Monitoring of Energy Metabolism by Organic Acid Profiling Analysis in Plasma of Type 2 Diabetic Mice

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Abstract: Objective: This study was conducted to investigate energy metabolism based on changes in organic acids in diabetes and to establish a correlation between metabolites or bone microarchitecture and the glucose index in type 2 diabetic mice.

Methods: Seven-week-old male C57BL/6 mice were randomly divided into a non-diabetic group and a diabetic group. The diabetic group was fed a high-fat diet (HFD) that induced insulin resistance for 5 weeks. Afterwards, diabetes was induced by a single streptozotocin injection. Both the groups were fed a normal diet and HFD diet for 9 weeks.

Results: The fasting blood glucose level glycosylated hemoglobin (HbA 1c) significantly increased in diabetic mice. Bone-alkaline phosphatase activity decreased in the diabetic group. Diabetes increased the levels of ketone bodies, including 3-hydroxybutyric, acetoacetic and butyric acid, whereas it decreased Krebs cycle components, including succinic acid and malic acid, as well as levels of glycolytic products, including lactic acid. Diabetes also induced a shortage of trabecular bone mineral density (BMD) by the regulation of trabecular morphometric parameters in the femur and tibia. Correlation analysis indicated that BMD, Krebs cycle components and lactic acid levels were negatively correlated with HbA 1c, whereas ketone bodies were positively correlated with HbA 1c.

Conclusion: This research suggested that uncontrolled HbA 1c can affect bone loss, production of ketone bodies and utilization of glucose metabolites for energy production in type 2 diabetes.

Keywords: Diabetes, organic acid, profiling analysis, HbA 1c, ketone bodies, bone.

1. INTRODUCTION

Diabetes is one of the most common chronic diseases, and its prevalence in Korea in 2016 was 14.4 % [1]. Diabetes not only decreases the quality of life and increases the economic strain for patients, but also significantly elevates the incidence of deaths [2]. Thus, research on the treatment and prevention of diabetes has become an important task. Although diabetes is largely predictable through lifestyle and clinical factors and preventable through eating habits and life pattern improvements, the metabolic pathways responsible for its progression are not completely understood [3]. Therefore, many studies have been conducted to investigate the pathogenic mechanism of diabetes by various methods, including transcriptomics, metabolomics, and proteomics [4-6]. However, knowledge regarding metabolic pathways and molecular mechanisms associated with the pathogenesis of diabetes and related complications is still insufficient. In contrast to other ‘omics’ technologies, metabolomic profiling reflects individual predisposition, which makes the technique very efficient for investigating the pathophysiology of diabetes [7]. Organic acids are crucial metabolites.
that play an integral role in energy metabolic pathways as transcriptional and immune response regulators [8]. Their importance is heightened by their association with illnesses such as diabetes [9]. Accordingly, organic acid profiling analysis may become a practical clinical tool for understanding the complexity of metabolic pathways in diabetes.

Diabetes can lead to serious complications affecting the nervous system, eyes, kidneys and bone [10]. Among many diseases leading to osteoporosis, diabetes is of particular importance because of its prevalence [11]. Some researchers have reported that diabetes is associated with an elevated risk of osteoporosis-associated fractures [12, 13]. Osteoporosis and diabetes are generally observed in the senior population [14]. Thus, there is a need for additional research on the relationship between diabetes and osteoporosis.

To date, no clear relationship between bone mass measurements and biochemical parameters of energy metabolism has been observed in diabetes [15]. Therefore, the current study was conducted to investigate changes in energy metabolism in diabetes as these changes are related to organic acids. To accomplish this, we conducted metabolic profiling analysis of the relationship between glucose parameters and organic acids or bone-related marker changes in diabetes.

2. MATERIALS AND METHODS

2.1. Animals and Experimental Design

Seven-week-old male C57BL/6 mice were purchased from Orient Inc. (Seoul, Republic of Korea). Mice were individually housed in polycarbonate cages at 22 ± 2°C and maintained on a 12 h light-dark cycle. The mice were allowed to acclimatize under laboratory conditions for seven days, then randomly divided into non-diabetic and diabetic groups that were either fed a normal diet (5% corn oil, wt/wt) or a high fat diet (HFD) with 37% of the calories from fat (3% corn oil and 18% lard, wt/wt) to induce obesity and insulin resistance for five weeks.

