Plasma amino acid abnormalities in calves with diarrhea

Kenji TSUKANO¹,² and Kazuyuki SUZUKI²*  

¹) Minami-Hokkaido Agricultural Mutual Relief Association, 74-2 Higashimae, Hokuto, Hokkaido, 041-1214, Japan  
²) School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimachi, Bunnkyoudai, Ebetsu, Hokkaido, 069-8501, Japan  

Correspondence to: Kazuyuki SUZUKI, DVM, Ph.D.  
School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimachi, Bunnkyoudai, Ebetsu, Hokkaido, 069-8501, JAPAN  
E-mail: kazuyuki@rakuno.ac.jp  
Phone and Fax: +81-11-388-4702  

RUNNING HEAD  
AMINO ACID ABNORMALITIES IN CALVES
Abstract

Since few studies have been published investigating plasma amino acid abnormalities in calves with illnesses, the aim of this study was to examine plasma amino acid abnormalities in calves with diarrhea. Forty-three Holstein calves aged 10.9 ± 5.6 days old were used for this study. Thirty-one of the 43 calves exhibited clinical signs of diarrhea without severe acidemia. The other 12 healthy calves were used as the control. Concentrations of plasma essential amino acids, non-essential amino acids, branched-chain amino acids, glucogenic amino acids, and ketogenic amino acids in diarrheic calves with hypoaminoacidemia were significantly lower than those in healthy calves. No significant differences were observed between diarrheic calves with normoaminoacidemia and healthy calves when looking at these parameters.

Keywords: amino acid, calf, diarrhea, hypoaminoacidemia
Diarrhea in calves leads to an overall loss not only of electrolytes and body fluids, but it also decreases the absorption of carbohydrates, lipids, and amino acids [5]. As a result, calves exhibit dehydration, electrolyte imbalance, metabolic acidosis, and negative energy balance (NEB). Metabolic acidosis stimulates proteolysis and increases the activity of branched-chain ketoacid dehydrogenase (BCKDH), and increased BCKDH activity accelerates the oxidation of amino acids. In addition, the ATP-dependent ubiquitin-proteasome system (UPS), the major pathway responsible for muscle protein degradation under catabolic conditions, may be upregulated in skeletal muscle by a number of factors, one being metabolic acidosis [2]. Our previous study showed that increases in plasma total amino acid (TAA) and branched-chain amino acid (BCAA) concentrations in diarrheic calves with severe acidemia were the result of an acceleration in proteolysis, similar to that in humans [24].

Hypoaminoacidemia, on the other hand, has been reported in the context of several diseases, such as in patients with glucagonoma syndrome [1], in dogs with hepatocutaneous syndrome [17], and in pigs undergoing hemodialysis [8]. Previous reports demonstrated that the principal response to hypoaminoacidemia was a reduction in the rate of protein synthesis [8]. Several studies have shown that protein synthesis is regulated by an extracellular signal, rather than by an intracellular signal [6, 8]. Amino acid supplementation in patients with amino acid abnormalities may be useful; however, it is not known which amino acids should be supplemented. Few studies have been published contributing to the understanding of plasma amino acid abnormalities in calves with diarrhea. Furthermore, current research on amino acid supplementation mainly focuses on feeding management for healthy calves [10, 26], while calves with illnesses are rarely studied.
The aim of this study was to investigate the plasma amino acid abnormalities in calves with diarrhea. Our hypothesis is that malabsorption of nutrients in the intestinal tract associated with diarrhea affects concentrations of some plasma amino acids. These data may help in understanding which amino acids preferentially become deficient in the presence of diarrhea.

All procedures in this study were performed in accordance with the Good for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University and the National Research Council [19]. Forty-three Holstein calves aged 10.9 ± 5.6 days old were recruited for this study. Thirty-one of the 43 calves were admitted to the Minami Hokkaido Agricultural Mutual Relief Association with clinical signs of diarrhea. The onset of diarrhea was unknown, but there was no treatment history, including farmer's treatment in all the calves on the initial examination. Cryptosporidium parvum (C. parvum) was detected in 20 (64.5%) calves by the C. parvum rapid test kit (BOX-BI0K-155-10TEST, COSMO BIO Co., Ltd., Tokyo, Japan). Ten (32.3%) calves exhibited clinical signs of mild dehydration and acidemia based on physical examination and blood gas analysis. The remaining 21 (67.8%) calves were clinically normal except for the presence of diarrhea, as assessed by vital signs, physical attributes, milk intake, and hematological analysis. The 12 healthy calves which had normal fecal production and were negative for C. parvum were used as the control. These calves were also kept on farms in the same area. All of the calves were initially housed in individual calf hutchs. Normally, all calves were given two feedings of milk at a rate of 5% of body weight per feeding in the morning (5:00 a.m.–7:00 a.m.) and the afternoon (5:00 p.m.–8:00 p.m.). No calves in this study exhibited anorexia. The calves also had ad libitum access to hay and water. Concentrate
feeding was not allowed during the study.

