CLINICAL TRIAL DOCUMENT

Study Protocol and Statistical Analysis

Substrate Oxidation Responses to Moderate-Intensity Exercise Under Fasted and Fed Conditions in Male Soccer Players

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Study Protocol and Statistical Analysis

Participants

Nineteen soccer players with a minimum of 10 years of training experience and a regular training frequency of 2–3 sessions per week were recruited. Four participants withdrew after the baseline assessment because of time constraints, and a further four participants were lost to follow-up during the intervention phase following the second experimental visit. Therefore, data from eleven participants who completed all study procedures were included in the final analyses (Figure 1). Participants were eligible for inclusion if they met the following criteria: (1) male soccer players aged 18–35 years, (2) minimum of 4 years of continuous training at a professional or semi-professional level, (3) body mass index between 18.5 and 24.9 $kg \cdot m^{-2}$, (4) non-smoker, and (5) free from musculoskeletal injuries or any diagnosed metabolic or cardiovascular diseases that could interfere with exercise performance or metabolic assessments. Prior to data collection, all participants provided written informed consent. The study protocol was approved by the institutional ethics committee (SBA 26/387) and conducted in accordance with the Declaration of Helsinki.

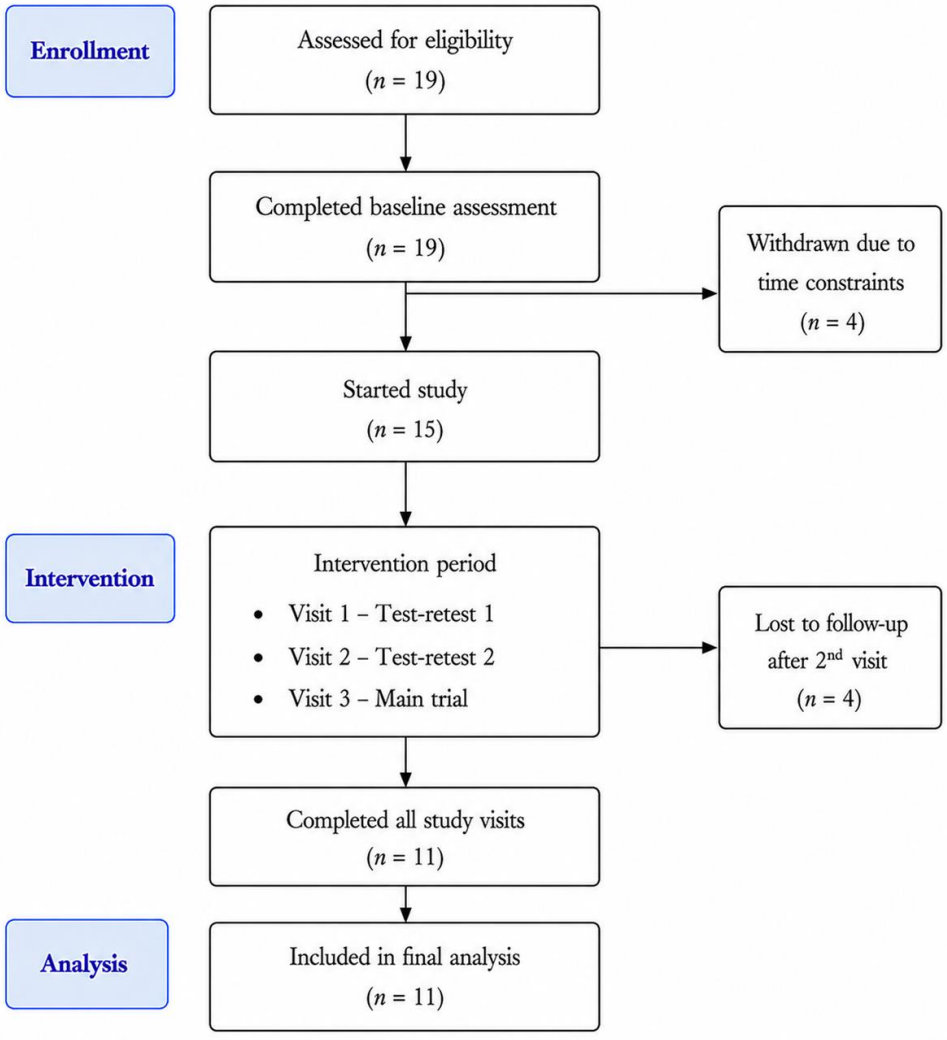


Figure 1. Consort participation flow from enrollment to analysis.

