Study Protocol and Statistical Analysis Plan
For Research Project:
"Safety and Efficacy of Combining Intranasal Insulin & Acute Exercise"

clinicaltrials.gov # NCT04292535

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Abstract: To determine if physical activity engagement alters the dose-response profile and safety of administration of insulin into the intranasal mucosa. Using a randomized placebo controlled double-blind pre-posttest design, participants were randomly assigned to receive a dose of either 0, 20, 40, 60, 80, 100, or 120 IU of NovoLog insulin aspart prior to being randomized into participating in a 20 minute session of either moderate-intensity aerobic exercise or a passive control condition. The safety of the protocol was assessed using a symptom questionnaire assessing common symptoms of altered blood glucose and common side effects of intranasal insulin. The efficacy of the intranasal insulin for inducing alterations in cognition was assessed using both behavioral and neuroelectric measures.

Aims:

1) To provide a preliminary assessment of the safety profile of combining intranasal insulin with exercise; to ensure that undue risks are not incurred through combination therapeutic approaches.

2) To provide a preliminary assessment of the efficacy of combining intranasal insulin with exercise for enhancing inhibitory aspects of cognitive control and sustained attention alongside neuroelectric indices of attention.

Design: Double blind, placebo controlled Phase 2 clinical trial using serial stratification randomization accounting for biological sex. Participants were first allocated to a dose of intranasal insulin (0 [placebo], 20, 40, 60, 80, 100, 120 IU), then allocated to either exercise or control activity.

Study Population: Given the potential risk of combining exercise with intranasal insulin the present investigation utilized a sample of healthy, non-diabetic, fasted college-aged adults to limit the potential for a serious adverse event. The sample consisted of college aged young adults from the mid-Michigan, USA area.

Eligibility Criteria:

Criteria for inclusion: All individuals that agreed to participate were selected on a first come, first serve basis. No individual was turned down due to sex, race, or ethnicity. The following inclusion criteria exist for all participants:

a. Participants must be over the age of 18.
b. Participants must have normal or corrected-to-normal vision in order to complete the cognitive task.

Exclusion criteria: The following exclusion criteria exist for all participants:

a. Lack of consent.
b. Presence of any major neurological health issues, brain trauma, or concussion with loss of consciousness assessed through a health history and demographics questionnaire.
c. Type I or Type II Diabetes.
d. Self-reported pregnancy.
e. Currently has any type of inflammation or blockage of the nasal passageways (i.e. allergies or a cold affecting the sinuses).
f. Inability to engage in treadmill-based exercise.

Recruitment: Participants were recruited from the mid-Michigan community population via flyers and list-serve announcements.

Protocol: This investigation was approved by the Michigan State University Human Research Protection Program.

Screening Day (day 1): On the screening day of the testing protocol, following consenting to participate in this investigation; participants completed a brief health and history demographics questionnaire, a physical activity readiness questionnaire (Thomas et al., 1992), a medical screening questionnaire, the Wechsler Abbreviated Scale of Intelligence – 2nd Edition (WASI-II; (Wechsler, 2011), and a test of maximal oxygen uptake (VO_2max; using the same protocols as reported in (Chandler et al., 2019; Pontifex et al., 2016)). The medical screening questionnaire ensured that all participants were free of medication for high blood pressure, cholesterol, asthma, heart issues, depression/anxiety; and were not currently on any beta-blockers, sulfonamide antibiotics, corticosteroids, protease inhibitors, or Clonidine.

Participants who were eligible to continue in the study were then randomly assigned using a serial stratification approach accounting for biological sex to either an exercise [active experimental group] condition or sitting [control experimental group] condition. Within each condition; participants were subsequently randomly assigned using a serial stratification approach accounting for biological sex to receive a dose of either 0, 20, 40, 60, 80, 100, or 120 IU of fast acting insulin (Novolog Insulin Aspart 100 mg/mL) administered into the intranasal mucosa. Participants were instructed to refrain from exercise or caffeine intake, and too fast for at least 3 hours prior to participating in the second day of the testing protocol.

Assessment day (day 2): See Figure 1 for an overview of the assessment activities.

