Lanthionine ketenimine (LK), a natural metabolite, improves muscle stem cell performance

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INTRODUCTION: Skeletal muscle possesses remarkable regenerative capabilities driven by muscle-resident stem cells (MuSCs), also known as satellite cells (SCs). Metabolic disorders, injuries, and aging can lead to muscle atrophy and functional decline. Injury or extended periods of muscle inactivity can also result in increased production of reactive oxygen species (ROS) accelerating protein breakdown and inhibiting synthesis. Autophagy, the process of degrading cytoplasmic components to maintain cellular homeostasis, plays an important role in the regenerative capacity of SCs. For example, aging is characterized by impaired autophagy in SC, resulting in overall muscle mass and strength loss. Pharmacological manipulation of autophagy relies on derivatives of the microbial macrolide rapamycin, which inhibits mTORC1. However, mTOR inhibitors affect protein, nucleotide, and lipid synthesis, independently of autophagy. Lanthionine ketenamine (LK), a metabolite found in mammalian brain tissue, mimics rapamycin effects despite its unknown natural function. LK and its more cell membrane-permeable analogue LK (ester)-phosphonate (ester) (LKE-PE), activate autophagy, protect against ROS, and act as potent antioxidants in neurons[1]. The purpose of this study was to assess the impact of LKE-PE as a novel therapeutic on the regenerative potential of SCs. Successful outcomes from this study may hold promise for the development of regenerative treatments for muscle aging and injuries.

METHODS: Six 6-months old Sprague Dawley male rats were obtained from Envigo LLC (IN) after IACUC approval. Hindlimb muscles were used for SCs isolations based on Allen et al. [2]. To determine the purity/% yield after isolation, SCs were identified for Pax7 expression using FACS flow cytometry. SCs were treated with pharmacological interventions during their proliferative stage (while exposed to growth medium; 20% FBS in DMEM) and during the differentiation phase (~80-90% confluent cells were switched to differentiation media composed of low glucose DMEM containing 2% horse serum for four days). Pharmacological drug preparation and application: Rapamycin was combined with culture media to obtain a final concentration of 100nM [3]. LKE-PE was synthesized using the standard Michaelis-Arbuzov reaction conditions as previously described [4]. The structure of LKE-PE was confirmed by ^{1}H, ^{13}C, and ^{31}P NMR, and liquid chromatography-tandem UV spectrophotometry mass spectrometry. Cell proliferation: An MTS assay was performed after exposing proliferating SCs to Rapamycin and various concentrations of LKE-PE. Briefly, SCs were either treated for 24hrs. (**1T**), 48hrs. (**2T**), 72hrs. (**3T**), or were left to recover (**R**) either 24hrs. or 48hrs. after treatment and before analyses. Hydrogen peroxide-induced oxidative stress: An MTS assay was first performed to evaluate SCs viability after being exposed to 500µM of hydrogen peroxide (H_2O_2). Then, various treatments were implemented, including simultaneous treatment with pharmacological drugs and H_2O_2, and pre-treatment of the SCs before exposing them to stress. RT-qPCR: Myogenic genes expression during proliferative and differentiative stages and after exposure to different treatment drugs was evaluated. Relative fold expression outcomes during these phases were evaluated by normalizing to the control group in each group. Migration/Scratch assay: Implemented in SCs under stress (H_2O_2) and with treatments to mimic and evaluate the regulation of cell migration in-vivo after an injury over a 24hr. period.

