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Multivariate Analysis of D- And L-Amino Acid Composition in Human Seminal Plasma Reveals Correlations with Sperm Concentration, Motility and Morphology

Masatoki Katayama	Department of Functional Bioanalysis, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo
Satoru Kaneko	Department of Obstetrics and Gynecology, Ichikawa General Hospital, Tokyo Dental College, 5-11-13, Sugano, Ichikawa, Chiba
Takahiro Tsukimura	Department of Functional Bioanalysis, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo
Kiyoshi Takamatsu	Department of Obstetrics and Gynecology, Ichikawa General Hospital, Tokyo Dental College, 5-11-13, Sugano, Ichikawa, Chiba
Tadayasu Togawa	Department of Functional Bioanalysis, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo

ABSTRACT

Objective: We present a first report of the concentrations of 33 D- and L-amino acids (AA) in human seminal plasma from IVF-ET patients with normo-, oligo- and azoospermia.

Method: We assess their relationship with sperm concentration, motility, and morphology by t-Test, regression analysis and PCA.

Results: Highest concentrations in L-AA were found in Gly (4611 µg/ml), L-Thr and L-His; in D-AA, D-Met (18.7 µg/ml), D-Leu, D-Val. L-Asn concentration differed significantly ($p = 0.05$) between normo- and oligozoospermia. L-Asn, D-Thr, and D-Leu were correlated with all three sperm properties. D-AA % of total AA differed significantly between normo- and oligozoospermia, particularly in D-Ala and D-Tyr. D-Phe and D-Leu concentrations were correlated with all three sperm properties, and D-Ala, D-Tyr, D-Met and D-Val with motility and morphology. Thus, D-Phe and D-Leu had the highest correlations to sperm properties among D-AA, and the ketogenic D-AA deamination metabolism was strongly correlated with these properties.

KEYWORDS: Seminal plasma, amino acid enantiomeres, multivariate analysis, ketogenic amino acid

INTRODUCTION

Prediction of male reproductive functions has been attempted by investigating the relationship between sperm parameters such as concentration and motility, and the concentrations of several amino acids such as arginine [1] and γ-ABA [2] in human semen/seminal plasma (SP) [2-6]. Katayama *et al*. [7] investigated 40 amino acids and related compounds in human SP and found that Asn and Gln were present at levels of less than one-tenth of those in human blood [8-10]. By comparison, NH_3 concentration was over 400 times higher than in blood and highly variable. This indicates that NH_3 accumulation might be a result of Gln and Asn catabolism.

It is generally accepted that most amino acids are L-racemic, however, recent analyses using ninhydrin post-column labelling have found mixtures of D- and L-amino acids. Several studies have recently drawn attention to the physiological significance of D-amino acids (D-AA) such as D-serine (D-Ser) and D-aspartic acid (D-Asp) in the brain [11,12] and in testes, where they might contribute to spermatogenesis in Leydig cells ([13,14]. These studies used pre-labelled duplicate HPLC [15,16], but due to the complexity of the method only a few amino acids, such as D-Ser and D-Asp, could be examined. We therefore developed a novel one-step differential simultaneous determination HPLC method for D- and L-amino acids in body fluids by modifying the o-phthalaldehyde (OPA) HPLC method [16,17]. Using this method, we here present a screening of 33 D- and L-amino acids in human seminal plasma (SP). In addition we examined correlations between the concentration of several D- and L-amino acids and human sperm parameters with the aim of developing new D-AA based parameters for clinical diagnosis of sperm properties.

MATERIALS AND METHODS

Semen collection and sperm analysis

Human ejaculate was obtained from patients who visited the Reproduction Center in the Ichikawa General Hospital, Tokyo Dental College. Consent was obtained from all study participants, who were briefed about the aims of the study and the parameters to be measured. The study was approved by the ethics committees of Meiji Pharmaceutical University and Ichikawa General Hospital. The WHO guidelines (WHO Manual 4th ed. 1999) [18] classify sperm into three categories based on concentration, namely normozoospermia ($\geq 20\times10^6$/ml), oligozoospermia ($< 20\times10^6$/ml), and azoospermia. Sperm-head morphology was evaluated according to the criteria of [19].

