after. The dehydration protocol will include up to four bouts of 15 min on a treadmill to achieve 1·5–2 % of BML (attributed to fluid/sweat loss) with 5-minute breaks between bouts to allow athletes to clean the accumulated sweat using a towel and to be weighed using an electronic scale connected to the plethysmograph computer (BOD POD®, Life Measurement, Inc., Concord). Throughout, no fluid or food intake will be allowed, and corrections will be performed for urine in case of any urine passed during the protocol[1].

To create heat stress of 26–30°C, the air temperature will be adjusted, using an air conditioning device, according to relative humidity. The temperature and humidity will be monitored using a temperature-humidity meter (BC25, Trotec Gmbh & Co). We will also assess the skin temperature (forehead, hands and feet) using a portable infrared thermometer (RI01-RD-LED, Alicn Medical) immediately before the individuals enter the heated room and after dehydration (after saliva, urine and blood collection and BIA analysis as described below). To ensure that all participants exercise at the same percentage of effort, treadmill workloads will be individualised based on 80–95 % of the first ventilatory threshold (VT1) assessed in the maximal graded exercise test performed during the baseline assessments (described below) and treadmill grade will be set as 1 %. The VT1 was chosen because it considers the metabolic and ventilatory responses of an individual, leading to a more personalised and potentially optimised dehydration protocol.

In contrast, using other approaches such as the percentage of maximal oxygen consumption or percentage of maximal heart rate as a basis for the dehydration protocol is a more standardised approach[33,34]. While it provides a general measure of exercise intensity, it may not account for individual variations in physiological responses to exercise. This can lead to less precise intensity, and therefore, utilising the VT1 in dehydration may offer a more individualised and effective approach.

Intervention

Over a 4-day period, participants assigned to the EG will be instructed to increase WI (≥ 45 ml/kg/d, maintaining regular solid food choices, but increasing fluid intake[30]), while the CG will be instructed to maintain WI (≤ 35 ml/kg/d, maintaining regular solid food choices and fluid intake). The 4 d of intervention was chosen based on previous studies,[12] where authors found that body mass, urine colour, urine specific gravity, 24-h urine and haematological variables are sensitive to 4 d of alterations in habitual fluid intake. For the EG, prepared bottles of water with the required amount will be given every morning and collected empty the following day. Instructions to drink small amounts of water every hour will be transmitted. Interactions through social media platforms (WhatsApp®) will be used to reinforce the intervention and share experiences (i.e. common difficulties and possible solutions) regarding changes in WI.

On the 1st and 4th day, participants will collect first and second urines of the day as well as a void sample of saliva[35]. Also on the 4th day, participants will perform the neuromuscular protocol described below in the 'Neuromuscular function tests' section.

Adherence to instructions regarding WI will be determined by the return of drinking bottles, analysis of daily food records, assessment of water turnover (i.e. analysis of deuterium elimination rate from collected second urines of the 1st and 4th day of the intervention[35] and daily screening questions.

Measurements

Participants will be asked to wear comfortable clothes during the dehydration protocol and neuromuscular function test. For the body composition assessment, participants will be asked to only wear underwear and remove metal accessories. Evaluations will be conducted at a room temperature of 21–23°C and relative humidity of 40–50 %, except for the dehydration protocol, as described. Only the researchers involved in this investigation will have access to the final data related to this protocol.

Anthropometric measurements

Participants will have their weight and height measured wearing a bathing suit and without shoes to the nearest 0·01 kg and 0·1 cm, respectively, with a scale and stadiometer (Seca, Hamburg, Germany). BMI will be calculated using the formula (weight(kg)/height^2(m^2)).