Type 2 diabetes (T2D) was then induced by STZ injection (100 mg/kg body weight in 0.1 M citrate buffer, pH 4.2; Sigma, St. Louis, MO, USA) administered to the peritoneum of mice. The non-diabetic mice were injected with citrate buffer or saline alone. After seven days, only the STZ treated mice that exhibited a fasting blood glucose level ≥11 mmol/L were used in the diabetic group. Both groups were then fed a normal or HFD diet for nine weeks. The composition of the experimental diet was based on the AIN-76 diet [16].

The mice had free access to food and water, and their food consumption was measured daily while their weight gain was measured weekly. Following the experimental period, food was withheld for 12 hours, after which the mice were anesthetized with ether and blood samples were taken from the inferior vena cava to determine plasma biomarkers.

The experimental protocols and procedures used in the present study were approved by the Sunchon National University Institutional Animal Care and Use Committee (SCNU-IACUC-2014-03).

2.2. Blood Glucose, Glycosylated Hemoglobin (HbA1c)

Fasting blood glucose was measured using a glucometer (G-doctor Supersensor, Allmedicus, Korea) and venous blood was drawn from the tail vein after a six-hour fast. The blood HbA1c level was determined after hemolysis of anticoagulated whole blood specimen with immune-turbidimetric method.

2.3. Plasma Biomarkers

Insulin levels were measured with a bio-plex pro mouse diabetes 2-plex kit (Bio-Rad, Hercules, CA, USA). The C-peptide level was determined using commercially available quantitative sandwich enzyme immunoassay kits (Shinayagi Co., Ltd. Gunma, Japan). Total alkaline phosphatase (total-ALP) activity was measured using an automated chemistry analyzer (Fuji-Dri-Chem 350, Fujifilm, Tokyo, Japan). Bone alkaline phosphatase (Bone-ALP) was measured using commercially available ELISA (enzyme-linked immunosorbent assay) kits (EIAab Science, Guanggu, China).

2.4. Sample Preparation for Organic Acid Profiling Analysis in Plasma by GC-MS

Profiling analysis of organic acid in plasma was conducted based on methoxime/tert butyldimethylsilyl (TBDMs) derivatives. Briefly, plasma (100 μL) was added to 0.9 mL of distilled water containing 0.1 μg of 3,4 dimethoxybenzoic
acid as an internal standard. The aqueous phase was then adjusted to pH ≥ 12 with 5.0 M sodium hydroxide and reacted at 60°C for 60 min with methoxyamine hydrochloride (10 mg). Samples were subsequently adjusted to pH ≤ 2 with 10% sulfuric acid and saturated with sodium chloride, after which they were subjected to solvent extraction sequentially with diethyl ether (3 mL) and ethyl acetate (2 mL). Next, the combined extracts were evaporated to dryness under a gentle stream of nitrogen. Dry residues containing organic acids and fatty acids were reacted at 60°C for 30 min with triethylamine (5 μL), toluene (20 μL), and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (20 μL) to form TBDMS derivatives for gas chromatography (GC) - mass spectrometry (MS) analysis [17, 18].

2.5. Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS analysis was conducted with a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) interfaced to a 5975B mass selective detector (70 eV, electron ionization mode; Agilent Technologies) as previously reported. Briefly, the mass spectra were scanned in a range of 50–650 u at a rate of 0.99 scans/s. The temperatures of the injector, interface and ion source were 260, 300 and 230°C, respectively. An HP Ultra-2 (Agilent Technologies, Santa Clara, CA, USA) cross-linked capillary column coated with a 5% phenyl/95% methylpolysiloxane bonded phase (25 m × 0.20 mm I.D.; 0.11 mm film thickness) was used for all the analyses. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in constant flow mode. Samples (1 mL) were introduced in split-injection mode (10:1). The oven temperature was initially set to 100°C (2 min), increased to 250°C at a rate of 5°C/min and finally to 300°C at a rate of 20°C/min (5 min). In a selected ion monitoring mode, three characteristic ions for each compound were used for peak confirmation, and one target ion was selected for quantification [19].

