RESEARCH PAPER | Medical Science | Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

Thirty-seven amino acids and three related nitrogen compounds in human seminal plasma: multivariate analyses reveal strong correlations among asparagine, glutamine, and ammonia with sperm parameters

KEYWORDS: Seminal plasma, Amino acids, Ammonia, Multivariate analyses

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 272-8513, Japan

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 272-8513, Japan

Tadayasu Togawa
Department of Functional Bioanalysis, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo

ABSTRACT *We assessed the relationship of 40 amino acids and nitrogen compounds in human seminal plasma with three sperm parameters – concentration, motility, and morphology. The amino acid content of seminal plasma was different from serum, for example, Sar and β-Ala at very low levels. In particular, Asn and Gln were found at levels below 1/20th of those of Asp and Glu, the opposite relationship in serum. Using ANOVA, we revealed significant differences in Asp, Glu, Gln, γ-ABA, NH_3, and Car. Next, we conducted a regression analysis and found that Asp, NH_3, γ-ABA, and Orn were highly correlated with sperm parameters. Gln and HCys showed negative correlations. Asp, Asn, Glu, Gln, and Orn showed correlations with NH_3, and were extracted by principal component analysis. The findings suggest that amino acids and sperm concentration could be used for diagnostic purposes, in particular amino acids related to NH3 metabolism, Asn, Gln, and NH_3.*

INTRODUCTION

Aside from their traditional role in nutrition, amino acids also play a significant role in a variety of physiological and pathological processes. For example, amino acids are the principle components of neurotransmitters (Siegel *et al.,* 1999); newborn babies are routinely screened for inherited aminoacidopathies such as phenylketonuria, maple syrup urine disease, homocystinuria, and tyrosinemia (Scriver *et al.,* 2006); and dysmetabolism of γ-aminobutyric acid (γ–ABA) causes neurological disorders (Siegel *et al.*, 1999). To date, the relationships between the concentration of amino acids in semen/seminal plasma and sperm parameters such as concentration and motility have been investigated to predict male reproductive functions (Aniello *et al.,* 2012; Calogero *et al.,* 1996; Hernvann *et al.,* 2009; Papp et al., 1983; Paulsen *et al.,* 1980; Silvestroni *et al.,* 1979). However, previous studies have typically only assessed the relationships of the 20 proteogenic amino acids with sperm parameters; furthermore, those studies are usually focused on particular amino acids, such as carnitine (Car) (Gürbüz *et al.,* 2003) and arginine (Arg) (Srivastava *et al.,* 2006), and γ-ABA (Calogero *et al.,* 1996). In contrast, the present study conducted a thorough screening of 37 amino acids and 3 related nitrogen compounds (NCs) in human seminal plasma (SP). Statistical comparisons were made between the amino acids and NCs with sperm parameters that were classified according to the World Health Organization (WHO) Manual (4th ed. 1999) by means of ANOVA and multivariate analyses including multivariate regression, principal component analysis (PCA), and cluster analysis.

MATERIALS AND METHODS

Semen collection and sperm analysiss

Human ejaculates were obtained from patients who visited the Reproduction Center, Ichikawa General Hospital, Tokyo Dental College. Consent was obtained from all study participants who provided ejaculates after they were briefed about the study aims and measurement items. The ethics committees of Meiji Pharmaceutical University and Ichikawa General Hospital specifically approved this study. The WHO guidelines (WHO Manual 4th ed. 1999) classify sperm into 3 categories based on concentration [normozoospermia (> 20×10^6/ml), oligozoospermia (< 20×10^6/ml), and azoospermia] and into 2 categories based on motility [normozoospermia (≧ 50% of total sperm) and asthenozoospermia (< 50%)]. Sperm head morphology was evaluated according to the strict criteria of Kruger et al., (1988). Sperm with oval-shaped heads were defined as having normal morphology and classified as normozoospermia (≧ 4.0% of total sperm) or teratozoospermia (< 4.0%).

Determination of amino acids and NCs in seminal plasma

Materials: All standard solutions and analysis buffers were purchased from Hitachi (Tokyo, Japan). Analytical conditions were set in accordance with the Physiological Fluids Analysis Method (Hitachi #2611). System: L-8900 (Amino Acid Analyzer). Column: for separation, a 4.6mm I.D. × 60mm [#2622] column was used; for ammonia trapping, a 4.6mm I.D. × 40mm [#2650L] column was used. Eluent: L-8500 PF-Kit, column temperature: 32–70 °C. Flow rate: 0.35 ml/min. Detector: VIS 570 nm, 440 nm. Each amino acid was derived using ninhydrin reagent.