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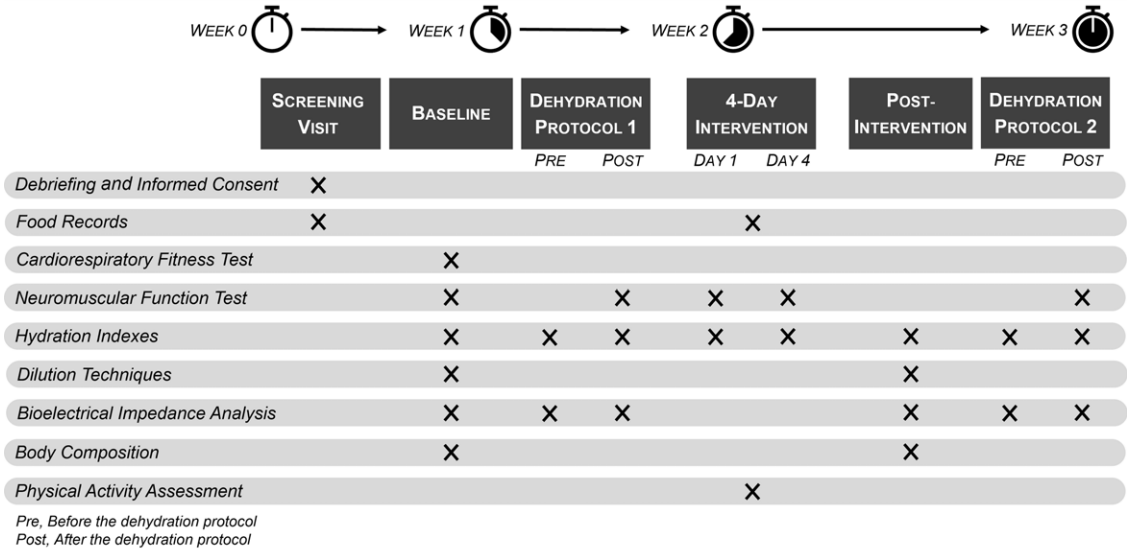

Fig. 3. Schematic description of the measurements that will be assessed and when they will be performed.

Graded exercise test

A trained exercise physiologist will perform a graded exercise test until exhaustion on a variable speed and incline treadmill (Pulsar 3p, HP Cosmos) to determine their VO_2peak and the VT1. The test will start with a 5-min seated period, then a 1-min standing followed by a 1-min at 8 kmh^{-1} with consecutive increases of 1 kmh^{-1} per minute until exhaustion and an inclination grade of 1 %. Recovery will include a 3-min walk at 2·4 kmh^{-1} speed and a grade of 2·5 % and 3-min sitting. Expired gas measurements will be taken using a breath-by-breath metabolic cart (QUARK RMR, version 9.1, Cosmed). The VO_2 and heart rate data will be displayed in 20-s averages. Participants will exercise until participants volitional fatigue or VO_2 does not increase despite increasing workload[36]. Peak oxygen will be determined as the highest 20-s average of the last minute. The highest oxygen value attained at the end of the test will be accepted as VO_2peak if a plateau in VO_2 with an increase in treadmill speed is observed.

The VT1 will be determined according to (1) the modified V-slope method; (2) the ventilatory equivalent method (ventilation (VE)/VO_2 method) and (3) the end-tidal O_2 pressure method[37].

Food records

Three-day food diaries will be used to assess the athlete's habitual total WI (i.e. the sum of the water in beverages and foods). Before filling, a certified dietitian will provide written and verbal instructions using specific guidelines, pictures of portion sizes and examples of common errors. The software package Food Processor Plus® (version 10.12.0.0, ESHA Research, USA) will be used to determine the contribution of water from beverages and foods. To identify under and over-reporting, the ratio between energy intake and resting energy expenditure (estimated by the Schoefield equations[38]) will be used[39]. The cut-offs used will be 1·1 for under-reporting[40] and 4·0 for the over-reporting[41], excluding values outside this range.

Hydration indexes

Serum, saliva and urine osmolality (mOsm/kg) will be assessed by using the osmometer (Mod OSMO1, Advanced Instruments, Canada) as a measure of the number of dissolved particles per unit of water in each fluid.