2.6. Star Symbol Plotting

18 OA levels were normalized to the corresponding mean values of the non-diabetic group, and the normalized values were calculated as follows:

\[
\text{Normalized value} = \frac{\text{mean values of diabetic group}}{\text{mean values of non-diabetic group}}
\]

Each normalized value was plotted as a line radiating from a common central point. The far ends of the lines were joined together to produce octadecagonal star patterns using Microsoft Excel, as described in another study [20].

2.7. Bone Microarchitecture Analysis by Micro CT

Morphological measurements, including bone surface/total volume (BS/TV), bone volume density (BV/TV), bone surface/bone volume (BS/BV), structure model index (SMI), trabecular number (Tb.N), pattern (Tb.Pf), separation (Tb.Sp), thickness factor (Tb.Th) and bone mineral density (BMD) were calculated from microcomputed tomography (micro CT) data using SkyScan 1172 (SkyScan, Belgium). Regions of interest for the analysis were the proximal tibia and distal femur metaphysis. User-defined contours were outlined on every fifth slice of a 150 slice region extending 2.5 mm distally from the growth plate, starting at the point where growth plate tissue was no longer visible in the grayscale CT slice. For the quantification of bone mineral density (BMD), micro CT was calibrated using two standard phantoms with a density of 0.25 and 0.75 g/cm². The image slices were reconstructed and analyzed using the CTAn analyzer software (SkyScan) [21].

2.8. Statistical Analysis

Experimental data were indicated as the mean ± standard error (SEM). Two-tailed p-values were calculated using unpaired (independent) t-tests in Social Science Software (SPSS, Chicago, IL). p-values ≤ 0.05 were considered significant. The significance of the difference between the variances of two samples was determined using a Levene’s test. If the resulting p-value of the Levene’s test was > 0.05, equal variances were assumed and the output of the equal variance t-test was adopted as the p-value. If the resulting p-value of the Levene’s test was ≤ 0.05, the variance was assumed to be unequal and the output of the unequal variance t-test was adopted as the p-value. Correlation analysis was performed using Pearson’s coefficient.

3. RESULTS AND DISCUSSION

3.1. Characteristic of T2D

We confirmed the characteristics of diabetic mice used in this study. Diabetes is mostly based
on the results of fasting glucose or HbA1c concentrations. The fasting blood glucose, HbA1c and bone-ALP are shown in Table 1. The fasting blood glucose levels and HbA1c significantly increased in diabetic mice, while the bone-ALP activity decreased in the diabetic group.

### 3.2. Organic Acid Metabolomic Profile

Changes in various metabolites are related to the physiological state of illness [22]. Thus, organic acids are specific metabolites that have given rise to scientific interest. The organic acids are metabolites of crucial metabolic pathways, such as the Krebs cycle, carbohydrate metabolism, ketone body metabolism, etc. [23]. As shown in Table 2, the levels of 3-hydroxybutyric and acetoacetic acid were significantly enhanced in the diabetic group by 6 fold and 10 fold, respectively. The levels of total ketone bodies were calculated from the sum of acetoacetic and

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**Table 1. Characteristics of diabetic and non-diabetic mice.**

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetes</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>32.68±0.70</td>
<td>22.78±0.32***</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>177.37±10.85</td>
<td>495.14±28.66***</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.13±0.06</td>
<td>9.07±0.22***</td>
</tr>
<tr>
<td>Insulin (pg/μL)</td>
<td>1730.00±330.5</td>
<td>596.28±103.35*</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>2.42±0.08</td>
<td>2.12±0.09*</td>
</tr>
<tr>
<td>Total-ALP (U/L)</td>
<td>163.25±14.15</td>
<td>409.85±20.98***</td>
</tr>
<tr>
<td>Bone-ALP (ng/mL)</td>
<td>1.54±0.28</td>
<td>0.71±0.09*</td>
</tr>
</tbody>
</table>

*Mean ± SEM. Values are significantly different between groups according to Student’s t-test. *p < 0.05, ***p < 0.001, Diabetes versus non-diabetes.

