Innovative (electro-driven) sample preparation tools for metabolomics study of muscle aging

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Addendum

Summary

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Acknowledgements

Curriculum Vitae

List of Publications

Summary

Sarcopenia is a leading cause of the loss of independence in the elderly globally, and effective medication for sarcopenia is still lacking. Oxidative stress, inflammation, energy production, and nutrition were reported to be highly related to sarcopenia, and dietary restriction (DR) is considered as an effective intervention to attenuate aging and retard aging-related diseases, including sarcopenia. Aging-related diseases have been widely investigated using a mouse model deficient in the DNA excision-repair gene *Ercc1* (*Ercc1*$^{\Delta/-}$) or *Xpg* (*Xpg*$^{-/-}$). To contribute to diagnosis and treatment of sarcopenia, we aim to use metabolomics approaches in this mouse model to explore the mechanism of dietary restriction effects on sarcopenia and try to identify important biomarkers for sarcopenia. However, the muscle tissue that can be isolated from *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice is limited, presenting a challenge for its analysis utilizing multiple metabolomics platforms profiling a broad range of metabolites related to oxidative stress, inflammation, energy production, and nutrition. Therefore, the scope of this thesis is to firstly develop innovative and miniaturized sample-preparation methods for small amounts of samples, and secondly to apply these developed methods to the minute muscle tissue samples from *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice for investigating the mechanisms involved in sarcopenia.

In **Chapter 2**, we developed a manual sample-preparation setup for basic analytes by using an emerging method; three-phase electroextraction (EE), which has advantages for extraction of charged analytes from small amounts of samples, especially samples with low concentration of analytes. Four model basic compounds, *i.e.*, propranolol, amitriptyline, bupivacaine, and oxeladin, were utilized to do the proof-of-principle study of the three-phase EE method by optimizing different parameters, *i.e.*, the types and composition of organic phase, pH of sample and acceptor phase, composition of acceptor phase, extraction voltage, and extraction time. The results revealed that the types of organic phase, pH of sample and acceptor phase, extraction voltage and time are crucial for three-phase EE method to achieve a high extraction efficiency for basic analytes. The optimized three-phase EE method was successfully applied to human urine and plasma samples consuming only a small amount of sample.

To thoroughly investigate the crucial parameters for the three-phase EE method, acidic model compounds, *i.e.*, naproxen, fenoprofen, flurbiprofen, and ibuprofen, were utilized

with an upgraded manual EE setup in **Chapter 3**. A Design of Experiment approach (Box-Behnken design) was used for optimization of the three-phase parameters, *i.e.*, the type of organic solvent, pH of the sample and acceptor phase, the extraction voltage and time and the optimized method was successfully applied to limited volume human plasma samples. Our results revealed that similar to the basic compounds investigated in Chapter 2, the type of organic solvent, pH of sample and acceptor phase, the extraction voltage and time are crucial parameters for three-phase EE method.

Because of the time and effort required, the manual three-phase EE setup is limited in its application to large numbers of minute sarcopenia samples. Automation is an effective solution for this challenge. However, the stability of the three-phase EE (acceptor droplet) on the automated platform, and how this affects the three-phase EE performance, needs to be investigated before applying the automated three-phase EE to sarcopenia samples. Therefore, in **the first phase of Chapter 4**, an automated 3-well plate three-phase EE module with machine vision was developed, and utilized for the acceptor droplet stability evaluation, and for the estimation of its extraction performance by adopting eight model compounds and a Design of Experiment approach (Box-Behnken design). A stable acceptor droplet was observed in the automated three-phase EE setup under different conditions, *i.e.*, different extraction voltage and pH of sample. The optimized automated three-phase EE method showed high extraction recovery for the model compounds and was successfully applied to human urine and plasma samples with low limits of detection (LODs). The sample plate for the automated three-phase EE setup with machine vision only contains three wells for clear visualization of acceptor droplet and easy fabrication of the plate. However, this limited the throughput and application of the setup. To apply the automated three-phase EE method to large numbers of minute sarcopenia samples, a high-throughput and fully automated three-phase EE setup was developed **in the second phase of Chapter 4** by using a modified 96-well plate with an integrated bottom electrode. Acylcarnitines play important roles in β-oxidation and energy production of fatty acids by transport fatty acids from cytoplasm into mitochondria, which is highly associated with muscle strength and function. Ten kinds of acylcarnitines were utilized as model analytes to optimize the fully automated and high-throughput three-phase EE setup by using a Design of Experiment approach (Box-Behnken design). The optimized setup showed good extraction performance for the acylcarnitines in both academic and human plasma samples. To supply reference

information for mouse muscle tissue isolation (after mouse dissection) for the sarcopenia study, the optimized setup was applied to the progeroid (*Ercc1*$^{\Delta/-}$) mouse muscle to deduce whether fast muscle tissue isolation is necessary for acylcarnitine stability. The results demonstrated that muscle tissue isolation speed does not affect acylcarnitine stability, and the fully automated and high-throughput three-phase EE setup can be used for the sarcopenia muscle tissue analysis.

Only charged analytes can be extracted by the automated and high-throughput three-phase EE setup, to analyze more metabolites for the sarcopenia mechanism study, a sample-preparation method for uncharged (polar and non-polar) metabolites is necessary. In **Chapter 5**, two developed liquid-liquid extraction (LLE) methods, BuOH-MTBE-Water (BMW) and BuOH-MTBE-More-Water (BMMW), and two previously reported LLE methods, Bligh-Dyer (BD) and BuOH-MTBE-Citrate (BMC), were compared and evaluated to obtain the optimal method. Internal standards (ISTDs) of metabolites related to sarcopenia, *i.e.*, oxidative stress, inflammation, energy production, and nutrition, were utilized for the evaluation with pig muscle as a surrogate matrix. BMMW method showed the best extraction performance and was applied to *Ercc1*$^{\Delta/-}$ mice muscle to investigate muscle tissue isolation speed on metabolite stability. The results revealed that fast sample isolation is critical for these metabolites stability, and for the sarcopenia mechanistic study.

The developed miniaturized sample-preparation method was utilized in the study of the effects of dietary restriction (DR) on sarcopenia in **Chapter 6**, together with multiple optimized liquid chromatography- mass spectrometry (LC-MS) metabolomics platforms. Here we demonstrated that oxidative stress related, pro-inflammatory, and muscle growth stimulation metabolites were notably down-modulated by DR, and the anti-inflammation and energy production pathways, and the energy source consumption were significantly upregulated by DR in *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice. The down-modulated muscle growth stimulation metabolites and the improved consumption of energy source may induce decreased mouse body weight (and potentially muscle mass) by DR. The downregulated oxidative stress, pro-inflammatory metabolites, and up-modulated anti-inflammatory metabolites contributed to a lower energy expenditure by DR, which may result in an improved muscle quality together with the contribution of upregulated energy production. The better muscle quality may be associated with the unaffected grip strength and improved

motor coordination and learning performance seen in the behavioral study of DR in *Ercc1*$^{\Delta/-}$ and *Xpg*$^{-/-}$ mice. These findings provide mechanistic insight of sarcopenia, supply potential biomarkers for future diagnosis, prevention, and treatment of sarcopenia, and may contribute to healthy aging in the world.