Blood samples will be collected to assess serum osmolality and to determine serum AVP through enzyme-linked immunosorbent assay and serum Na concentration by ion-selective electrode – indirect. Blood samples will be drawn from an antecubital vein via single venepuncture by a certified phlebotomist and collected in tubes of serum gel. After collection, the tube will be left for 2 hours at room temperature for clot retraction and then centrifuged for 10 min at 3500 rpm. The serum will be separated into two tubes for freezing at −80°C and −20°C, respectively, for serum AVP and serum Na.

For saliva sample collection, subjects will provide unstimulated saliva by sitting quietly for 2 min, allowing saliva to passively accumulate in the mouth. Then, participants will collect 3–4 ml of saliva in salivettes or until they feel that they are not able to. The CV calculated in our laboratory using ten subjects is 0·2 %.

Urine specific gravity will be determined using a refractometer (PAL-10S, ATAGO) from a fasting urine sample. All the mentioned samples will be analysed in duplicate. Based on ten subjects, the CV is 0·02 % for urine specific gravity.

Additionally, visual analog rating scales of thirst and mouth dryness will be obtained. Participants will answer questions by placing a mark on a 10 cm line according to their subjective analyses.

Dilution techniques

Deuterium dilution will be used to measure TBW using a hydra stable isotope ratio mass spectrometer (PDZ, Europa Scientific). After a 12 h fast, participants will be weighed and collected their first morning urine sample. Immediately after, each participant will take an oral dose of 0·1 g of 99·9 % ^2H_2O per kg of body weight (Sigma-Aldrich) diluted in 50 ml of tap water. During the equilibration period, during which no food or beverage will be consumed, subjects will be asked to void 2 h after the oral dose and collect urine sample after 4 h and 5 h. Collected samples will be prepared for $^2H/^1H$ analysis, as described elsewhere[35]. The CV based on ten repeated measures for TBW with the stable isotope ratio mass spectrometry in our laboratory corresponds to 0·3 %[42].

Through the dilution of sodium bromide (NaBr), ECW will be determined. After collection of a saliva sample, each participant will be asked to drink 0·030 g of 99·0 % NaBr (Sigma-Aldrich) per kg of body weight diluted in 50 ml of ultrapure water. During a 5 h equilibration period, during which no food or beverage will be consumed, saliva samples will be collected at hour 3, 4 and 5 into salivettes. The samples will be centrifuged and frozen for posterior analyses through high-performance liquid chromatography (Dionex Corporation). Details are described elsewhere[43]. The CV in our laboratory based on ten repeated measures for ECW using high-performance liquid chromatography is 0·4 %[44]. ICW will be determined as the difference between TBW and ECW (ICW = TBW-ECW).

For both analyses (TBW and ECW), single-use containers will be used throughout the collection and analysis process, being stored in adequate conditions to avoid any contamination. Moreover, during the analysis process, duplicates, blanks and multiple standards will be used, helping to identify if any contamination occurred.

Bioelectrical impedance analysis

Whole-body and segmental BIA will be applied using the AKERN BIA 101/BIVA PRO, a phase-sensitive single-frequency BIA (SF-BIA) device that measures PhA and Z, and calculates R and Xc. Subjects will be instructed to lie in a supine position for 2 min[45] before measurement with their legs abducted at 45° compared with the median line of the body and arms at 30° from the trunk. After cleaning participants' skin with isopropyl alcohol, eight low-impedance electrodes (Biatrodes, Akern Srl) will be used for measuring the raw parameters by placing them on the dorsal surfaces of both feet and ankles and at both wrists and hands, keeping a distance of 6 cm between each electrode[46]. Classic and specific bioimpedance vector analysis will be performed, i.e. normalising R and Xc parameters for stature (H) in meters (R/H and Xc/H)[47] and for cross-sectional area[48], respectively. Then, the values will be plotted inside a graph (RXc-graph). The measurement will be done around 20–25 min after the dehydration protocol with control for skin temperature at around 36°C (cleaned from sweat) with a room temperature of 21–23°C[28]. The CV of these measurements in our laboratory corresponds to 0·6 and 1·5 % for R and Xc at 50 kHz, respectively[49].