Determination of D- and L-amino acids in human seminal plasma

Materials: All standard solutions and analysis buffers were purchased from Hitachi (Tokyo, Japan). The HPLC equipment consisted of an LC-10A LC pump and controller, a DSU-20A degasser, and a RF-535 detector (ex. 245 nm, em. 430 nm) (all Shimadzu, Kyoto, Japan). HPLC conditions included a flow rate of 1.0 ml/min and the following gradient program: solvent B, 0–75min (2–55%), 85–95min (55–2%), and termination at 95 min.

Sample preparation: An equal volume of 10 % (w/v) trichloroacetic acid was added to the SP samples. The mixture was vortexed and centrifuged at 4000 g for 10 min. The supernatant was collected and filtered through a 0.45 µm pore filter, and a subsample of 50 µl was examined.

Pre-label method: To each subsample of 50 µl SP preparation we added 20 µl norleucine (Nle), 0.1 mol/l HCl, and 50 µl of 1.0 mol/l Borate buffer [pH 10.4, containing OPA and *N*-isobutyryl-*L*-cysteine (IBLC)], which were vortexed to mix. We then injected 10 µl of the reaction solution into the HPLC.

Statistical analyses: *t*-tests, multivariate regression analysis, and principal component analyses (PCA) were performed using JMP 10.0.2 software (SAS Institute, Japan).

RESULTS

The concentrations of the examined 33 D- and L-amino acids in human SP differed between normozoospermia, oligozoospermia, and azoospermia specimens **(Tables 1, last of manuscript)**.

L-Amino acids: The amino acids with highest concentrations were glycogenic AA. In particular, AA that had been metabolized to oxaloacetic acid occurred in high concentrations. The highest concentration was detected for Glycine (Gly) (4611 µg/ml), while the L-amino acid with the highest concentration was L-Threonine (L-Thr) (1464 µg/ml) followed by L-Histidine (L-His) (1105 µg/ml).

D-amino acids: We also found high concentrations of glycogenic D-AA, and in particular high concentrations of D-Methionine (D-Met) (18.7 mg/ml), which had been metabolized to succinyl-CoA, followed by D-Phenylalanine (D-Phe) (10.6 mg/ml), which had been metabolized to acetyl-CoA. The simultaneous determination of D- and L-enantiomeres revealed that concentrations differed between these. In general, D-AA were found to occur at levels of 0.3-33 % (D-AA / (D-AA + L-AA)) × 100 of total AA.

Comparison of D- and L-AA concentrations in normo- and oligozoospermia

Since we only found two samples of azoospermic SP, we restricted our comparisons to normo- and oligozoospermia.

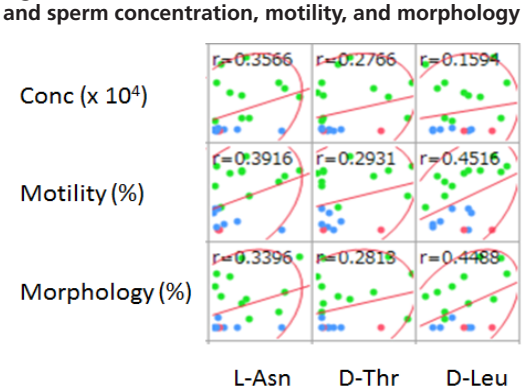
L-AA: Normozoospermia had significantly higher concentrations of L-Asn (p = 0.0431), L-Leu (p = 0.0294), L-Phe (p = 0.0380) and L-Lys (p = 0.0327) than oligozoospermia. As in our previous report, both NH_3-related L-Asn and ketogenic L-Leu and L-Lys gave significant ($p < 0.05$) values.