**Table 2. Levels of 18 organic acids related to glucose metabolism in diabetic and non-diabetic mice (μg/100μl plasma).**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Non-diabetes</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoacetic acid</td>
<td>3.72±0.65</td>
<td>36.07±9.47*</td>
</tr>
<tr>
<td>cis-Aconitic acid</td>
<td>0.19±&lt;0.01</td>
<td>0.19±&lt;0.01</td>
</tr>
<tr>
<td>2-Hydroxybutyric acid</td>
<td>0.18±0.02</td>
<td>0.43±0.08*</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.91±0.17</td>
<td>1.31±0.23</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>0.08±0.02</td>
<td>0.03±&lt;0.01</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>10.52±0.67</td>
<td>10.51±0.98</td>
</tr>
<tr>
<td>3-Hydroxybutyric acid</td>
<td>6.98±1.27</td>
<td>40.65±11.66*</td>
</tr>
<tr>
<td>2-Hydroxyglutaric acid</td>
<td>0.05±&lt;0.01</td>
<td>0.05±&lt;0.01</td>
</tr>
<tr>
<td>4-Hydroxyphenyllactic acid</td>
<td>0.04±&lt;0.01</td>
<td>0.03±&lt;0.01</td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>0.15±&lt;0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>α-Ketoglutaric acid</td>
<td>0.20±0.03</td>
<td>0.13±&lt;0.01</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>13.19±1.57</td>
<td>7.00±0.66**</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.22±0.03</td>
<td>0.09±&lt;0.01</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>0.08±&lt;0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>Oxaloacetic acid</td>
<td>0.21±&lt;0.01</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>0.09±&lt;0.01</td>
<td>0.09±&lt;0.01</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>1.94±0.11</td>
<td>2.00±0.41</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.34±0.06</td>
<td>0.14±&lt;0.01*</td>
</tr>
</tbody>
</table>

*Mean ± SEM. Values are significantly different between groups according to Student’s t-test. *p < 0.05, **p < 0.01, Diabetes versus non-diabetes.
3-hydroxybutyric acid in plasma. Total ketone bodies were elevated in the diabetic group by 15 fold compared to the non-diabetic group (Fig. 1). Ketone bodies were released into the blood from the liver when hepatic lipid metabolism changed to a state of increased ketogenesis [24]. Yared et al. showed that hyperketonemia or diabetic ketoacidosis can co-exist with hyperglycemia in diabetes [25]. The levels of ketone bodies observed in this study were insignificant compared to diabetic ketoacidosis but indicated that diabetes induced ketosis progression.

![Total-ketone bodies](image)

**Fig. (1).** Level of total-ketone bodies and correlation between HbA1c and total-ketone bodies in mice. Values are expressed as means ± SEM. Values are significantly different between groups according to Student’s t-test. *p < 0.05, Diabetic group versus non-diabetic group.

The levels of lactic, succinic and malic acid are described in Table 2. The lactic, succinic and malic acid levels significantly decreased in diabetes, while no differences were observed in the levels of pyruvic and glycolic acids. In the energy metabolic process, lactic acid is normally related to the glycolytic pathway, whereas succinic and malic acids play important roles in the Krebs cycle. Gaster reported that reduction of the Krebs cycle may be responsible for a portion of the diabetic phenotype [26]. Succinic acid in the cycle is known to facilitate diabetes treatment [27] and can be up-regulated and metabolized by pancreatic cells, leading to increased proinsulin biosynthesis and insulin secretion coupled with lower blood glucose [28].

The normalized levels of 18 organic acids to the corresponding mean values of the non-diabetic group were used to construct the star symbol patterns, and these star symbol patterns showed a clear difference as illustrated in Fig. (2). The star pattern of diabetes was distorted, allowing it to be readily distinguished from the octadecagonal shape of non-diabetic plasma. The star symbol plots, which were based on the concentrations of 18 organic acids after normalization to the corresponding mean values of non-diabetes, were found to be effective for the observation of diabetes because of the observed transformation of octadecagonal patterns. Therefore, the current method of metabolomic profiling combined with the star pattern recognition method might be helpful for understanding biochemical states of energy metabolism in diabetes.