Body composition – four-compartment model

Fat mass, a molecular component, is calculated from mathematical models, a reference four-compartment model, as described below:

$$FM(kg) = 2.748 \times BV - 0.699 \times TBW + 1.129 \times Mo - 2.051 \times BW$$

BV is the body volume (L), and it will be assessed by ADP (BOD POD, Life Measurement Inc., Concord). Each subject will wear a swimsuit, and their body mass will be measured to the nearest 0·001 kg by an electronic scale connected to the plethysmograph computer. BV will be computed based on the initial BV corrected for thoracic gas volume and a surface area artifact computed automatically. The measured thoracic gas volume will be obtained in all subjects. The CV for BV, based on test-retest using 10 subjects was 0·5 %[23].

Bone mineral content (BMC, kg) will be determined using DXA (Hologic Explorer-W, QDR for Windows version 12.4). Considering that bone mineral content represents ashed bone, bone mineral content will be converted to total-body mineral osseous (Mo) via multiplying it by 1·0436[50]. Based on test-retest using 10 subjects, the CV for BMC in our laboratory was 1·6 %[23].

After the determination of fat mass from the four-compartment model, FFM is obtained by subtracting fat mass from body mass. Hydration of the FFM can be calculated as TBW/FFM.

Neuromuscular function tests

Using a portable hand dynamometer (Jamar Plus+, Sammons Preston, Rolyon), handgrip strength will be measured through maximum voluntary isometric contraction (MVIC) of the hand and forearm muscles for 5 s with participant sitting in a straight-back chair, with feet flat on the floor, shoulders abducted and neutrally rotated, elbow flexed at 90°, forearm in neutral position with the wrist self-selected between 0–30° extension and between 0–15° ulnar deviation. Prior to the test, the grip dynamometer will be adjusted to the size of the hand. Each participant will be assessed three times on both hands alternately. After each attempt, there will be a resting period of 60 s[51].

For the lower body strength, participants will be assessed on a Biodex System 3 Pro isokinetic dynamometer (Biodex Medical Systems). The participants will remain seated with the belts positioned on the thorax, abdomen, thigh and above the knee, on the side, that is being evaluated to limit knee movement. The knee centre of rotation will be carefully aligned with the dynamometer axis of rotation, and the lever arm will be firmly attached to the lower leg with inextensible straps 2 cm above the medial malleolus. A familiarisation session will be performed. First, the participants will complete a questionnaire to identify their dominant leg[52]. Then, the participants will be asked to perform one set of fifteen sub-maximal repetitions of isokinetic contractions performed at 60° s^{-1} and then two sets of maximal isometric knee extension (knee at 70° for the extension) and knee flexion (30° for the flexion)[53]. This will be performed to minimise the learning effect associated with this specific motor task[54].

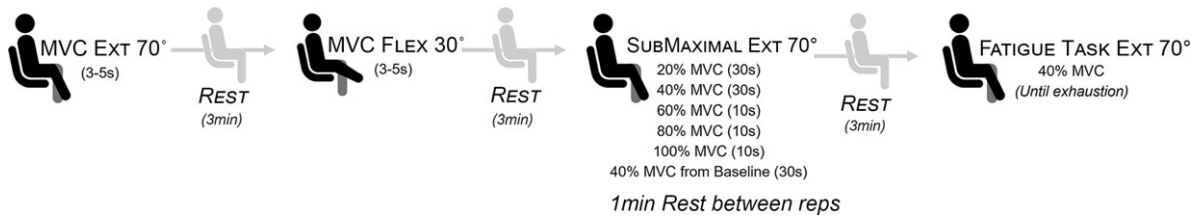

Fig. 4. Schematic description of the neuromuscular measurements using the isokinetic dynamometer.