Blood collection from all calves was conducted between January and March of 2016. Blood collections were done at least 3 hours after providing the calves with milk. Venous blood samples were collected by jugular venipuncture from all calves upon the first medical examination. Non-heparinized blood samples were stored in EDTA-2K coated vacuumed tubes and plain tubes and then centrifuged for 15 min at 3000 g. using a standardized procedure to harvest plasma and serum. Free amino acid concentrations in plasma were measured by high-performance liquid chromatography (HPLC) using a commercial amino acid analysis kit (EZ: faast, Shimadzu, Kyoto, Japan) and an automated amino acid analysis system (Shimadzu Prominence and LCMS-2020, Shimadzu). Amino acid measurements were taken at the same time and in the same laboratory. In the serum biochemical analysis, total cholesterol was measured using enzyme methods with the DRI-CHEM 3500V (FUJIFILM Corporation, Tokyo, Japan). Serum total protein concentrations were measured using the Biuret and Bromocresol Green methods. Heparinized blood samples were analyzed for blood pH, base excess (BE), and glucose concentrations using an automatic gas analyzer (i-STAT 1, Abbott Lab, Princeton, IL, U.S.A.) and i-STAT cartridge (i-STAT EC8+ Cartridge, Abbott Lab).

All detectable amino acids and their abbreviations are listed in Table 1. We calculated essential amino acid (EAA: Thr + Val + Met + Ile + Leu + Phe + His + Lys + Arg + Trp), non-essential amino acid (NEAA: Ser + Glu + Asp + Gln + Asn + Pro + Gly + Ala + Cys-Cys + Tyr), TAA (EAA + NEAA), glucogenic amino acid (GAA; Thr + Val + Met + His + Arg + Ser + Glu + Gly + Ala + Pro + Asp + Asn + Gln + Cys-Cys), ketogenic amino acid (KAA; Leu + Lys) and BCAA (Val + Ile + Leu) levels. Statistical analysis was performed using Excel Toukei 2010 (SSRI, Osaka, Japan). The data were
summarized and described as mean ± standard deviation (SD). After confirming that plasma TAA concentrations in healthy calves were normally distributed, a mean ± SD of 1.96 was set as the reference value for normal plasma TAA concentrations with a 95% confidence interval. The Steel-Dwass test was employed for comparison among groups. The significance level was $p < 0.05$.

The reference range for plasma TAA concentrations in healthy calves was 2,089.1-3,194.8 nM. Thus, we adopted cut-off values for hypoaminoacidemia and hyperaminoacidemia in diarrheic calves of <2,089.1 nM and >3,194.8 nM, respectively. Based on the reference range for plasma TAA concentrations used in this study, 6/31 (19.4%) diarrheic calves were classified as hypoaminoacidemic, and 25/31 (80.6%) diarrheic calves were classified as normoaminoacidemic. Three of the 6 (50.0%) diarrheic calves with hypoaminoacidemia and 17 of the 25 (68.0%) diarrheic calves with normoaminoacidemia were infected with *C. parvum*. There were no hyperaminoacidemic diarrheic calves in this study. Blood pH values in the hypoaminoacidemic and normoaminoacidemic diarrheic calves and the healthy calves were 7.35±0.08, 7.36±0.05, and 7.42±0.02, respectively. Values of BE in the hypoaminoacidemic and normoaminoacidemic diarrheic calves and the healthy calves were 4.4±4.6, 6.2±5.5, and 12.3±3.0, respectively. Blood pH values were significantly lower ($p<0.01$) in normoaminoacidemic diarrheic calves than in healthy calves, and BE values were significantly lower ($p<0.001$) in hypoaminoacidemic and normoaminoacidemic diarrheic calves than in healthy calves. However, there were no diarrheic calves with severe acidemia (blood pH < 7.20) [4] in this study.