![Star symbol plots](image)

**Fig. (2).** Star symbol plots of diabetes data were constructed based on the levels of glucose-related metabolome after normalization to the corresponding mean values of non-diabetic group. The following rays are shown: 1, 3-hydroxybutyric acid; 2, pyruvic acid; 3, acetoacetic acid; 4, lactic acid; 5, glycolic acid; 6, 2-hydroxybutyric acid; 7, malonic acid; 8, succinic acid; 9, fumaric acid; 10, oxaloacetic acid; 11, α-ketoglutaric acid; 12, 4-hydroxyphenylacetic acid; 13, malic acid; 14, 2-hydroxyglutaric acid; 15, cis-aconitic acid; 16, 4-hydroxyphenyllactic acid; 17, citric acid; 18, isocitric acid.
3.3. Changes in Trabecular Bone Microarchitecture by T2D

Osteoporosis is characterized by low BMD and microarchitectural deterioration of bone tissue [29]. Deterioration of bone microstructure has been shown to be related to an imbalance between bone resorption and bone formation [30].

In the proximal tibia and distal femur, BV/TV, BS/TV and Tb.Th decreased in the diabetic mice relative to the non-diabetic group. In addition, BS/BV increased in the diabetic group compared with the non-diabetic group. In the distal femur, SMI and Tb.Pf were higher than in the non-diabetic group, whereas Tb.N was reduced compared to the non-diabetic group. The Tb.Sp, indicating trabecular separation, increased in the diabetic tibia.

BMD is considered to be the standard measure for the diagnosis of osteoporosis, while postmenopausal bone loss is characterized by a decrease in BMD [31]. The trabecular BMD of the femur and tibia decreased in the diabetic group by 72% and 41%, respectively, when compared with the non-diabetic group. Two-dimensional images of the proximal tibia and distal femoral metaphysis showed that bone loss was slightly induced by T2D. The results are displayed in Fig. (3).

3.4. Correlation Between HbA1c and Organic Acid or Bone Microarchitecture

The glucose metabolites (lactic, succinic, fumaric and malic acid) showed a negative correlation with HbA1c. In this study, higher HbA1c was associated with a significant decrease in lactic acid or other intermediate products of the Krebs cycle for energy production, which resulted in a significant increase in ketone bodies. Acetoacetic, 3-hydroxybutyric and 2-hydroxybutyric acid were positively correlated with HbA1c. This study showed that increasing ketone bodies were significantly correlated with high HbA1c levels, but not with fasting blood glucose, suggesting that higher HbA1c levels may be a critical factor driving the
formation of ketone bodies in diabetes. Zhu et al. showed that plasma ketone concentration, which is directly associated with HbA1c, is also directly associated with the risk of ketosis in diabetic patients [32]. Thus, HbA1c is an effective factor for the identification of diabetic patients at high risk of ketosis.

The trabecular BMD of both femur and tibia was strongly negatively correlated with HbA1c. In addition, HbA1c was inversely correlated with BV/TV, BS/TV and Tb.N, while it was positively correlated with BS/BV, SMI and Tb.Pf in the femur. In the tibia, only BS/BV was positively correlated with HbA1c. We did not find an immediate correlation between ketone bodies and bone loss. Ma et al. reported that higher HbA1c is associated with higher BMD in diabetes, which is consonant with the results of the present study [33].

The results are shown in Fig. (4). Our findings indicate an association between HbA1c and the progression of osteoporosis by T2D. However, the mechanisms underlying these relationships remain complex; therefore, it is recommended that various influential factors be considered while investigating the correlation between diabetes and BMD.

**CONCLUSION**

We demonstrated that changes in organic acid induced by T2D lead to changes in energy metabolism, such as the Krebs cycle and ketone bodies production.

Moreover, alterations in organic acid by diabetes were readily distinguishable by star pattern analysis. Therefore, metabolic profiling combined with star pattern analyses will be useful for understanding the biochemical state in diabetes.

This study showed that the components of plasma Krebs cycle and trabecular BMD were negatively correlated with HbA1c, whereas ketone bodies were positively correlated with HbA1c, indicating that uncontrolled blood HbA1c can affect bone loss, production of ketone bodies and utilization of glucose metabolites for energy production in T2D.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

The experimental protocols and procedures used in the present study were approved by the Sunchon National University Institutional Animal Care and Use Committee (SCNU-IACUC-2014-03).
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CONSENT FOR PUBLICATION
Not applicable.

AVAILABILITY OF DATA AND MATERIALS
Not applicable.

FUNDING
None.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS
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