Each testing session will begin with a warm-up of the dominant leg, consisting of three sets of 3 s knee extension and flexion at 50 %, 75 % and 1 set at 90 % of athletes' perceived maximum effort[55]. Afterward, an MVIC 5-s voluntary knee extension (knee at 70°) and knee flexion (knee at 30°) will be performed with a 3 min pause between trials. The participants will be instructed to avoid any countermovement and will be asked to exert their maximum force as hard and fast as possible. After a 3 min rest, participants will perform one set of four submaximal isometric repetitions of knee extension from MVIC of that day using visual feedback provided by the software: (1) 30 s at 20 % of MVIC; (2) 30 s at 40 % of MVIC; (3) 10 s at 60 % of MVIC and (4) 10 s at 80 % of MVIC; and then 1 maximal set of 10 s at 100 % of MVIC. In the measurements after the baseline, participants will be asked to perform one additional set at 40 % of MVIC from the baseline to calculate the percentage change in MVIC and pRTD across time points. Between repetitions, a pause of 1 min will be performed between repetitions. Last, and after a 3 min rest, participants will perform an isometric contraction at 40 % of MVIC (measured on the day) until exhaustion. Exhaustion will be considered if a decrease of more than 10 % of 40 % of MVIC for more than 10 s is observed[56]. Throughout, verbal encouragement and audible feedback from the dynamometer software will be provided to each participant. The protocol is presented in Fig. 4.

Both maximal torque in Newtons (N) and rate of torque development (RTD) will be obtained[53,57]. The torque onset will be considered 6Nm above the baseline. Contractions associated with pre-tension or countermovement will be discarded. The greatest instantaneous torque achieved during any repetition will be considered the MVIC for both knee extension and flexion. The RTD will be obtained from the slope of the force–time curve (Δforce/Δtime) expressed in $N.s^{-1}$. Then, the instantaneous RTD peak (pRTD) will be obtained as the highest slope of the curve[58] using a 20-ms time window. Sequential RTD will be calculated in incremental epochs of 50 ms from torque onset (0 ms) up to 250 ms[53]. Explosive torque (Etorque) will be defined as the % aMVIC attained at specific time points (50, 100, 150 and 200 ms). Torque, pRTD and sequential RTD will be measured in absolute and normalised terms (relative to MVIC) as described elsewhere[53].

During the lower body assessments, EMG signals will be recorded (EMG Delsys Trigno Avanti, Delsys Incorporated) from the vastus lateralis, rectus femoris, vastus medialis and biceps femoris muscles in accordance with the guidelines of the Surface EMG for the Non-invasive Assessment of Muscles[59]. The electrodes will be placed before the 5 min of resting after the dynamic warm-up. EMG signals from each muscle will be pre-amplified (gain 1000), band-pass filtered (20–450 Hz) and A/D converted at 1 kHz (MP100, BIOPAC Systems Inc.). AcqKnowledge 3.9.1 software will be used for data collection and processing (BIOPAC Systems Inc.). For MVIC, the amplitude of background surface EMG will be derived from the average rectified value analysed over an epoch of 500 ms (250 ms either side) around MVIC (MATLAB version R2014b) and through fast Fourier transformation the median power frequency will be calculated[7]. For submaximal repetitions, from each recorded contraction, a 500 ms period of stable torque at approximately the prescribed level will be identified and used to calculate mean knee extension torque. The torque/EMG of each agonist (quadriceps) and antagonist (hamstring) EMG site will be measured for each of these epochs for both maximal and submaximal knee extension repetitions. The relationships of the torque-agonist EMG and torque-antagonist EMG will be assessed with: (1) relationship slope ('m' constant of the linear function); (2) EMG at the highest common torque achieved by all participants (be derived by solving the individual linear function for the relationship between isometric knee extension torque and either agonist or antagonist EMG); (3) the slope of the antagonist EMG-agonist EMG relationship will be also calculated and then (4) the relationships of the torque-agonist EMG and torque-antagonist EMG will be plotted.

The CV for repeated within-day measurement of strength was based on ten adult participants, with the values corresponding to 1·5 % and 2·8 % for CVM of leg extension and flexion, respectively.