The concentrations of plasma amino acids in calves with hypoaminoacidemia and normoaminoacidemia are summarized in Table 1. Amino acid analysis
demonstrated significant decreases in TAA ($p<0.01$), EAA ($p<0.01$), NEAA ($p<0.05$), GAA ($p<0.01$), KAA ($p<0.05$), and BCAA ($p<0.05$) levels in calves with hypoaminoacidemia compared with healthy calves. In addition, plasma concentrations of TAA ($p<0.01$), EAA ($p<0.05$), NEAA ($p<0.01$), GAA ($p<0.01$), and KAA ($p<0.05$) in calves with hypoaminoacidemia were also significantly lower than those in calves with normoaminoacidemia. Amino acid analysis demonstrated significant decreases in cystine ($p<0.01$), phenylalanine ($p<0.05$), histidine ($p<0.05$), isoleucine ($p<0.01$), leucine ($p<0.05$), methionine ($p<0.05$), asparagine ($p<0.01$), proline ($p<0.01$), glutamine ($p<0.01$), arginine ($p<0.05$), serine ($p<0.05$), and threonine ($p<0.01$) levels in calves with hypoaminoacidemia compared with healthy calves. There were no significant differences between calves with hypoaminoacidemia and controls in the levels of the 8 remaining amino acids.

There were no significant differences in serum total cholesterol (61.5±15.0 mg/dl) or total protein (5.6±0.7 g/dl) levels in calves with hypoaminoacidemia compared to calves with normoaminoacidemia (total cholesterol: 56.9±21.8 mg/dl, total protein: 5.8±0.8 g/dl) and healthy calves (total cholesterol: 58.4±17.8 mg/dl, total protein: 5.7±0.5 g/dl). However, blood glucose concentrations in calves with hypoaminoacidemia (75.0±8.1 g/dl) were significantly lower than those in calves with normoaminoacidemia (98.2±19.1 g/dl, $p<0.01$) and in healthy calves (105.2±13.0 g/dl, $p<0.01$) (Table 2).

Essential amino acids are primarily responsible for the amino acid-induced stimulation of muscle protein anabolism in the elderly [25]. Plasma EAA concentration was reported to be more important than intracellular EAA concentration for protein synthesis [3, 7]. Increased plasma EAA concentrations result in increased protein
synthesis in human muscle [6]. Leucine is a BCAA that plays an important role in
protein synthesis via the mammalian target of the rapamycin (mTOR) signaling pathway
[16]. In addition, BCAAs inhibit protein degradation and are important nutrient signals
that act through direct and indirect effects [9, 18]. Glucose produced in the liver during
fasting is converted to pyruvate in skeletal muscle, transaminated with amino nitrogen
derived from BCAAs to produce alanine, and then converted back to glucose in the liver
by gluconeogenesis (dextrose-alanine-BCAA cycle) [21]. When the availability of
 glucose in the body is insufficient, animals require gluconeogenesis to produce glucose,
and gluconeogenesis from GAA has been suggested to be quantitatively important.
Blood glucose concentrations in hypoaminoacidemic calves were significantly lower
than those in calves with normoaminoacidemia and in healthy calves in this study.
Diarrhea leads to decreases in the absorption not only of carbohydrates but also of lipids
and amino acids in calves [5]. One potential association between hypoaminoacidemia
and decreases in blood glucose concentrations is that diarrheic calves with
hypoaminoacidemia cannot maintain gluconeogenesis due to deficiencies in glucose and
amino acids. Trefz et al. [23] demonstrated that hypoglycemia was not easily diagnosed
based on clinical signs but should be suspected in calves with clinical evidence of
septicemia, hypothermia, acute abdominal emergencies, and history or clinical evidence
of malnutrition. Indeed, diagnosis based on clinical signs in diarrheic calves with
hypoaminoacidemia was also difficult in this study.

No significant differences were observed in this study in the concentrations of
plasma TAA, EAA, NEAA, GAA, KAA, and BCAA between calves with
normoaminoacidemia and healthy calves. However, plasma concentrations of cystine
($p<0.01$), histidine ($p<0.05$), methionine ($p<0.05$), glutamine ($p<0.01$), and arginine
(p<0.01) were significantly lower in calves with normoaminoacidemia than in healthy calves. The limiting amino acid causes inefficient nitrogen utilization [11], and it becomes easily depleted in the body. Methionine, lysine, and threonine are often considered the limiting amino acids in calves [26]. In addition, the efficiency of arginine and cysteine utilization in calves was high, at 90.0% and 74.0%, respectively [10]. In calves with diarrhea, amino acids with a low basal level and high utilization in the body may be preferentially depleted. As another explanation, these amino acid variations may have been caused by diarrhea. C. parvum is one of the more commonly isolated gastrointestinal pathogens in dairy and beef calves, and it is associated with mucosal inflammation [14]. A previous study demonstrated that plasma histidine concentrations are lower in the presence of intestinal inflammatory disorders [13]. Oxidative stress is one of the major factors impairing the integrity of the gastrointestinal tract barrier and increases intestinal permeability [15]. Cystine and methionine are most susceptible to oxidative changes due to the high reactivity of their sulfhydryl groups [29]. Furthermore, the intestines require a large amount of energy for repair and replication of the mucosal barriers. Arginine and glutamine are well-known energy sources for enterocytes [27]. Therefore, these amino acid variations may be specific to calves with diarrhea, especially those with intestinal mucosal injury.