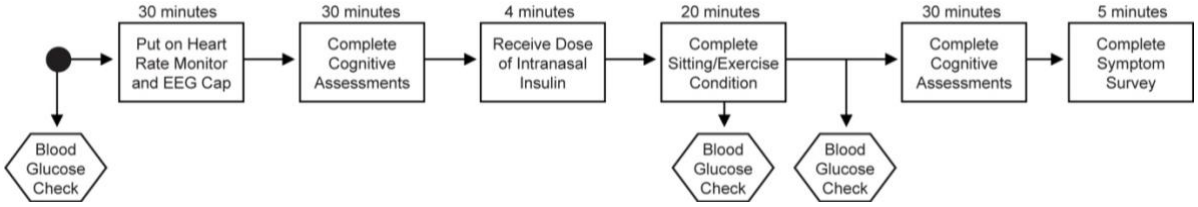

Figure 1. Diagram of experimental protocol.

Upon arriving to start the second day of testing, participants' blood glucose level was assessed via a glucometer (CVS Health Advanced Blood Glucose Meter, United States) to ensure that their blood glucose fell within the 70-115 mg/dL guideline to exercise safely. Following this, participants were outfitted with a polar heart rate monitor (Model H7, Polar Electro, Finland) to assess heart rate during the experimental condition and then were prepared for neuroelectric testing. Participants were subsequently seated in a sound-attenuated testing chamber approximately 1 m away from the computer monitor where they completed tests of inhibitory control and sustained attention while behavioral and neuroelectric measures were assessed. Once the pretest cognitive assessment was completed, participants were administered solution into their intranasal mucosa.

Using a double-blind, placebo-controlled approach; identical vials of saline solution and Novolog insulin aspart 100 mg/mL were obtained differentiated only by the color of the stopper top, and labeled as investigational drug A and investigational drug B. All participants were administered 6 doses of 0.2 mL of solution via the LMA MAD Nasal Intranasal Mucosal Atomization device into alternating nostrils with a total administration volume of 1.2 mL. The dose of intranasal insulin was controlled by varying the number of doses which contained saline relative to the doses which contained Novolog insulin aspart 100 mg/mL (see Figure 2). The experimenters were aware of the number of doses of investigational drug A and investigational drug B administered; but were unaware which solution or stopper top was associated with insulin or saline. Following administration of the solution, participants completed the sitting/exercise experimental conditions.

Figure 2. Illustration of the intranasal insulin dosing protocol.

Active Comparator: Passive Control. The sitting experimental condition consisted of a 20 min duration where participants sat in a chair (HR = 73.2 bpm [95% CI: 70.3 to 76.2]).

Intervention. The exercise experimental condition consisted of similar 20 min duration of exercise on a motor-driven treadmill at an intensity between 60 and 65% of maximum heart rate achieved during the test of maximal oxygen uptake (HR = 118.1 bpm [95% CI: 116.2 to 120.1]).

During both the sitting and exercise experimental conditions, participants watched an emotionally neutral video (minutes 65-85 from Wonders of the Universe (*Wonders of the Universe*, 2011)). Midway through the sitting and exercise experimental conditions and again 5 minutes following the completion of the experimental conditions, peripheral blood glucose was assessed via a glucometer. If peripheral blood glucose fell below 70 mg/dL, testing was immediately discontinued for all measures. Following the peripheral blood glucose assessment, participants again completed the tests of inhibitory control and sustained attention while behavioral and neuroelectric measures were assessed. Finally, upon completion of the cognitive assessments, participants completed a symptom survey which assessed potential side effects of intranasal insulin that participants might have experienced at any time during the experimental protocol and if participants were still experiencing those symptoms.

Measures:

Safety of Intranasal Insulin: The safety of intranasal insulin in combination with exercise was assessed by examining the incidence of participants reporting potential side effects of intranasal insulin (Schmid et al., 2018) at any time during the experimental protocol and the persistence of those symptoms. Additionally, the extent to which peripheral blood glucose was altered was also assessed via glucometer at the beginning of the session, during the middle of the exercise/sitting condition, and following the exercise/sitting condition

Inhibitory Control Task: Inhibitory control was assessed using a letter version of the Eriksen flanker task (Eriksen & Eriksen, 1974; McGowan et al., 2019), which is classified as a behavioral response selection construct of the inhibition/suppression focus and the performance monitoring focus of cognitive control according to the NIMH Research Domain Criteria (RDoC) classification system. Participants were instructed to attend to and to respond as accurately as possible to a centrally presented stimulus nested amid either congruous (‘MMMMM’) or incongruous (‘NNMNN’) flanking stimuli. Participants completed 80 practice trials at pretest followed by 160 trials grouped into two blocks of 80 trials, each consisting of equiprobable congruency and directionality; at each assessment period (160 trials pre-experimental condition and 160 trials post-experimental condition). For each block of trials, participants were presented with perceptually similar letter pairs (e.g., pretest block 1: M – N, pretest block 2: E – F, posttest block 1: I – T, posttest block 2: U – V) and were instructed to respond by pressing the button assigned to the letter presented in the middle of the flanking letters.