Profile of mood states

The profile of mood states questionnaire will be employed to assess distinct mood states. This questionnaire will be completed before and after the dehydration protocols. The profile of mood states is a five-point self-administered scale that assesses various mood states, including sixty-five items that measure six different factors: tension-anxiety, depression – dejection, anger hostility, fatigue-inertia, vigor activity and confusion bewilderment.

Statistics

Statistical analysis will be performed using IBM-SPSS® Statistics for Windows version 27.0, 2017 (SPSS Inc., an IBM Company). The normality of the variables will be assessed by using the Kolmogorov–Smirnov test. Data will be presented as mean ± SE. For demographic categorical variables (e.g. sex), data will be displayed as percentage (%) of *n*. To test the effects of the intervention on neuromuscular (torque, RTD, EMG signal) and hydration variables (serum, saliva and urine osmolality, urine-

specific gravity, TBW, ECW, ICW, FFM hydration and raw BIA parameters), linear mixed models including randomised group and time as fixed effects with sex, baseline values and type of sports as covariates will be performed, assessing the impact of the group (0 – CG; 1 – EG), time (0 – Baseline; 1 – Post-intervention), and group-by–time interaction on the primary and secondary outcomes. If possible, a sub-analysis for the oral contraceptive use, menstrual cycle phase and type of sports (team sports, power and endurance sports) will be performed. For all models, the covariance matrix for repeated measures within subjects over time will be modelled as unstructured or, if necessary, compound symmetry. Difference-in-differences will be calculated between the CG and EG before and after the intervention as: ($Outcome_{post\text{-}intervention}EG - Outcome_{Baseline}EG$) – ($Outcome_{post\text{-}intervention}CG - Outcome_{Baseline}CG$). Since our intervention includes repeated measurements, reliability of the dependent variables will be tested over time using test-retest analysis and across different researchers (inter-rater reliability). Linear mixed regression models will also be used to explore the association between the neuromuscular function (dependent variable) and other variables namely hydration state, water compartments and raw BIA parameters (independent variables). A linear mixed regression model will be created for each independent variable. All analyses will be intention-to-treat, including data from all participants who will be randomly assigned. Sensibility analysis will be performed on the primary outcomes (strength and power variables), by performing a single imputation of missing data based on multivariate linear regression to predict missing outcomes. Statistical significance will be set at $P < 0{\cdot}05$. For sample size calculations, we considered a type I error of 5 % and a power of 95 % (using Anova repeated measurements on the software GPower® version 3.1.9.2) to detect an effect size of 1·1 for statistically significant differences based on changes in MVIC of knee extension after acute dehydration as reported elsewhere[60]. A total of nineteen participants per group would be required. Thus, we will enroll a total of thirty-eight participants. As the study is short for each participant, and we have several contact times with participants, it is not necessary to include a dropout rate. Also, based on a previous pilot study (n 3), we do not anticipate difficulties in maintaining the participants enrolled in the study given its nature and time course. Still, if any participant withdraws, the results of the analyses already collected will be delivered to the respective participant.

Discussion

Overall, the H_2OAthletes project will allow us to understand if athletes with a low habitual WI can benefit in their performance and hydration status from increasing WI for 4 d.

Regarding neuromuscular function, we will be able to analyse if fatigue is due to the neural system. In this sense, after the dehydration protocols, we expect to observe EMG changes associated with muscular fatigue, such as a decrease in MPF towards lower frequencies. A reduction of MPF has been linked to reductions in muscle fibre conduction velocity[7,61]. Therefore, it can be hypothesised that a shift in the MPF towards lower frequencies could reflect a reduced membrane excitability[7]. We also expect that participants will be able to increase their motor command to counteract dehydration effects and muscle fatigue, observing an increase in average rectified value as a result of centrally mediated regulation of the motor unit activity resulting from an increase in firing rates of motor units and/or an increase in the motor unit recruitment[62]. These results are expected especially for lower levels of contractions (altering the relation torque/EMG), as at the higher values the possibility for recruitment of additional motor units is smaller than at lower levels of contraction[63]. We also expect a decline in MVIC and RTD after dehydration due to the reduced possibility of recruitment of additional motor units at maximal intensities and a reduction of the frequency discharge rate[63]. Additionally, psychological factors may affect neuromuscular function, as dehydration has been related to increased perceptional fatigue, tension and anxiety, possibly affecting participants' behaviour and reducing their motivation[64]. In this trial, the inclusion of the profile of mood states questionnaire will be used to explore if these variables are associated with neuromuscular function.

