PROTOCOL

Physiological and sensory responses to prolonged fasting in humans

REACH:

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1. BACKGROUND

The human genome has evolved around periods of restricted food intake and is highly adapted with the relevant pathways to cope with this. Yet, there are still gaps in our understanding of these physiological responses. In the absence of energy intake, the body

maintains life by using stored energy, which is sufficient to sustain life over a prolonged period (> 1 year) if energy stores are sufficient [1]. In particular, the shift in metabolic substrate initially acts to maintain glucose before then acting to try and spare muscle tissue. However, there is also a significant shift in amino acid metabolism, and little research has explored this in human skeletal muscle and or adipose tissue. Furthermore, to the best of our knowledge, little or no research has investigated changes in the genes/proteins that regulate lipid/glucose metabolism in adipose tissue in response to prolonged fasting.

Furthermore, it has been established that periods of prolonged fasting result in a reduction in skeletal muscle insulin sensitivity [2], perhaps due to the excess of fatty acids resultant from severe energy deficit. However, this has primarily been established using proof of principle methods (e.g. oral, or intravenous tolerance tests). Whilst these have been invaluable in establishing effects, it is also important to employ more ecologically valid models, such as mixed meal tolerance tests.

2. OVERVIEW OF PROPOSED RESEARCH

2.1 Hypothesis

Due to the exploratory/novel nature of the model this is not considered hypothesis driven research.

2.2 Aims and Objectives

Aims

1. To establish the metabolic/molecular response in both adipose tissue and skeletal muscle to acute starvation (~82 h).
2. To establish the sensory experience to prolonged fasting (pain, fatigue, drive).

Objectives

Assess Amino acids in adipose, skeletal muscle and circulation in response to prolonged fasting
Assess postprandial response to a meal pre and post prolonged fast
Assess anthropometric changes in response to prolonged fasting
Assess the sensory experience in response to prolonged fasting

2.3 Rationale

We plan to study participants before, after and during ~82 hours of no energy intake. Whilst few studies have extended starvation beyond 48-72 h, this duration is sufficient to observe the established shift towards greater reliance on lipids to fuel metabolism [2-5]. Necessarily, extension of the starvation beyond this duration therefore guarantees stability in the reliance on endogenous fuels (e.g. NEFAs, and ketones). At the level of the adipose tissue, an increase in the efflux of NEFA from adipose tissue as well as a reduction in glucose uptake is apparent during the early stages of a prolonged fast (~20 h) [5-7]. Necessarily, given that changes in metabolism within the adipose tissue occur this rapidly, it is surprising that so few studies have included measures of this tissue under conditions of prolonged fasting. In particular, assessment of genes and proteins responsible for regulating substrate metabolism in the adipose tissue would provide interesting insight into the metabolic flexibility of this tissue under severe energy deficit. Furthermore, measurement of amino acids and their respective ketoacids in adipose, skeletal muscle, and the circulation would further our understanding of how these metabolically important tissues respond in order to preserve/maintain skeletal muscle during energy deficit [2, 8].

Surprisingly, only one study to date has investigated changes in post-prandial metabolism following mixed meal ingestion, as opposed to oral or intravenous tolerance tests [2, 9, 10]. Notably, whilst this study aimed to achieve a 72-h starve, provision of the mixed meal tolerance test immediately prior to beginning the 72-h starvation period resulted in an actual starve duration closer to ~2 days. Given the dramatic effect of acute starvation on gastric emptying [9] it is especially important to investigate the meal responses. Furthermore, given that insulin resistance following prolonged fasting persists even following a 24 h refeed, it would be insightful to include measures of sequential meals (i.e. the response to the standardised lunch and dinner will be assessed via continual glucose monitors).
Finally, few, if any, studies have assessed the physical activity response to periods of prolonged fasting. Necessarily, methods such as doubly labelled water would not be appropriate due to the underlying assumptions with RQ as well as only giving a total energy expenditure value. Alternatively, employment of combined heart rate/accelerometery (Actiheart), would be appropriate in order to assess the change in physical activity levels, whilst giving a detailed breakdown in changes in intensity.