D-AA: Normozoospermia differed from oligozoospermia most significantly in the concentrations of D-Thr (p = 0.0273) and D-Lys (p = 0.0327). L-Thr was metabolized as both glycogenic and ketogenic AA while D-Lys was metabolized only as ketogenic AA.

Regression analysis

L-AA: Strong positive correlations were observed between L-Asn concentration and sperm concentration (r = 0.3201), motility (r = 0.3916), and morphology (r = 0.3396; Figure 1). Similarly, the concentrations of L-Asp (r = 0.1597), Gly (r = 0.1662), and L-Val (r = 0.2337) were correlated with sperm concentration **(Figure 1)**.

Figure 1: Correlations between amino-acid concentrations and sperm concentration, motility, and morphology



D-AA: The concentrations of D-Thr and D-Leu were correlated with sperm concentration (r = 0.3336 and r = 0.2416, respectively), motility (r = 0.2931 and r = 0.4516), and morphology (r = 0.2818 and r = 0.4489; Figure 1). Furthermore, we found correlations between the concentrations of D-Met (r = 0.1898), D-Val (r = 0.2557), D-Phe (r = 0.3867), and D-Ile (r = 0.2554) and sperm motility, and between D-Thr (r = 0.2818), D-Ala (r = 0.2833), D-Met (r = 0.2533), D-Val (r = 0.2334), D-Phe (r = 0.2334), and D-Leu (r = 0.4488) and sperm morphology **(Figure 1)**.

As previously reported, L-Asn, which is involved in NH_3 metabolism, showed strong correlations with sperm parameters in regression analyses. In contrast, glycol and ketogenic D-Thr and ketogenic D-Leu showed strong correlations among three sperm properties, respectively.

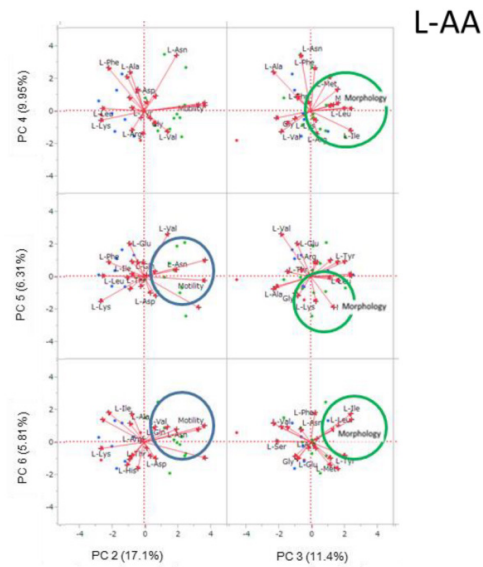
PCA of D- and L-amino acids in human seminal plasma

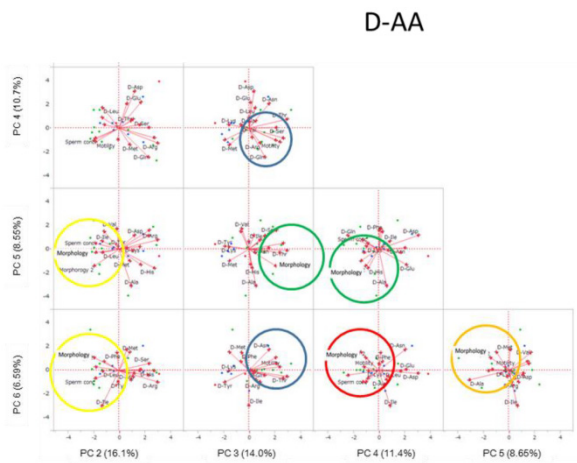
We used PCA to extract the main contributing factors that relate D- and L-amino acid concentrations to sperm parameters. The eigenvalues and biplots for selected D- and L-AA against sperm concentration and motility/morphology are presented in **Figure 2**. Over six principal components (PC6), the cumulative eigenvalues for sperm concentration, motility, and morphology exceeded 82% and 79% for L-AA and D-AA, respectively. In the following, location in the biplot matrix is given as PC*y*-*x*, where *y* and *x* are the matrix axes.