As mentioned, some athletes have a low WI[15]. Evidence has demonstrated differences between LOW and HIGH in thirst, urine indexes and AVP[12]. In fact, LOW have higher AVP values, suggesting intracellular dehydration. Moreover, although some studies[16–18] have highlighted the importance of assessing ICW and ECW for athletes, there are no studies linking the water compartments to neuromuscular function in athletes. Thus, this study would show how body water compartments respond to a WI and how they are related to AVP, which has not yet been reported. For instance, if those exposed to 4 days of increased WI increased their ICW, a reduction in cellular shrinkage would be expected, as supported by the AVP changes reported by previous studies[12].

Finally, there is no consensus regarding the best protocol for hydration assessment in athletes[22]. In this project, as AVP levels will be assessed before and after the dehydration protocols, we expect an increase in AVP levels after the acute dehydration protocols, but we also expect a fall in serum AVP in the pre-dehydration assessment following the 4-day intervention in the EG. Alternatively, it has been previously pointed out that raw BIA parameters are useful predictors of body water compartments[43,65]. However, the usefulness of these parameters as markers of fluid-related shifts requires further research, especially in tracking acute hydration changes. This trial will employ broad hydration status-related variables assessments, helping to understand which methods might be more appropriate for each situation (i.e. acute hydration and after increased WI). Despite the strengths of the H2OAthletes project, some limitations should be addressed. Regarding female athletes, no sexual hormone analysis will be performed. We are fully cognizant of the significance of the higher strains experienced during the luteal phase of the natural menstrual cycle, primarily attributed to progesterone's stimulatory influence on the autonomic nervous system[66]. Additionally, we recognise that exogenous hormone use can significantly impact day-to-day recovery, particularly the increased cardiovascular strain when compared with the natural menstrual cycle, possibly affecting the dehydration protocols[66]. While we acknowledge the importance of studying these specific factors, we must address the challenges we face with the recruitment process. Our study requires athletes who engage

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in more than 6 h of training per week, have a regular low water intake and are available for a highly demanding study in terms of time commitment. Consequently, it is not feasible for us to guarantee that all participants will start in the same menstrual phase or be free from the use of birth control pills. Nonetheless, we emphasise that we will diligently capture and analyse the available data on menstrual cycle phase, hormone usage and any relevant factors that may influence the study outcomes. This comprehensive approach will enable us to gain insights and analyse the relationships between hormonal fluctuations, hydration status and athletic performance as far as our sample permits[66,67]. However, considering the study time required to evaluate each athlete, it is not possible to always evaluate the female athlete in the same phase and thus it must be mentioned as a limitation. Although we recognised that a hormonal sex evaluation would be extremely useful in our project, it would make our project more expensive, and it is beyond our possibilities.

Additionally, we are aware that other techniques such as the interpolated twitch technique could provide more insights as a method used to assess voluntary muscle activation, and it has been performed to study the effects of dehydration on neuromuscular function[68,69]. This method involves delivering an electrical stimulus to the motor nerve during a maximal voluntary contraction, allowing for the estimation of the level of voluntary muscle activation[68,69]. While interpolated twitch technique has its merits, we are not able to include this method in our study because we do not have access to this technique during the time course of this project. Still, EMG offers distinct advantages in terms of its ability to provide continuous, real-time, detailed and differentiated measurement of muscle activation.

