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The difference of metabolic profile between male and female zebrafish

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Abstract Various experiments using zebrafish have been highlighted recently in the scientific community. Because it is possible to conduct practical experiment from various neurological research to area of genetic study or toxicity experiment. However, gender difference effects are nearly not considered. If the gender differences of zebrafish are considered it is possible to obtain more accurate data. In this study, zebrafish which have different genders were compared each other with NMR-based metabolomics. The extracts of male and female zebrafish were measured by 600 MHz NMR spectrometer. Statistical analysis and target profiling were conducted. As a result, muscle related metabolites were observed in male zebrafish and nerve related metabolites were observed in female zebrafish.

Keywords NMR spectroscopy, Metabolomics, Zebrafish, Pattern recognition, Target profiling

Introduction

Various species of fish are used in experiments. Among them, zebrafish is evaluated as an important model organism for the many scientific fields. The genetic information of zebrafish is similar to human.[1] The entire nucleotide sequence of the zebrafish genome has been completed.[2]

Metabolomics is the study of the lowest step of metabolites in biological central dogma. The metabolites are the end products of metabolism in the body. Metabolites can be affected by the external environment.[3] Therefore metabolomics is applied in the many experiment fields.[4] Mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most common tools which are used in metabolomics research. Even though the sensitivity of MS is higher than NMR spectroscopy, NMR spectroscopy has many advantages such as minimal sample preparation, non-destructive technique and high reproducibility.[5] Recently, It has been reported that technical development to improve sensitivity of NMR spectroscopy like cryogenic probe or now tension upgrade such increase has been reported.[6]

In order to obtain useful information from the complex NMR data, pattern recognition method such as a multivariate statistical analysis and target profiling are used in metabolomics study.

Experimental Methods

Zebrafish (Danio rerio) were obtained from Green Fish (Seoul, Korea). The fish, that ranged in weight

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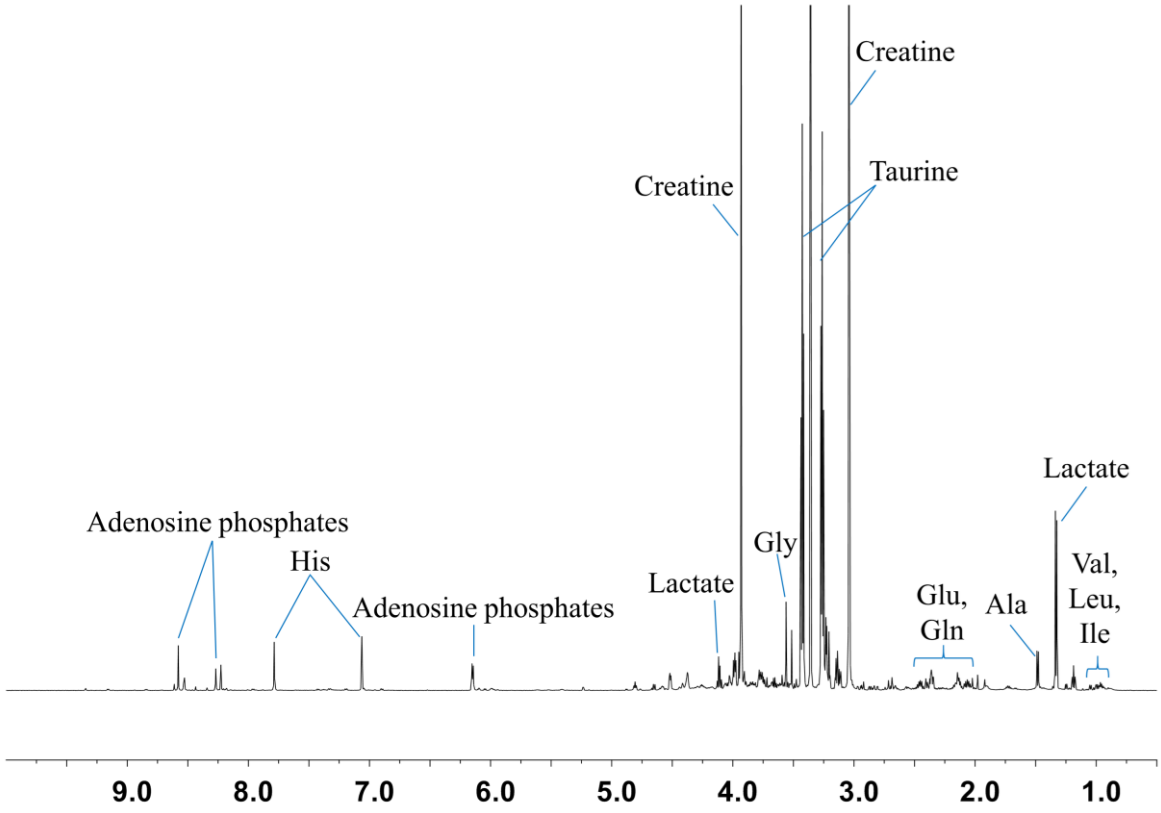

Figure 1. Representative ^{1}H NMR spectrum of zebrafish aqueous extract

from 0.8 ± 0.3 g body mass, were raised in dechlorinated water at 25 ± 1 ℃ for 1 week to acclimate before the experiment. The photoperiod was kept a constant cycle (14h light:10h dark). The fish were fed twice a day during the acclimation period except one day before the experiment. 10 of zebrafish were selected randomly in each gender. They were weighed their body mass, quickly frozen with liquid nitrogen and lyophilized overnight. Completely dried zebrafish were homogenized with mortar and pestle. Aqueous metabolites were extracted by chloroform, distilled water, and methanol. This extraction protocol consulted Bligh and Dyer's method and it was made most suitable to this experiment.[7] The samples were centrifuged at 3,000 rpm for 10 min at 4 ℃. After the centrifugation, aqueous layers were transferred to vials and they were lyophilized overnight for eliminating the solvent which was using extraction step. After the lyophilization, 700 µl deuterium oxide (D_2O, 99.9% in D), that includes 2 mM 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d4) as a reference of 0 ppm and quantification, was added into the vial to re-dissolve the metabolites. All samples were transferred to 5 mm NMR tubes.