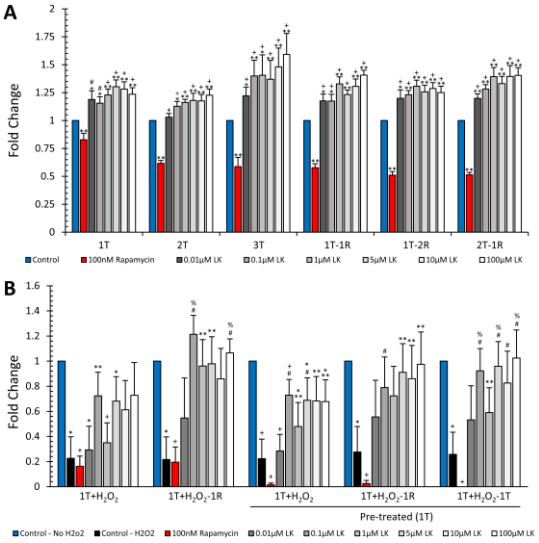

Fig.1 A) SC proliferation with various treatments. * = different from control at p<0.05; ** = different from control at p<0.01; + = different from RAP at p<0.01; # = different from RAP at p<0.05. **T**: treatment; **R**: recovery. **B)** SCs exposed to H_2O_2 and various treatments. * = different from control at p<0.05; + = different from control at p<0.01; ** = different from RAP at p<0.05; # = different from RAP at p<0.01; % = different from control+H_2O_2 at p<0.05.

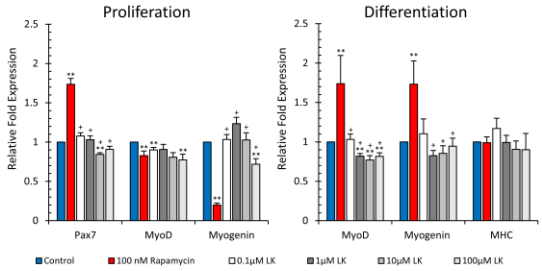

Fig.2 RT-qPCR of SCs while exposed to the various treatment groups. * = different from control at p<0.05; ** = different from control at p<0.01; + = different from RAP at p<0.05; # = different from RAP at p<0.01.

RESULTS: The average number of cells positive for Pax7 was ~96%. Rapamycin significantly decreased the proliferation of cells when compared to controls. LKE-PE significantly increased proliferation of SCs when compared to the controls, regardless of treatment approach (**Fig. 1A**). SCs showed a significant decrease in viability, up to ~46%, when exposed to 500µM of H_2O_2 and compared to controls (*p<0.0001*). Regardless of the treatment approach, LKE-PE protected SCs in an induced oxidative stress environment by significantly increasing viability as compared to SCs treated with H_2O_2 or Rapamycin (**Fig. 1B**). Depending on the treatment approach, LKE-PE was able to either restore viability/proliferation close to normal (control) values or surpass the outcomes from this group. Rapamycin increased Pax7 gene expression when compared to the control group or other treatment groups (**Fig. 2**). Similarly, treatment with Rapamycin resulted in a significant increase in MyoD and Myogenin during the differentiation phase when compared to control. While an increase in MHC outcomes was observed during the differentiation stage when treated with LKE-PE this was not significantly different from controls. No significant differences were found between Rapamycin and controls in cell migration when exposed to oxidative stress over the 24hr period. However, LKE-PE improved cell migration, with differences between the control group during the initial 6 hrs. (p<0.05) or after 6hrs (p<0.05), depending on the implemented dose.

DISCUSSION: Skeletal muscle atrophy is a multi-factorial catabolic process resulting in a decline in muscle mass and function, characterized by decreased protein synthesis, fiber diameter, and force production, among some. This phenomenon is triggered by various stimuli, including disuse (e.g., immobilization, denervation), aging, injury, starvation, and pathological conditions (i.e., cachexia). In this study we showed that LKE-PE increased the proliferative capability of SCs, and upregulated genes associated with myogenesis, which suggests it may have the potential to counteract the natural effects associated with aged SCs or other conditions. Since oxidative stress influences the cell's ability to function, we also showed the protective effect of LKE-PE against ROS.

SIGNIFICANCE/CLINICAL RELEVANCE: In summary, our in vitro studies demonstrated promising results using LKE-PE to establish a base for future studies and a potential use in regenerative medicine for muscle pathologies (atrophy, sarcopenia, injury, cachexia, aging, etc.).

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REFERENCES: 1. Hensley & Denton, 2015. 2. Allen et al., 1997 3. Bibee et al., 2014. 4. Shen et al., 2018.