L-AA: L-Met, L-Leu, and L-Ile were clustered close to sperm morphology in PC4–3, L-Leu and L-Lys in PC5-3, and L-Leu and L-Lle in PC6-3 plots (green circles). In PC5-2, L-Asn clustered close to sperm concentration, as did L-Asn, L-Gln, and L-Val in PC6-2 (blue circles).

D-AA: D-Lys and D-Phe clustered close to sperm concentration, motility, and morphology in PC6-4 (red circle). In PC5-2, D-Ile, D-Tyr, and D-Leu clustered close to sperm concentration and morphology, as did D-Phe, D-Leu, D-Tyr, and D-Ile in PC6-2 (yellow circles). D-Met, D-Lys and D-Ala clustered close to sperm motility and morphology in PC6-5 (orange circle). D-Ser, D-Arg, and D-Gln in PC4-3, and D-Asn, D-Gln, and D-Thr in PC6-3 clustered close to sperm motility (blue circles), and D-Asn, D-Thr in PC5-3 and D-Lys, D-His, and D-Ala in PC5-4 clustered close to sperm morphology (green circles).

Figure 2: PCA biplot of sperm parameters and D- and L-amino acid concentration, motility, and morphology





In the preceding regression analyses, the concentration of L-Asn was correlated to all three sperm properties, and those of L-Asp, L-Val, and Gly were correlated with sperm concentration. Amongst D-AA, D-Thr and D-Leu were correlated with three sperm properties and D-Val, D-Ile, D-Met, and D-Phe with two sperm properties each. Based on the PCA analyses we thus determined that L-Met, L-Met, L-Tyr, and L-Glu levels reflected sperm concentration, and that L-Leu, L-Ile, L-Asp, L-Asn and the D-AA D-Leu, D-Ile, D-His, D-Lys, D-Met reflected sperm morphology. D-Thr reflected all three sperm properties. PCA results also suggested a relationship between sperm properties and amino acids involved in NH_3 metabolism (L-Asp, L-Asn).

Determination of sperm quality from D-amino acid content and its correlation with sperm properties

Given the correlations between several D-AA and various sperm parameters, we examined the use of sample D-AA content as a novel diagnostic parameter for sperm pathogenesis. We calculated D-amino acid concentration (%) as (D-AA / (D-AA + L-AA)) × 100 **(Table 2, last of manuscript)**. For normozoospermia the D-amino acid with the highest concentration was D-Asn (33.5 %), followed by D-Gln (22.3 %), and D-Arg (3.9 %). Consequently, D-Asn and D-Gln may play an important role in spermatogenesis.

Comparison of D-AA concentration among sperm categories: A comparison of D-AA content between normozoospermia and oligozoospermia, specimens revealed that normozoospermia had significantly higher concentrations of D-Ala (p = 0.1000), D-Tyr (p = 0.0699), D-Phe (p = 0.0817), and D-Leu (p = 0.0274) than oligozoospermia.

Correlations between D-AA concentration and sperm parameters: Regression analysis identified strong positive correlations between the concentration of D-Phe and sperm concentration (r = 0.3807), motility (r = 0.5187), and morphology (r = 0.3033). Similarly, D-Leu concentration was correlated with all three parameters (r = 0.4169, 0.4796, 0.4529 for sperm concentration, motility, and morphology, respectively), as was D-Ile (r = 0.2843, 0.2292, 0.2229). The concentrations of D-Glu (r = 0.1928, 0.2509, 0.2852), D-Ala, D-Tyr, D-Met, D-Val and D-Ile were correlated with sperm motility (r = 0.1743–2843) and morphology (r = 0.2210-0.3666). Leu is a ketogenic AA and metabolized to Acetyl–CoA. Phe and Ile are both ketogenic and glycogenic AA.