2.4 Study design

This study will employ a single-arm, non-randomised design in which participants will act as their own control prior to undergoing 3 days of starvation. The control phase includes ~3 days of habitual energy balance assessment before coming into the laboratory for baseline metabolic and anthropometric assessment. Participants will be provided with monitoring equipment throughout the 3 days of starvation and will be asked to provide a blood sample as well as record their blood pressure, weight, and urinary ketones each day. On the middle day participants will also be asked to come into the lab to provide a blood sample at 2000 h.

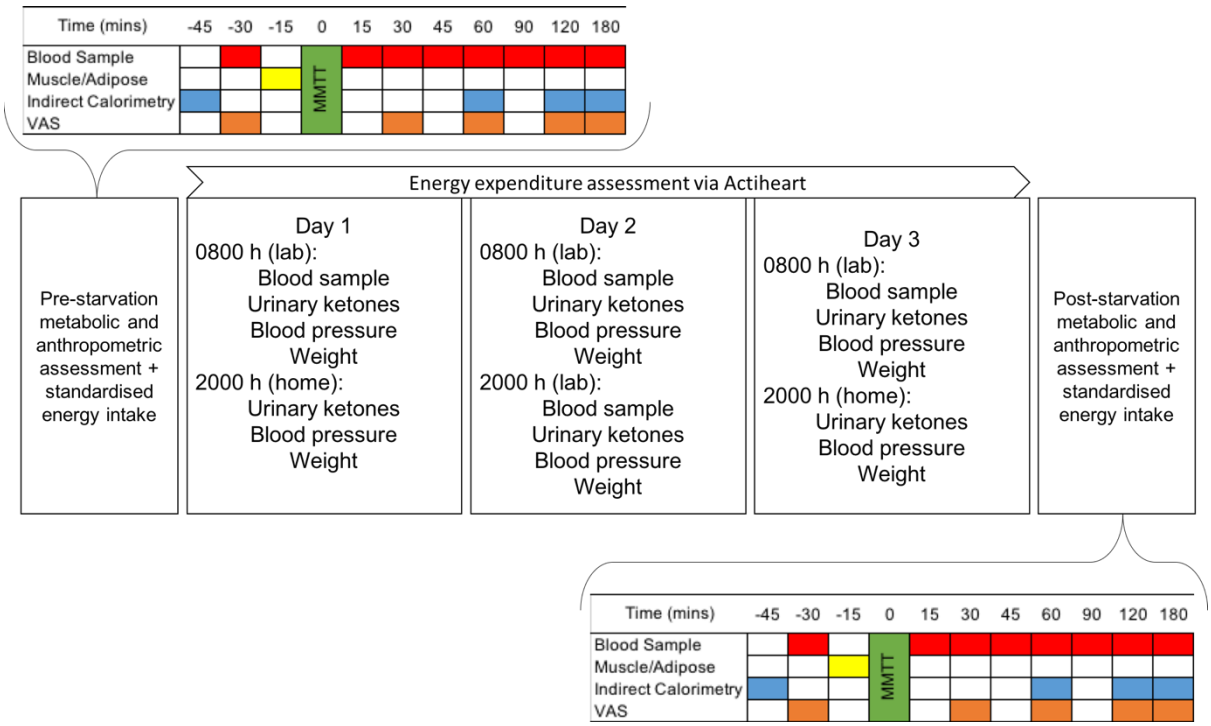

Figure 1. Protocol schematic depicting the study design.

2.5 Outcome measures

Primary

Metabolic response to feeding before and after a prolonged fast
Change in amino acid metabolism (i.e. BCAAs and ketoacids) in skeletal muscle, adipose tissue, and in the circulation

Secondary

Change in circulating glycerol and FFAs across the prolonged fast (indices of lipolysis from adipose tissue)
Change in Autophagy (in skeletal muscle and neutrophils)
Total nitrogen (or at least urea nitrogen) in urine (24h collection) throughout the fast (or at least 1st vs last day)
Change in plasma lactate and pyruvate in response to prolonged fasting (indices of Cori cycle)
Change in urinary (acetoacetate), and circulating (beta-hydroxybutyrate) ketones in response to prolonged fasting.
Change in body mass, pre-post, and during the prolonged fast.
Change in blood pressure pre-post, and during the prolonged fast.
Change in waist-hip circumferences pre-post the prolonged fast.
Change in sensory experience (pain, fatigue, drive) in response to prolonged fasting.
Change in haematocrit/haemoglobin pre-post the prolonged fast.
Change in grip strength pre-post the prolonged fast.
Change in reaction time pre-post the prolonged fast.
Change in aspects of immune function and infection risk
Change in interstitial fluid glucose concentrations pre-, post-, and during the prolonged fast (assessed via continual glucose monitoring)
Occurrence of adverse events (e.g. nausea, vomiting, migraines)