Plasma concentrations of aspartic acid (p<0.01) and glutamic acid (p<0.05), on the other hand, were significantly higher in calves with normoaminoacidemia than in healthy calves. It is well known that histidine, glutamine, and arginine can be converted to glutamic acid via specific pathways [20, 22, 28]. In addition, high aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in the intestinal mucosa was confirmed [20]. In this study, variations in aspartic and glutamic acid in
diarrheic calves with normoaminoacidemia could be explained by the fact that degradation of histidine, glutamine, and arginine is accelerated in the intestines in the presence of diarrhea, and then the resulting glutamic acid is converted to alpha-ketoglutarate, alanine, or aspartic acid by AST and ALT. However, there were no significant differences between calves with hypoaminoacidemia and controls in the levels of aspartic and glutamic acids. The presence of NEB causes decreases in plasma glucogenic amino acids in dairy cattle, including aspartic and glutamic amino acids [12]. In this study, in diarrheic calves with normoaminoacidemia, aspartic acid and glutamic acid levels increased due to the activation of gluconeogenesis by AST and ALT. Because diarrheic calves with hypoaminoacidemia cannot maintain gluconeogenesis due to the diarrhea-induced severe malabsorption of nutrients in the intestinal tract, it seems that plasma aspartic acid and glutamic acid decreased. Our hypothesis is that the first reaction seen in some amino acid abnormalities in diarrheic calves is associated with intestinal damage, and then NEB associated with malabsorption of nutrients causes further systemic amino acid abnormalities. In this study, plasma concentrations of phenylalanine ($p<0.05$), isoleucine ($p<0.05$), asparagine ($p<0.01$), proline ($p<0.01$), and serine ($p<0.01$) were significantly lower in calves with hypoaminoacidemia than those in calves with normoaminoacidemia. These data may support our hypothesis.

To the best of our knowledge, clinical cases of hypoaminoacidemia in livestock, including calves, have not been reported. The amino acid profiles of diarrheic calves with normoaminoacidemia and hypoaminoacidemia aid in our understanding of which amino acids are preferentially depleted due to intestinal damage and NEB. Hypoaminoacidemia in diarrheic calves can be caused by many factors such as age of onset, differences in pathogens present, severity of disease, duration of disease, and
disease management. Cases of normoaminoacidemia in diarrheic calves could shift to
hypoaminoacidemia if these factors are altered. Our results indicate that it may be
necessary to add amino acids as energy supplementation in diarrheic calves.

Conflicts of interest
The authors have no conflicts of interest directly relevant to the content of this
article.

REFERENCES

   Endotext [Internet]. www.endotext.org. [accessed on July 29, 2018]

   Soc. Nephrol. 9: 2030-2032.

3. Bennet, W. M., Connacher, A. A., Scrimgeour, C. M., Smith, K. and Rennie, M. J.
   1989. Increase in anterior tibialis muscle protein synthesis in healthy men during


   synthesis is modulated by extracellular, not intramuscular amino acid availability: a


objective, multivariate biomarkers composed of plasma amino acid profiles for the
and Wu, G. 2014. Effects of L-proline on the Growth Performance, and Blood
2007. Use of herbal preparations in the treatment of oxidant-mediated inflammatory
17. Loftus, J. P., Center, S. A., Lucy, J. M., Stanton, J. A., McDonough, S. P.,
Peters-Kennedy, J., Arceneaux, K. A., Bechtold, M. A., Bennett, C. L., Bradbury, C.
A., Cline, M. G., Hall-Fonte, D. L., Hammer-Landrum, J. F., Huntingford, J. L.,
Characterization of aminoaciduria and hypoaminoacidemia in dogs with