To ensure a high degree of task difficulty, at the midpoint of each block participants were given instructions to reverse the button-letter assignments (e.g., left button press for “M” through the first 40 trials and then right button press for “M” through the last 40 trials). Flanking letters were presented 300 ms prior to the onset of the target letter, and all five letters remained on the screen for a subsequent 100 ms (for a total stimulus duration of 400 ms) with a response window of 1000 ms and a variable inter-trial interval of 2300, 2400, 2500, 2600, or 2700 ms. All stimuli were 1.5 cm tall white block letters presented focally on a black background. Stimulus presentation and timing were controlled using PsychoPy 1.86 (Peirce, 2009). Reaction time was quantified within each congruency as the mean speed of responding following the onset of the stimulus only for correct trials. Response accuracy was quantified within each congruency as the proportion of correct responses relative to the number of trials administered.

Sustained Attention Task: Sustained attention was assessed using the rapid visual information processing task (Chandler et al., 2020; Neale et al., 2015) which is classified as a behavioral construct of attention according to the NIMH Research Domain Criteria (RDoC) classification system. Participants were presented with a series of single digits (1-9) in a box in the center of the screen at a rate of 100 digits/min and were instructed to make a button response with their right thumb as soon as they detected any of the three target sequences: ‘2-4-6,’ ‘3-5-7,’ or ‘4-6-8.’ To minimize working memory load, the three target sequences were presented on the bottom of the screen throughout the duration of the task. At pretest, participants completed a 1 min practice period prior to beginning the test trials which contained a series of 402 digits with 64 target sequences embedded. At posttest, participants completed another series of 402 digits with 64 target sequences embedded. Reaction time was quantified as the mean speed of responding following the presentation of the final digit of the target sequences only for correct sequence identifications. Response accuracy was quantified as the proportion of responses that correctly coincided with the 64 target sequences presented.

Neuroelectric Indices of Attention: During completion of the inhibitory control and sustained attention tasks, EEG activity was recorded from 32 electrode sites (Fpz, Fz, FCz, Cz, CPz, Pz, POz, Oz, F7/3/4/8, FT7/8, FC3/4, T7/8, C3/4, M1/2, CP3/4, TP7/8, P7/3/4/8, PO5/6) arranged in an extended montage based on the International 10-10 system (Chatrian, Lettich, & Nelson, 1985) using a Neuroscan Quik-Cap (Compumedics, Inc., Charlotte, NC). Recordings were referenced to averaged mastoids (M1, M2), with AFz serving as the ground electrode. In addition, electrodes were placed above and below the left orbit and on the outer canthus of both eyes to monitor electrooculographic (EOG) activity with a bipolar recording. Continuous data was digitized at a sampling rate of 2048 Hz and amplified 500 times with a DC to 70Hz filter using a Neuroscan Grael amplifier. The EEG data were then imported into EEGLAB (Delorme & Makeig, 2004), downsampled to 1024 Hz and prepared for temporal ICA decomposition. Data more than 2 s prior to the first event marker and 2 s after the final event marker were removed to restrict computation of ICA components to task-related activity. The continuous data was filtered using a 0.05 Hz high-pass 2nd order Butterworth IIR filter to remove slow drifts (Pontifex, Gwizdala, et al., 2017), and the mastoids electrodes were removed prior to ICA

decomposition. ICA decomposition was performed using the extended infomax algorithm to extract sub-Gaussian components using the default settings called in the MATLAB implementation of this function in EEGLAB with the block size heuristic (floor[sqrt(EEG.pnts/3)]) drawn from MNE-Python (Gramfort et al., 2013). Following the ICA decomposition, the eyeblink artifact components were identified using the icablinkmetrics function (Pontifex, Miskovic, et al., 2017) and the EEG data was reconstructed without the eyeblink artifact.