2.6 Statistical analysis

Sample estimate:

Due to the drastic nature of the intervention outlined (i.e. the complete abstinence from energy intake), a relatively small sample size will be required. As such, based on previous research, a sample size of 6-10 participants should be sufficient to observe changes in metabolism with this duration of fast.

Descriptive statistics will be calculated using Microsoft Excel. Normality of data will be assessed using GraphPad Prism 8.0. Repeated measures ANOVA Will be employed to identify significant effects of TREATMENT & TIME for post-prandial data. Paired t-test or Wilcoxon will be used to compare variables assessed only pre-and post-intervention. One-way ANOVA will be used to assess changes in variables assessed each day. Bonferroni post-hoc tests will locate significant differences where they exist. Normalised confidence intervals will be calculated to plot as error bars on figures. The magnitude of these CIs directly infers the difference between means at each time point (i.e. statistical significance) rather than variance of individual values around the mean.

3. Recruitment

Principally this study will recruit from within the department of health however of needed then participants will be recruited from the University of Bath campus a by word of mouth and by poster advertisement. The full enrolment protocol is outlined below, however participation

will be centred on the provision of obtaining informed consent prior the study and throughout participation.

Inclusion criteria

- Body mass index 20.0-29.9 kg·m-2
- Age 18-45 years
- Willing to abstain from food intake for just over 3 days (~82 hours)
- Able and willing to provide informed consent and safely comply with study procedures
- Females to maintain record of regular menstrual cycle phase or contraceptive use
- No anticipated changes in lifestyle during the study (e.g. holidays or exercise programmes)

Exclusion criteria

- Any reported condition or behaviour deemed either to pose undue personal risk to the participant or introduce bias
- Any diagnosed metabolic disease (e.g. type 1 or type 2 diabetes)
- Any reported use of substances which may pose undue personal risk to the participants or introduce bias into the experiment
- Pregnancy
- Breastfeeding
- Lifestyle not conforming to standard sleep-wake cycle (e.g. shift worker)
- History of eating disorders (e.g. anorexia)
- Any reported recent (<6 months) change in body mass (± 3%)
- Uncontrolled hyperthyroidism
- Advanced cerebrovascular insufficiency or dementia
- Advanced liver or kidney insufficiency
- History of migraine and or headache
- Psychotic disorders
- Unstable or severe coronary artery disease
- Retinal detachment
- Duodenal or stomach ulcer
- Cancer and malignant disease
- Regular use of Non-steroidal anti-inflammatory drugs (NSAIDs)
- Use of Systemic corticoids
- Use of Antihypertensives (especially beta-blockers and diuretics)
- Use of Antidiabetics
- Use of Anti-coagulants
- Use of Psychotropics (especially neuroleptics and lithium)
- Use of Anticonvulsants.

Participants will provide informed consent both verbally and in written form. Upon expression of initial interest in the study, potential participants will be provided with the relevant appended ‘Participant information sheet’ to read in their own time, dependent on which phase(s) of the study they wish to take part in. Should they then decide they would like to participate in the study, they will be invited to a pre-enrolment meeting at the University, whereby a member of the research team, who is experienced in taking informed consent, will explain the study protocols and procedures. Potential participants are encouraged to ask any questions they may have, in order for them to fully understand what participating in the study

entails. Following this, if the individual is still willing to participate, they will fill out 2 copies of the appended 'Informed Consent Form' - – (V1.0 18/08/20), and eligibility screening will occur.

