Report on Ex Vivo Testing of H2PLUS Technologies Proprietary Water on Skeletal Muscle Mitochondrial Function

Introduction

H2PLUS Technologies has developed a water treatment process that is intended to improve the general cellular function of living organisms that are provided with this water as a hydration source. The purpose of this study was to evaluation the effects of this proprietary water on skeletal muscle mitochondrial function when mitochondria were incubated in the proprietary water after harvesting from fresh skeletal muscle biopsies.

Results

Incubation of freshly harvested skeletal muscle mitochondria in H2PLUS-produced water resulted in a 13% increase in maximal rates of phosphorylating respiration compared to standard ultrafiltered laboratory water (134.4 vs 119.1 pmol O_2/(s*ml), p = 0.0653). This effect was driven primarily by an effect on Complex II-based respiration, which was increased by 21% (85.67 vs 70.66 pmol O_2/(s*ml), p = 0.0285). In contrast, respiration supported by Complex I was not significantly affected (80.64 vs 76.07 pmol O_2/(s*ml), p = 0.4501). Incubation of mitochondria in H2PLUS-produced water resulted in a significant increase in calculated mitochondrial efficiency compared to standard ultrafiltered laboratory water (96.50 vs 96.05%, p = 0.0416). This increase in efficiency was the result of the increase in maximal phosphorylating respiration, as there was no effect of water source on leak respiration (4.444 vs 4.505 pmol O_2/(s*ml), p = 0.6667) or leak respiration flux control ratio (6.467 vs 6.679%, p = 0.6909).

The maximal rate of ATP synthesis was significantly increased by 25% through incubation of mitochondria in H2PLUS-produced water compared to standard ultrafiltered laboratory water (419.65 vs 335.43 pmol ATP/(s*ml), p = 0.005). The efficiency of oxidative phosphorylation (the ratio of ATP produced to oxygen consumed) was not affected by the incubation of mitochondria in H2PLUS-produced water (7.25 vs 7.05 pmol ATP/pmol O_2, p = 0.346).

The rate of hydrogen peroxide production was minimally affected by incubation with H2PLUS-produced water. There was no effect of H2PLUS water compared to standard ultrafiltered laboratory water on hydrogen peroxide production in leak state (0.02078 vs 0.02709 nmol H_2O_2/pmol O_2, p = 0.200), respiration supported by Complex I (0.00097 vs 0.00078 nmol H_2O_2/pmol O_2, p = 0.320), or maximal phosphorylating respiration (0.00043 vs 0.00050 nmol H_2O_2/pmol O_2, p = 0.345). However, there was a trend towards a decrease in hydrogen peroxide production during respiration supported by Complex II (0.00106 vs 0.00202 nmol H_2O_2/pmol O_2, p = 0.089).