Following removal of the eye blink components, stimulus-locked epochs were created for correct trials from -500 to 1,500 ms around the stimulus, baseline corrected using the -100 to 0 ms pre-stimulus period, and filtered using a zero phase shift low-pass filter at 30 Hz. Trials with artifact exceeding ±100 μV were rejected. To ensure the integrity of the signal, stimulus-locked epochs were visually inspected blind to the experimental condition and dose prior to computing mean waveforms. Following visual inspection, the mean number of trials included in the waveforms was 112.0 [95% CI: 107.2 to 116.7] trials stimulus-locked to the congruent and incongruent trials (separately) of the Inhibitory Control task and 41.6 [95% CI: 40.1 to 43.2] trials stimulus-locked to the final stimulus of the target sequences of the Sustained Attention task.

Attention was indexed by the P3 ERP component. Attentional engagement was evaluated as the mean amplitude within a 50 ms interval surrounding the largest positive going peak within a 275 to 700 ms latency window following stimulus onset for the flanker task, and a 275 to 600 ms window for the RVIP task (Chandler et al., 2020; McGowan et al., 2019; Pontifex et al., 2015). Attentional processing speed was evaluated using ERP latency which was was quantified as the time at which maximum peak amplitude occurred. Given the well-established nature of the P3 ERP component, analyses were conducted using a nine-channel region-of-interest approach centering around the topographic maxima of the P3 (i.e., the CP3/Z/4, P3/Z/4, PO5/Z/6 electrodes).

Aims Assessment:

1) *To provide a preliminary assessment of the safety profile of combining intranasal insulin with exercise; to ensure that undue risks are not incurred through combination therapeutic approaches.*

   As the present investigation was a Phase II Clinical Trial, the research design is not sufficiently powered for hypothesis testing. Accordingly, this aim was satisfied by:

   A) Examining the frequency of participants reporting potential side effects of intranasal insulin at any time during the experimental protocol.

   B) Examining the frequency of participants reporting potential side effects of intranasal insulin that persisted at the end of the experimental protocol.

   C) Examining the frequency of study discontinuations due to violations of the safety thresholds (e.g., blood glucose outside the range of 70 to 150 mg/dL, feeling dizzy, or unable to safety continue the experimental protocol).

2) *To provide a preliminary assessment of the efficacy of combining intranasal insulin with exercise for enhancing inhibitory aspects of cognitive control and sustained attention alongside neuroelectric indices of attention.*

   As the present investigation was a Phase II Clinical Trial, the research design is not sufficiently powered for hypothesis testing. Accordingly, this aim was satisfied by computing the within subject effect size for each participant as the standardized change relative to the pretest assessment using the within-subject (d_{rm}) variance correction for Cohen's d (Lakens, 2013). To ensure the integrity of the effect size estimates, within-subject effect sizes exceeding 3 times the interquartile range were identified as outliers and removed from analysis. For each measure, within each experimental condition, the effect of intranasal insulin was corrected by subtracting the effect size observed for the 0 IU placebo group to isolate the effects of intranasal insulin. Preliminary assessments of the efficacy were thus quantified using:

   A) Effect size estimates with 95% confidence intervals for both control and exercise conditions collapsed across congruencies of the Flanker task to quantify the effects on **Behavioral Indices of Inhibitory Control**. Reaction time effects and response accuracy effects were separately extracted.

   B) Effect size estimates with 95% confidence intervals for both control and exercise conditions in response to the target trial of the Rapid Visual Information Processing task to quantify the effects on **Behavioral Indices of Sustained Attention**. Reaction time effects and response accuracy effects were separately extracted.

C) Effect size estimates with 95% confidence intervals for both control and exercise conditions for P3 amplitude at the nine-channel region-of-interest to quantify the effects on **Neuroelectric Indices of Attentional Engagement**. Effects collapsed across congruencies of the Flanker task and effects for the target trial of the Rapid Visual Information Processing task were separately extracted.

D) Effect size estimates with 95% confidence intervals for both control and exercise conditions for P3 latency at the nine-channel region-of-interest to quantify the effects on **Neuroelectric Indices of Attentional Processing Speed**. Effects collapsed across congruencies of the Flanker task and effects for the target trial of the Rapid Visual Information Processing task were separately extracted.

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