Another methodological limitation in our dehydration protocol is the combined use of heat stress and exercise, resulting in the possible effects of hypohydration being masked or exacerbated by exercise-induced fatigue and heat. It has been shown that heat exposure affects EMG signal[70] and thus it is not likely that dehydration affects neural drive, but may still act synergistically with skin temperature, core temperature or both. Still, we will control the skin temperature of the individuals and allow a recovery (while the athletes perform the post-dehydration data collection) before the neuromuscular tests. Core temperature would be interesting to analyse in our study due to the information provided about the temperature of the body's internal organs[71], while skin temperature refers to the temperature of the outer layer of the skin and may not provide the same level of accuracy[72]. However, the core temperature generally involves invasive methods such as rectal temperature or gastrointestinal pills[71]. Rectal temperature is widely accepted as an accurate measurement, stable when compared with other areas of the body and has small individual variability[71]. Despite these advantages, it is important to consider ethical considerations and individual consent when using rectal temperature measurement in research settings[71]. We found it not feasible due to its invasiveness, discomfort, hygiene, safety concerns and potential social desirability bias, possibly discouraging volunteers from participating in the study. Considering our demanding dehydration protocol that already includes invasive measurements such as blood collection, assessing rectal temperature would be too onerous for our participants. Another option would be gastrointestinal pills that are less invasive and seem to achieve the best balance between practicability, comfort and user acceptance and scientific validity and reliability[71]. However, due to funding constraints, we are not able to include it in the research project.

Also, the exercise session will occur below the VT1 to avoid or minimise fatigue. Although passive dehydration could be an option, it is important to note that while it may occur *in* situations like hot weather or saunas, it should not be considered a substitute for active hydration and cannot replicate the physiological demands experienced during exercise such as the imbalance in fluid and electrolyte levels[9]. Nevertheless, as the dehydration protocol will be performed twice (before and after the 4-day intervention), we will be able to identify if the EG presents a better response to the dehydration protocol compared with the CG. Moreover, although we cannot isolate the effect of heat-induced dehydration, exercise-induced dehydration is more realistic to the athletes' experience, providing more practical outcomes easily transposed to the athletes' training and performance. Also, some studies[73–75] were able to test the effects of dehydration on exercise performance with their subjects blinded to their hydration status given the acute nature of their study design, in our study this will not be possible given the nature of our study (two acute dehydration and 4-day intervention).

We also expect that long-distance athletes frequently exposed to training in heat may have a different response to our dehydration protocol[76]. It is known that repeated heat exposure induces adaptations that abate cardiovascular strain and likely contribute to attenuating exercise performance impairment and reducing the risk of heat illness. The cardiovascular adaptations include an increase in TBW, plasma volume expansion and enhanced skin blood flow and sweating responses. The magnitude of these adaptations is dependent on several factors such as exercise intensity, duration, frequency and number of exposures to the heat, as well as to the environmental conditions (e.g. dry *v.* humid heat) and protocol in which acclimation occurs[77]. Also, long-distance runners often experience plasma volume adaptation as part of their physiological response to endurance training. Plasma volume adaptation refers to the increase in blood plasma volume that occurs because of regular endurance exercise. During long-distance running, the body's blood volume, including plasma volume, may have several performance benefits, such as improved cardiovascular function and thermoregulation[78]. Although our research team is aware of these limitations, our recruitment is demanding and very specific, and thus we are not able to limit our study to a specific type of sports. Thus, if possible, we will analyse our data adjusted for the type of sports.

Finally, seasonal variations may influence the amount of water intake. Factors such as temperature, humidity and physical activity levels can vary significantly between seasons and affect an individual's hydration needs. Thus, we cannot ignore that categorising individuals as high or low drinkers may be dependent on seasonal variations.

Overall, we anticipate that the H_2OAtletes project will advance and clarify the effects of hydration changes (i.e. acute

dehydration and a 4-day intervention to increase WI) on neuromuscular function (with EMG analysis) using state-of-the-art methodologies.

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