Discussion

Previous studies of human SP have usually identified around 20 proteogenic L-AA [1,20], and a subset of authors have drawn attention to particular AA such as L-Arg and L-Car [1,3,5]. Our previous study [7] increased sampling to 40 AA and reported the novel metabolomic findings that NH_3 concentration in human SP was approximately 400 times higher than in blood plasma, and that amino groups transfer, so that e.g., L-Asp, L-Asn, L-Glu, and L-Gln translate into NH_3. It is commonly accepted that most AA have an L-racemic body, but most reports have disregarded the concurrent presence of D-AA in samples examined for L-AA. This is therefore the first report of successful simultaneous determination of 33 D- and L-AA in SP. Previous studies reported L-Asn and L-Gln concentrations in human SP of less than one-tenth the concentration in human blood [7-10]. By comparison, we here report high L-Asp and L-Glu concentrations in human SP. Although D-AA are generally considered rare in living organisms, our most interesting results were D-AA concentrations of 0.3–33% in SP, particularly of D-Methionine (D-Met; 18.7 µg/ml) and D-Phenylalanine (D-Phe; 10.6 µg/ml). In addition, several D-amino acids involved in glycogenesis (D-Ala, D-Ile, D-Leu) were shown to be related to the examined sperm properties. The concentrations of D-Asn and D-Gln in SP were approximately half of those of D-Asp and D-Glu, and differed from the corresponding concentrations of L-AA in SP. We suspect that those ketogenic L- and D-AA are metabolized by different deamination factors in the Leydig cells. In our PCA analyses, L-Asp, L-Asn, L-Ile, and L-Leu showed strong contributions to sperm morphology while L-Met and L-Tyr contributed to both sperm concentration and motility. Several D-AA, namely D-Leu, D-Ile, D-His, D-Lys, and D-Met contributed to morphology. In addition, D-Ala was correlated with motility and D-Thr with sperm concentration, motility, and morphology. The concentration of D-AA was strongly correlated with several sperm properties. In particular, D-Phe, D-Leu, and D-Ile showed strong correlations with sperm concentration (r = 0.2843-0.4169), motility (0.2292-0.5187), and morphology (0.2229-0.4529).

Conclusion

This is the first report of D-AA concentrations in human SP. The D-AA with the highest concentration was D-Met (18.7 µg/ml) followed by D-Phe (10.6 mg/ml). D-AA were found at concentrations of 0.3-33 % of total AA content. Comparisons of normozoospermia and oligozoospermia revealed significant differences in L-Asn, L-Ser, and L-Lys content and in D-Ala, D-Leu, D-Tyr, and D-Phe content. L-Asn, D-Thr, and D-Leu were strongly correlated with all three examined sperm properties (concentration, motility, and morphology), and L-Asp, L-Asn, Gly, and L-Val with sperm concentration. D-Ala, D-Asp, D-Val, and D-Met were correlated with at least one sperm property each. In PCA analysis, L-Met, L-Tyr, L-Glu, L-Asn, and L-Asp were identified as characteristic AA that related to at least one sperm property. D-Thr related to all three sperm properties while D-Leu, D-Ile, D-Ala, D-Lys, and D-Met related to at least one sperm property each. Based on these results, we propose the concentration of D-AA in human SP as a new parameter for diagnosis of sperm properties.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 1: Amino acid concentrations (µg/ml) in human seminal plasma (mean ± standard deviation)

	Total specimens (n=22)	Normozoospermia (n=11)	Oligozoospermia (n=10)	Azoospermia (n=2)
L-Asp	505.3±245.0	517.6±167.6	449.2±234.4	689.6
L-Thr	1464±1200	1566±1373	1343±1112	1390
L-Ser	895.3±956	738.3±575.2	772.3±597.3	2310
L-Asn	1.86±2.03	2.71±2.19	1.10±1.67	0.55
L-Glu	692.3±594.3	559.3±466.6	844.6±755	735.3
L-Gln	6.72±6.19	6.96±5.20	5.04±4.40	12.94
Gly	4611±3711	4545±3331	3800±3246	8621
L-Ala	513.3±229.2	499.0±300.7	495.1±114.5	675.4
L-Val	599.6±300.3	631.3±53.80	537.3±164.8	705.6
L-Met	687.6±361.3	563.3±372.3	744.6±387.7	418.7
L-Ile	899±593	835±549	1043±700.7	601