All ^{1}H-NMR spectra were acquired using a 600 MHz Agilent NMR spectrometer (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler 7600-AS. A Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence containing preset pulse was used to reduce the signals of water and macromolecule. Using 9.8 µs 90° pulse, 1.5 s relaxation delay, 3 s acquisition time and 128 scans, the ^{1}H-NMR spectra were measured. The TSP-d4 peak at 0.0 ppm is used

for reference to calibrate the chemical shifts.

Each ^{1}H-NMR spectrum was manually phased and baseline corrected. Each spectrum was binned from 0.5 ppm to 10 ppm except water peak area (from 4.7 ppm to 4.9 ppm) and the binning size was 0.001 ppm with total area normalization. These processes were conducted with the Chenomx NMR suite 7.1 (Chenomx Inc.,Edmonton,AB,Canada). The binning results were imported to Matlab icoshift tool for the alignment and then imported to SIMCA-P+ 12.0.1 (Umetrics, Umeå, Sweden). Principal component analysis (PCA), Partial leasts squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were achieved using SIMCA-P+ software.

Assignment of ^{1}H-NMR spectra and quantification of metabolites were accomplished by Chenomx NMR suite 7.1 software (Chenomx Inc., Edmonton, AB, Canada). Each peak was matched with the Chenomx NMR suite 600 MHz library database and compared to the peak of 2 mM TSP-d_4 at 0 ppm.

Results and Discussion

Figure 1 is a representative 1H NMR spectrum of the zebrafish. The result of statistical analysis, OPLS-DA score plot (Figure 2) shows a good separation of male and female based on the t[1] axis. OPLS-DA loading plot (Figure 3) shows the metabolite peaks that contributed the separation in the score plot. In this result, the concentrations of creatine, taurine and lactate were high in the male, and the concentrations of glycine, oxalacetate, ethanol and sn-glycero-3-phosphocholin were high in the female.

The muscle more exists in the male compare to female. So, male has a large amount of metablites associated with muscle than females. Creatine is an organic acid containing nitrogen. It is synthesis in vivo and plays an important role in the energy supply of the vertebrate muscle. The more than 95% of the creatine in the body is stored in skeletal muscle.[8] Taurine is an β-amino acid that the amino group, it has the behavior that are easily ionized in a lower pH than the other amino acids since sulfonic acid group

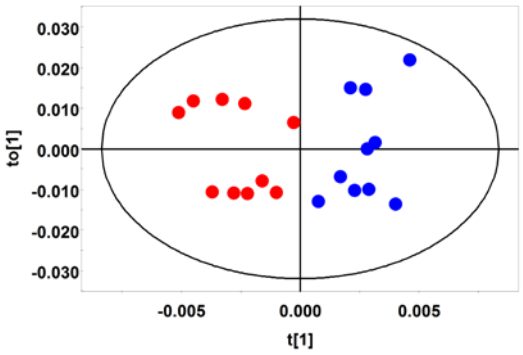

Figure 2. OPLS-DA score plot ●, Female; ●, Male

which has lower pK value is substituted with a α-carbon in place of carboxy group. It is not used in protein synthesis, and present in abundance in the form of free amino acids in most of the animal tissues and biological fluids has a very small amount or is present in almost non-existent. In particular, skeletal muscle, organs such as the mammalian brain, heart, liver and blood cells have high concentration of taurine. It exists very high concentration in the intracellular fluid as compared to the extracellular fluid. So taurine is involved in numerous biological processes.[9] Lactate is a substance that is generated as decomposition of glycogen by using muscles to make the energy in the body. Lactate can enter into the TCA cycle as an energy source through oxidation by a simple reaction of lactate dehydrogenase in slow muscle fiber.[10] Female has more metabolites related to nervous system than male. Glycine is used as constituents in a number of species. In addition, it is

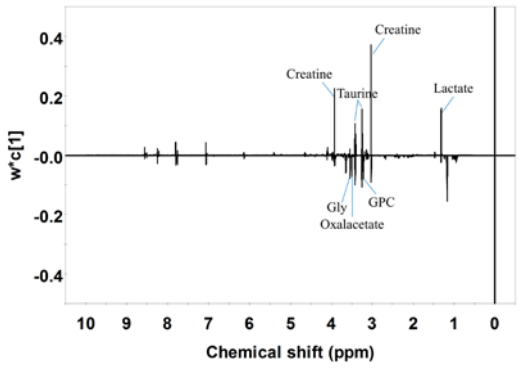

Figure 3. OPLS-DA loading plot

inhibitory neurotransmitter in the central nervous system, particularly the spinal cord, brain stem, optic nerves it has been shown that Glycine has beneficial effects in various animal models of peripheral inflammation.[11] sn-Glycero-3-phosphocholine is the intermediate of acetylcholine as well as phosphatidylcholine synthesis precursor and muscarinic receptors. In addition, it is also effective against the symptoms of degenerative dementia, vascular origin and disability.[12] It seems that using a female zebrafish in the neurological diseases such as Alzheimer's disease is more effective.

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