Discussion

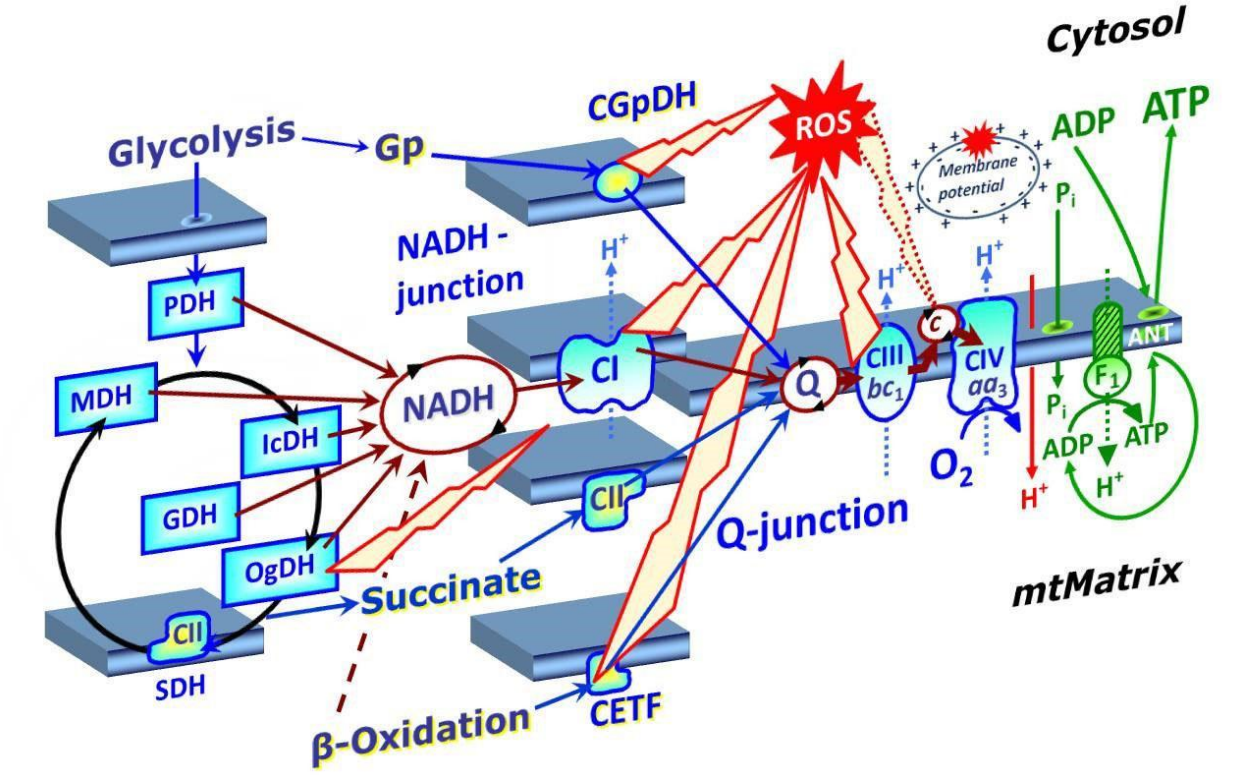

Figure 1: Schematic of mitochondrial respiration (adapted from Gnaiger E (2014) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis.). See text for relevant details.

Maximum phosphorylating respiration is the product of many different individual chemical reactions that occur in both serial and parallel arrangements, with multiple primary electron sources converging in parallel at the quinone junction of the mitochondrial electron transfer system (ETS), and further reactions occurring in a serial arrangement through Complex III, cytochrome c, Complex IV, and ATP synthase (See Figure 1). The entire process is tightly coupled normally (with the possible exception of ATP synthase); thus a difference in maximum phosphorylating respiration can result from a change in any single reaction if that reaction is sufficiently rate-limiting to the entire process. Conversely, a treatment can have a profound effect on a particular reaction, but if that reaction is not sufficiently rate-limiting then it will not be detected as a change in maximum phosphorylating respiration. In addition, the entire system operates against the backpressure created by the chemiosmotic membrane potential created by the pumping of protons across the inner mitochondrial membrane. These basic principles have a strong influence on the interpretation of these data. Complex I and Complex II are typically the electron sources with the greatest capacities, and thus the combination of the two complexes is often sufficient functionally to saturate the remaining downstream complexes. Evidence for this is seen in the fact that the sum of respiration through the two complexes individually ($P_N + P_S$) is larger than when both complexes are active concurrently (P_{N+S}). Therefore, the current evidence suggests that the primary effect of H2PLUS-produced water is increased activity of Complex II,

with a smaller effect on one or more of the elements of the common pathway (ubiquinone, Complex III, cytochrome c, or Complex IV). Due to the greater complexity of the reactions supporting respiration through Complex I, it is difficult to completely exclude an effect of H2PLUS-produced water on this arm of the ETS, as changes may be present but undetectable due to the highly-parallel nature of this portion of the oxidative phosphorylation process.

The measured increase in efficiency of the oxidative phosphorylation system can be due to either an increase in the efficiency by which protons are pumped across the inner mitochondrial membrane or a decrease in proton leakage back into the mitochondrial matrix. We did not detect a change in leak respiration, making it likely that the increase in efficiency is due to improved function of the specific elements that create proton movement as part of their function (i.e., Complex III and/or Complex IV). The increase in the rate of ATP synthesis appears to be the simple result of greater energy processing and creation of proton motive force to drive ATP synthase, rather than a change in ATP synthase itself.

There was limited impact of the H2PLUS-produced water on reactive oxygen species (ROS) generation during mitochondrial respiration. ROS production is a common side reaction in mitochondria, and current opinion supports a limited amount of ROS production as necessary for normal long-term mitochondrial health due to the function of ROS as signaling molecules. However, increased ROS production is also believed to be responsible for tissue injury during periods of high mitochondrial respiration (such as during exercise). The specific mechanisms for ROS generation are not completely understood but are believed to be linked to defective proton movement, particularly by Complexes I and III.

The primary limitation of this study is the low statistical power that is inherent in a study with a small number of subjects. With only 4 subjects, there is a chance that relevant biological effects of the H2PLUS water were not detected statistically, despite the use of several technical replicates and the ability to use the individual subjects as their own controls. It is possible that differences in hydrogen peroxide production and P/O ratio – all of which had p-values less than 0.35 - could be detected with a larger number of subjects. Conversely, the detection of statistically significant changes in mitochondrial respiration with n=4 subjects highlights the magnitude and relative uniformity of these changes.