L-Leu	341±358.3	207.3±153.3	523±493.3	260.3
L-Tyr	670±497.7	600.7±423	793.3±622.3	512
L-Phe	1003±678.3	768.7±570.3	1333±770	806.3
L-Lys	539±341	397.3±253.8	604.3±209.2	1023
L-His	1105±432.7	975.3±540.3	1293±224	969.7
L-Arg	443±356.3	608.7±663.7	434.7±375.3	489
D-Asp	2.01±3.63	1.33±3.1	1.67±2.3	6.97
D-Thr	2.03±2.31	2.83±2.8	0.9±0.5	2.8
D-Ser	1.54±2.13	1.68±2.23	1.42±2.46	1.32
D-Asn	1.75±3.40	1.48±2.22	2.23±4.9	2.22
D-Glu	2.42±3.11	2.01±2.21	1.95±1.84	6.83
D-Gln	1.60±1.77	1.96±2.11	1.41±1.46	0.52
D-Ala	9.06±11.33	10.49±14.43	5.23±5.07	18.41
D-Val	10.43±9.90	12.25±12.76	10.96±3.83	18.44
D-Met	18.70±19.98	19.12±20.39	18.85±28.97	16.55
D-Ile	10.51±8.78	10.73±9.92	12.77±8.39	14.02
D-Leu	11.11±6.61	12.51±7.44	10.82±4.42	17.39
D-Tyr	7.38±5.00	7.16±3.96	7.93±8.18	14.78
D-Phe	10.61±11.42	12.82±14.95	7.93±6.89	10.48
D-Lys	7.10±8.23	9.02±8.10	6.02±5.69	16.48
D-His	2.92±3.02	3.03±3.78	2.70±2.88	3.30
D-Arg	9.27±11.1	9.21±12.2	8.54±9.37	12.3

Table 2: Percentages of D-AA content in human seminal plasma (mean ± standard deviation), calculated from total AA concentrations as (D-AA / (D-AA + L-AA)) x 100

	Total specimens (n=22)	Normozoospermia (n=11)	Oligozoospermia (n=10)	Azozoospermia (n=2)
D-Asp %	0.487±0.959	0.456±1.227	0.446±0.653	0.744
D-Thr %	0.355±0.432	0.436±0.465	0.434±0.467	0.569
D-Ser %	1.141±2.360	1.251±2.677	1.724±3.073	1.997
D-Asn %	44.28±30.16	33.52±35.73	52.44±20.81	66.67
D-Glu %	1.567±4.102	2.261±5.741	0.668±0.803	1.763
D-Gln %	22.89±15.46	22.31±16.24	22.68±15.74	10.60
D-Ala %	1.615±1.732	1.977±2.194	0.960±0.959	2.578
D-Val %	1.714±1.379	1.873±1.734	1.311±0.697	2.660
D-Met %	2.944±3.832	3.280±4.914	2.263±2.409	4.157
D-Ile %	2.515±1.667	2.755±4.287	1.236±1.719	2.515
D-Leu %	5.192±4.624	6.930±5.510	2.846±2.466	6.191
D-Tyr %	2.591±4.090	3.831±5.402	0.955±0.953	3.142
D-Phe %	1.484±2.106	2.137±2.792	0.732±0.809	1.280
D-Lys %	1.496±1.265	1.718±1.281	1.057±1.183	2.246
D-His %	0.267±0.270	0.297±0.280	0.199±0.225	0.408
D-Arg %	3.890±6.640	2.869±3.703	4.955±9.696	4.712

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