Table 1. Plasma amino acid concentrations of calves with diarrhea

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Abbreviations</th>
<th>Control (n=12)</th>
<th>Normoaminoacidemia (n=25)</th>
<th>Hypoaminoacidemia (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amino acid</td>
<td>TAA</td>
<td>242.0 ± 282.1a</td>
<td>2564.7 ± 298.5**</td>
<td>1936.4 ± 151.3c**</td>
</tr>
<tr>
<td>Essential amino acid</td>
<td>EAA</td>
<td>1253.0 ± 182.1a</td>
<td>1122.3 ± 212.2*</td>
<td>885.5 ± 67.7c*</td>
</tr>
<tr>
<td>Non-essential amino acid</td>
<td>NEAA</td>
<td>1335.7 ± 236.8a</td>
<td>1392.6 ± 230.5**</td>
<td>1009.0 ± 139.8b**</td>
</tr>
<tr>
<td>Glucogenic amino acid</td>
<td>GAA</td>
<td>2101.0 ± 270.7a</td>
<td>2022.4 ± 266.1**</td>
<td>1512.3 ± 158.7c**</td>
</tr>
<tr>
<td>Ketogenic amino acid</td>
<td>KAA</td>
<td>286.6 ± 62.0a</td>
<td>294.9 ± 59.5</td>
<td>220.6 ± 18.6b*</td>
</tr>
<tr>
<td>Branched-chain amino acid</td>
<td>BCAA</td>
<td>488.6 ± 102.4a</td>
<td>445.8 ± 101.9</td>
<td>363.8 ± 45.9b</td>
</tr>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>270.9 ± 100.2</td>
<td>300.9 ± 64.5*</td>
<td>209.5 ± 63.3c*</td>
</tr>
<tr>
<td>Cystine</td>
<td>Cys-Cys</td>
<td>9.06 ± 2.83a</td>
<td>0.20 ± 0.00c**</td>
<td>0.21 ± 0.01c**</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>10.2 ± 2.8a</td>
<td>15.5 ± 4.8**</td>
<td>7.9 ± 2.8**</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>94.1 ± 30.5a</td>
<td>131.5 ± 48.6b</td>
<td>88.9 ± 43.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>63.3 ± 8.0</td>
<td>64.2 ± 10.8*</td>
<td>53.9 ± 3.9*</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>294.3 ± 85.9</td>
<td>355.6 ± 147.2</td>
<td>284.6 ± 63.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>110.9 ± 42.4a</td>
<td>65.5 ± 38.0b</td>
<td>58.8 ± 12.5b</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>144.1 ± 19.4a</td>
<td>96.2 ± 21.8*</td>
<td>74.2 ± 10.6*</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>134.8 ± 53.7</td>
<td>156.9 ± 39.2*</td>
<td>109.6 ± 14.6*</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>151.9 ± 31.9a</td>
<td>138.0 ± 30.8</td>
<td>111.0 ± 10.8b</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>53.1 ± 33.4a</td>
<td>32.8 ± 9.4b</td>
<td>25.3 ± 7.0b</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>78.5 ± 12.8a</td>
<td>74.4 ± 31.4*</td>
<td>46.6 ± 15.3c*</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>165.8 ± 30.3a</td>
<td>146.9 ± 39.5**</td>
<td>94.9 ± 20.1c**</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>258.0 ± 47.5a</td>
<td>184.9 ± 59.3e</td>
<td>158.3 ± 57.9c</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>184.6 ± 42.4a</td>
<td>131.8 ± 42.8b</td>
<td>126.9 ± 37.1b</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>154.9 ± 26.1a</td>
<td>182.8 ± 66.1**</td>
<td>118.0 ± 13.2b**</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>184.1 ± 71.4a</td>
<td>188.1 ± 82.2</td>
<td>113.8 ± 15.1c</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>232.6 ± 57.0</td>
<td>211.7 ± 59.7</td>
<td>178.5 ± 27.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>33.6 ± 5.7</td>
<td>37.1 ± 10.5</td>
<td>33.4 ± 5.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>53.3 ± 10.2</td>
<td>49.8 ± 21.9</td>
<td>41.9 ± 10.2</td>
</tr>
</tbody>
</table>

Mean ± SD, Units: nM

Control vs normoaminoacidemia or hypoaminoacidemia a-b: p<0.05, a-c: p<0.01 by Steel- Dwass test
Normoaminoacidemia vs hypoaminoacidemia *: p<0.05, **: p<0.01 by Steel- Dwass test
The lower limit of detection for plasma cystine concentrations is 0.20 nM. Thus, the cut-off value for plasma cystine concentrations was set to 0.20 nM.
Table 2. Blood biochemical analysis in diarrheic calves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=12)</th>
<th>Normoaminoacidemia (n=25)</th>
<th>Hypoaminoacidemia (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>58.4 ± 17.8</td>
<td>56.9 ± 21.8</td>
<td>61.5 ± 15.0</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.7 ± 0.5</td>
<td>5.8 ± 0.8</td>
<td>5.6 ± 0.7</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>105.2 ± 13.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.2 ± 19.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.0 ± 8.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD

a-b: p<0.01 by Steel-Dwass test