Study Design

This study employed a repeated-measures, within-subject design to examine the reproducibility of exercise substrate oxidation measurements and to compare substrate oxidation during exercise responses between fasted and fed conditions in soccer players. The study was conducted during the off-season to minimize the influence of competitive match congestion and training load variability. Each participant attended the laboratory on four separate occasions following an overnight fast of 10–12 hours. All laboratory visits were conducted under standardized environmental conditions at the same time of the day to reduce the potential influence of circadian variation. Participants were instructed to avoid strenuous exercise, alcohol, and caffeine for at least 24 hours before each visit and to maintain similar dietary intake before experimental trials. During the first visit, participants underwent body composition assessment using dual-energy X-ray absorptiometry (DXA). Following this assessment, participants completed a maximal incremental exercise test on an electronically braked cycle ergometer to determine $\dot{V}O_2max$. After a 2-hour recovery period, participants performed a 20-minute familiarization trial at 60% of $\dot{V}O_2max$ to minimize potential learning or habituation effects during the subsequent experimental trials. The second and third visits served as reproducibility trials, during which participants completed a 30-minute submaximal cycling test at 60% of $\dot{V}O_2max$ on an electronically braked cycle ergometer under overnight-fasted conditions. These two trials were used to assess the test–retest reproducibility of substrate oxidation measurements during steady-state submaximal exercise. The fourth visit was conducted at least 72 hours after the previous trial. Participants arrived at the laboratory at 08:00 following a 10–12-hour overnight fast and completed a 30-minute submaximal cycling test at 60% of $\dot{V}O_2max$ in the fasted condition. One hour after completion of the first exercise bout, participants consumed an *ad libitum* meal consisting of pasta and meatballs. Two hours after the first exercise bout, corresponding to one hour after meal ingestion, participants completed a second 30-minute submaximal cycling test at the same relative intensity under the fed condition (Figure 2). During all 30-minute submaximal exercise trials, respiratory gases were measured using a Quark CPET system (COSMED, Rome, Italy). Gas exchange data were analyzed during predefined steady-state periods, specifically minutes 10–15 and 25–30 of exercise, to calculate rates of CHOx and FOx.

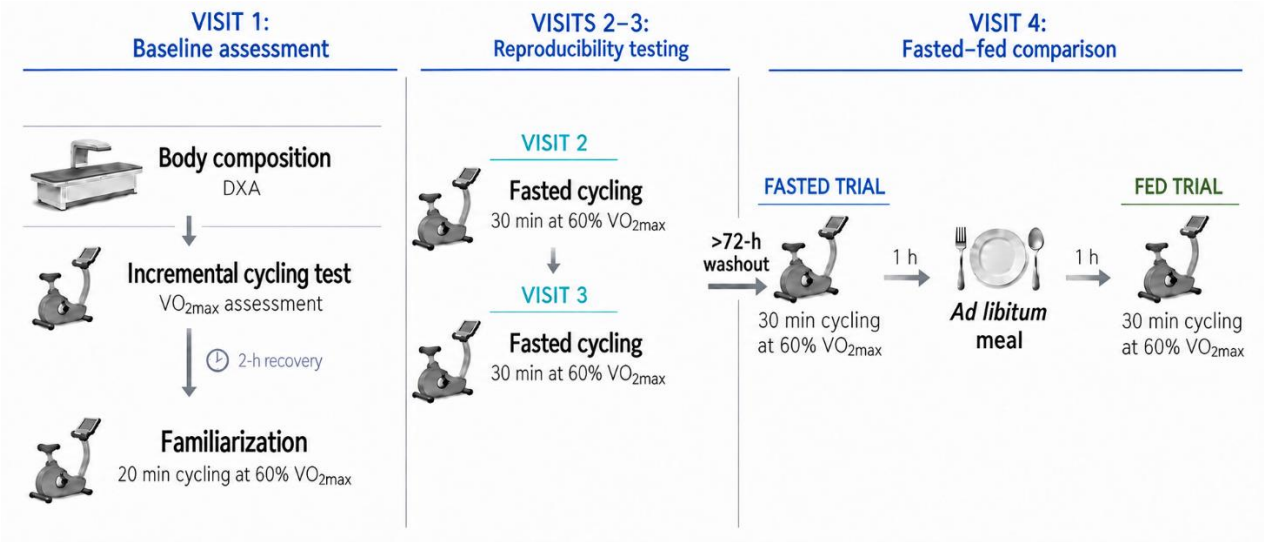


Figure 2. Study design.

Measurements

Body composition measurement

Body composition was assessed using DXA (Lunar Prodigy Pro Narrow Fan Beam (4.5º), GE Health Care, Madison, Wisconsin, USA in the morning following an overnight fast of 10–12 hours to minimize the potential influence of recent food or fluid intake on measurement accuracy. Prior to each assessment session, DXA was calibrated in accordance with the manufacturer's standard operating procedures to ensure measurement precision and consistency. All scans were performed by the same trained researcher in a standardized manner, with participants wearing light clothing and no metal accessories.