3. Confidentiality

Each participant will be allocated a unique study identification code upon enrolment to the study. This will be noted alongside their contact information in a designated file which will be kept locked in a separate office. All subsequent documents used throughout the study will be marked with this identification code rather than any identifiable information to prevent any association between personal data and recorded outcome measures by any unauthorised personnel or in any presentation of the data. Any electronic identifiable information will be kept in a password protected computer in a locked office of which only the members of the research team can access. Additionally, no identifiable information will be included in the presentation and dissemination of the results of this study.

4. Trial days

3 days prior to the first experimental visit participants will visit the laboratory to be fitted with a continual glucose monitor, an actiheart activity monitor, as well as a food diary and food scales. They will be asked to record energy intake and to standardise physical activity, caffeine, and alcohol intake.

Upon finishing the 3 days of lifestyle standardisation participants will enter the laboratory for where weight, height, waist:hip circumference, sagittal-abdominal diameter, and skin-fold will be assessed prior to assessment of resting metabolic rate, blood pressure and urinary ketones. Participants will also undergo grip strength, reaction time, and sensory experience assessment. Following this, samples of skeletal muscle and adipose tissue will be collected from the *vastus lateralis* and abdominal adipose tissue respectively. An intravenous cannula will then be placed a 10 mL blood samples will be drawn before ingestion of a carbohydrate rich porridge breakfast (7.3 mg carbohydrate·kJ^{-1}).

A 3-hour postprandial period follows the ingestion of breakfast with blood samples will be collected at 15, 30- and 60-minute intervals during the first, second and third hour respectively alongside hourly expired gas samples and assessment of subjective appetite and mood. Upon completion of the 3-hour post-breakfast period, participants will have completed the visit and will be provided with a standardised lunch and dinner. Participants will also be provided with urinary ketone sticks, a blood pressure monitor, a set of weighing scales, as well as an actiheart monitor.

Participants will be asked to assess urinary ketones using provided assessment sticks in the evening at 2000 h.

5. Intervention

Participants will be provided with their last meal at ~1900 h on the day of the meal testing. From this point onwards, participants will be asked to refrain from eating for ~82 hours until their return visit to the laboratory. Throughout this period of extended fasting participants will be provided with 80 mmol sodium and 40mmol potassium daily (to consume in the form of slow-release tablets) to minimize the confounding effects of fluid deprivation and intravascular volume depletion on cardiovascular reflexes and sympathetic nervous system activity. Subjects will also be permitted to consume water, non-sugared carbonated drinks

and, with the exception of the day of the pre/post measurements, non-sugared decaffeinated coffee and tea (if they normally consume tea and coffee).

Upon waking each day participants will be asked to assess their urinary ketone levels, blood pressure, and weight. Participants will be asked to repeat this in the evening at 2000 h.

Each day participants will be asked to return to the laboratory to provide a blood sample. On the second day of starvation participants will also be asked to provide a blood sample at 2000 h.

Furthermore, throughout the intervention participants will be asked to wear an actiheart monitor in order to assess physical activity levels in response to prolonged fasting.

6. Follow up visit

Upon completion of the intervention, participants will visit the laboratory at the University of Bath, and repeat the pre-intervention visit with the porridge breakfast. Following this repeated visit, participants will again be provided with a standardised lunch and dinner.

7. Ethical considerations of risk and discomfort

Acute starvation may cause discomfort for participants including headaches and lack of energy. All participants will be supervised by a member of the research team throughout the protocol. Participants who drop out after one day of fasting will not be included in follow up testing, but will be provided with food to break their fast. Conversely, participants who decide to not continue with study following 2 days of fasting will be asked to come in for follow up testing/standardised meal provision. Biopsies also introduce a risk for discomfort for participants but will only be performed by trained and experienced members of staff who follow the guidelines for safe practice. Intravenous cannulation has the potential to cause minor discomfort and bruising. Furthermore, there is a small associated risk of infection and/or embolism, where blood flow to the vein becomes partially restricted. In order to minimise these risks, cannulation will only be carried out by qualified and experienced staff members. The cannula is an essential aspect will only be in the vein for a maximum of 3 hours per laboratory visit, whereas the venepuncture needle on days 1,2, and 3 of the fast will be removed as soon as the sample is obtained.

8. Benefits to participants

Participants will be provided with standardised lunches as well as dinners on the pre and post intervention days.

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