Materials and Methods

This study was approved by the Oklahoma State University Institutional Animal Care and Use Committee. Four healthy (as determined by routine physical examination) adult Thoroughbred geldings were used. Horses were at minimal aerobic fitness at the time of the study, having been housed together in pasture with no compulsory exercise for at least 6 months. Body condition was maintained with free access to pasture, supplemented with hay and commercial grain ration as needed to maintain a score of 5/9. Skeletal muscle biopsies were obtained using sterile technique from the center of the triceps muscle (2-3 cm deep) using a 10 ga percutaneous UCH biopsy needle while under light sedation and using local anesthesia. Three passes of the biopsy needle typically yielded 180-300 mg of wet muscle. Biopsies were immediately transferred into vials with ice-cold BIOPS solution (2.77 mM CaK_2-EGTA, 7.23 mM K_2-EGTA, 20 mM

imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine, adjusted to pH 7.1, prepared using ultrapurified laboratory water) and transported to the laboratory for analysis. Mitochondria were isolated using standard techniques as described in a commercial kit for mitochondria isolation (MITOISO1 Mitochondrial Isolation Kit, Sigma-Aldrich) to produce approximately 80 ul of mitochondrial suspension per 100 mg of fresh tissue used for mitochondrial isolation. Samples were kept at 0-4°C throughout the processing until 15 ul of mitochondria suspension was added to each respirometry chamber, and all analysis was completed within 12 hr of the initial biopsy.

High resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to analyze the effects of solvent on mitochondrial oxygen consumption, ATP synthesis, and hydrogen peroxide production. The mitochondrial respiration media and all substrates were prepared using either H2PLUS-produced water or control solvent (ultrapurified laboratory water produced by the central reverse-osmosis water system and further filtered using a series of filter cartridges in the PI's laboratory). Basic mitochondrial respiration media contained 0.5 mM EGTA, 1 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA (essentially fatty acid free), adjusted to pH 7.1. Samples were analyzed in duplicate for each assay and tested solvent. Oxygen consumption was calculated as the negative slope of the oxygen measurement and reported as pmol O_2/(s*ml). ATP synthesis was measured using Magnesium Green according to the technique of Chinopolous et al 2014. Hydrogen peroxide production was measured using Amplex UltraRed according to the technique of Krumschnabel et al 2015.

A single Substrate/Uncoupler/Inhibitor Titration (SUIT) protocol was used to provide a general assessment of mitochondrial function. After injection of the mitochondrial suspension and stabilization of the measurement signals, pyruvate (5 mM), glutamate (10 mM), and malate (0.5 mM) were titrated into each chamber to produce NADH and stimulate non-phosphorylating (leak) respiration supported by NADH oxidized through Complex I (L_N). ADP was then added to stimulate phosphorylating respiration through Complex I (P_N). The addition of succinate (10 mM) resulted in phosphorylating respiration through the combination of Complex I and Complex II (P_{N+S}). Finally, rotenone (0.5 μM) was added to block Complex I, with the resulting oxygen flux representing the capacity of Complex II to support mitochondrial oxygen consumption through the oxidation of succinate alone (P_S). All oxygen flux measurements were corrected for residual oxygen consumption (measured after stabilization of the mitochondrial suspension and prior to the addition of any substrates). In addition to the ROX-corrected oxygen flux measurements, several flux control ratios were calculated, including FCR_{Leak} (leak respiration as a ratio of the corresponding phosphorylating respiration), FCR_N and FCR_S (NADH-supported and succinate-supported respiration as the proportion of maximal respiration supported by Complex I and Complex II combined, respectively) and oxidative phosphorylation efficiency ($1-L_N/P_{N+S}$). Results for each parameter of interest were calculated for individual respirometer chambers, then duplicates were averaged by subject for each experimental condition. Maximal ATP synthesis rate was calculated using the change in Magnesium Green fluorescence during the period of the assay corresponding to P_{N+S}, using a $MgCl_2$ calibration obtained prior to each individual chamber assay and Kd values for ADP and ATP binding with Mg^{2+} derived for those

specific experimental conditions. Hydrogen peroxide production rates were calculated for the L_N, P_N, P_{N+S}, and P_S using Amplex UltraRed calibration values obtained in each chamber periodically throughout the individual assays. Oxygen consumption data for each horse was analyzed using repeated measures two-way ANOVA (Prism 9.1.0, GraphPad Software, San Diego, CA), with horse as the repeating variable and solvent and fluorophore as the independent variables. If the overall ANOVA yielded $p<0.1$, then post-hoc pairwise comparisons were performed using Fisher's Least Squares Difference on both independent variables. ATP synthesis rates and hydrogen peroxide production rates were compared using paired student's t-test.