$\dot{V}O_2$max measurement

Following DXA body composition assessment, $\dot{V}O_2$max was assessed using a graded incremental exercise test performed on a cycle ergometer (COSMED E200, Rome, Italy) at the first visit. The test protocol began with three consecutive 2-min stages at 60 W, 120 W, and 150 W, respectively. Thereafter, the workload increased by 30 W every minute until the participant reached voluntary exhaustion. Heart rate (HR) was continuously monitored throughout the test using a heart rate monitor (Garmin International Inc., Olathe, KS, USA). Expired gases were collected breath-by-breath using an online gas analysis system (Quark CPET, COSMED, Rome, Italy), calibrated before each test according to the manufacturer's specific directions. $\dot{V}O_2$max was defined as the highest 30-sec average value recorded during the test. The test was considered valid if at least two of the following criteria were met: (a) a plateau in oxygen uptake despite increasing workload (i.e., $\leq$2 mL·kg^{-1}·min^{-1} increase), (b) a respiratory exchange ratio (RER) exceeding 1.10, and (c) a heart rate within 10 beats·min^{-1} of the age-predicted maximum (220 − age).

Reproducibility of submaximal exercise tests

During visits 2 and 3, two submaximal exercise tests that consisted of a 30-min cycling session performed at 60% of individual $\dot{V}O_{2max}$ on an electronically braked cycle ergometer (COSMED E200, Rome, Italy) were performed under identical conditions (*i.e.*, in the morning after an overnight fast) to evaluate the reproducibility of substrate oxidation during exercise. Respiratory gases were collected during two steady-state periods: from 10 to 15 minutes and 25 to 30 minutes, using the Quark CPET system (Quark Cardio Pulmonary Exercise Testing, COSMED, Rome, Italy). To minimize participant discomfort from the breath-by-breath mask, respiratory data were collected only during these specified periods of the 30-min submaximal test. FOx and CHOx rates were calculated using stoichiometric equations based on respiratory gas exchange, as proposed by Jeukendrup and Wallis [20].

Measurement of substrate oxidation during submaximal exercise

During visit 4, following a 3-min warm-up at 60 W, participants performed two 30-min cycling tests, with one in the overnight fasted state and one in the postprandial state, at a power output corresponding to 60% of their predetermined $\dot{V}O_{2max}$ on a cycle ergometer (COSMED E200, Italy). Participants were instructed to maintain a self-selected but consistent cycling cadence (±5 rpm) across both trials. During the tests, $\dot{V}O_2$ and $\dot{V}CO_2$ data were collected on a breath-by-breath basis using an online gas analysis during two steady-state periods, involving 10–15 minutes and 25–30 minutes (Quark Cardio Pulmonary Exercise Testing, COSMED, Rome, Italy). FOx and CHOx were calculated using the $\dot{V}O_2$ and $\dot{V}CO_2$ data collected during the tests with the equations proposed by Jeukendrup and Wallis [20]. For each trial, substrate oxidation rates were averaged across the two steady-state periods and expressed as absolute ($g \cdot min^{-1}$).

Ad libitum energy intake

At the fourth laboratory visit, following completion of the 30-min submaximal exercise test performed in the fasting state, participants were provided with *ad libitum* access to a standardized meal consisting of pasta and meatballs. All meals were freshly prepared on the day of testing by a trained dietitian according to standardized recipes, using weighed ingredients, and controlled cooking procedures. The nutritional composition, calculated per 100 g of cooked food, was 239 kcal, 11.2 g CHO, 20.1 g protein, and 12.7 g fat for the meatballs, whereas the pasta provided 157 kcal, 25.2 g CHO, 4.5 g protein, and 4.2 g fat. Participants were allowed to choose freely between meatballs, pasta, or a combination of both. They were instructed to consume the provided meal until they felt completely satiated, with no restrictions on the amount consumed, within a 20-min period. During the meal, approximately 250 mL of water was provided to standardize fluid intake.

Statistical analysis

Model assumptions were assessed using visual inspection of Q–Q plots of residuals and homogeneity tests. Main effects of condition and time, as well as condition × time interactions, were examined using two-way repeated-measures ANOVA. Relative reliability for substrate oxidation during exercise was assessed using the intraclass correlation coefficient (ICC) from a two-way mixed-effects model with absolute agreement for single measurements (ICC[3,1]) as proposed by Shrout and Fleiss (1979) [21], with results reported alongside 95% CI. Absolute reliability was quantified using the typical error (TE), calculated as the SD of the difference between test and retest measurements divided by the square root of 2. The coefficient of variation (CV%) was then calculated by expressing the typical error relative to the grand mean of test and retest values, where the grand mean represents the mean of all test and retest observations combined. The standard error of measurement (SEM) was calculated to reflect the absolute precision of individual scores using SEM = SD pooled × $\sqrt{}$ (1 − ICC), where SD pooled is the pooled SD derived from the combined test and retest data. The smallest detectable change at the 95% confidence level (SDC95) was calculated to determine the minimum change required to exceed measurement error using SDC95 = 1.96 × $\sqrt{2}$ × SEM. Values are presented as estimated marginal means (EMMs) ± standard error (SE) or SD with 95% confidence intervals (95% CI) for the 10–15 and 25–30 min exercise intervals. Effect sizes are reported as partial eta squared (ηp^2). Statistical significance was set at $p<0.05$. Statistical analyses were conducted with jamovi (version 2.6.44, Sydney, Australia).