Sample preparation: An equal volume of 10 % (w/v) trichloroacetic acid was added to the SP collected. The mixture was vortex-mixed and centrifuged at 4000 g for 10 min. The supernatant was collected and filtered through a 0.45-mm pore filter, and 5 ml was then used as the injection volume.

Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

Statistical analyses: ANOVA and multivariate statistical methods (regression analysis, PCA, and cluster analysis) were performed using JMP 10.0.2 software (SAS Institute, Japan).

Results

The concentrations of the 37 amino acids and 3 NCs in human SP were compared among normozoospermia, oligozoospermia, and azoospermia specimens (Table 1, last of manuscript).

A subset of the amino acids and NCs was not detectable in all the specimens. The number of specimens in which they were detected are expressed within the parentheses adjacent to the mean ± SD. In normozoospermia, the highest amino acid concentration detected was from glutamic acid (Glu) (3820 mg/ml), followed by lysine (Lys) (1909 mg/ml) and serine (Ser) (1796 mg/ml). In contrast, sarco sine (Sar) and methionine (Met) were found at very low concentrations, each detected at under 100 mg/ml. In BP, concentrations of asparagine (Asn) and glutamine (Gln) were more than 10 times higher than those of aspartic acid (Asp) and Glu (Nelson *et al.*, 1997; Scriver *et al.*, 2001; Venta *et al.*, 2001). In contrast, in human SP, Asn and Gln were present at under one-tenth of the levels of Asp and Glu. The concentration of NH_3 in human BP (0.1–0.8 mg/ml) (Kratz *et al.*, 2004; Nelson *et al.*, 1997) is tightly controlled. However, in SP, it was found to be over 400 times higher and showed a wider fluctuation range.

Next, we excluded 7 amino acids and NCs with missing values in at least 1 group and proceeded to compare the remaining 33 items between the 3 groups by means of ANOVA. In normozoospermia, the compounds that were found to be significantly higher than those in oligozoospermia and azoospermia included Asp (*p*-value: 0.0001), Glu (0.0132), γ-ABA (0.0015), and ornithine (Orn) (0.0152), with NH_3 (0.0001) giving the highest significant difference. In contrast, Gln (0.0003), Asn (0.0026), Car (0.0079), and homocysteine (HCys) (0.0092) were significantly lower in normozoospermia than in the other two groups.

Scattergram and regression analysis of amino acids and NCs in human seminal plasma

We then evaluated the correlations of 24 amino acids and 3 NCs with 3 sperm parameters (concentration, motility, and morphology) using multivariate statistical methods (regression analysis, PCA, and cluster analysis). Each parameter in which the number of samples was less than nine was excluded from the test objects (Fig. 1).

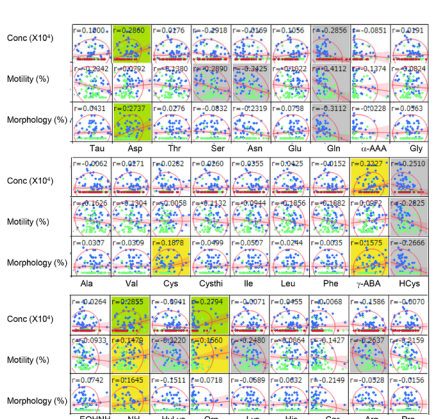


Figure 1: Correlation among 27 amino acids and sperm parameters (sperm concentration, motility, morphology)

Figure 2 summarizes the items indicative of positive and negative correlations. Positive correlations were observed for Asp, γ-ABA, and Orn with at least two parameters, whereas with NH_3, positive correlations with all three parameters were observed (r = 0.1479–0.2855).

In contrast, negative correlations were observed for Gln and HCys with all three sperm parameters (r = -0.4112 and -0.2510, respectively). Cysteine (Cys) was found to be positively correlated with one parameter; however, Ser, Asn, hydroxylysine (HyLys), Lys, and Arg were found to be negatively correlated.

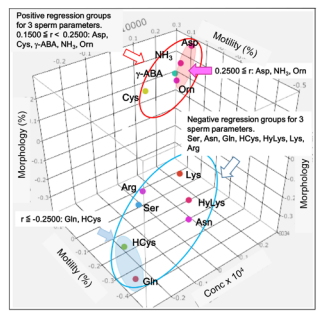


Figure 2: Amino acids showed positive (0.1500 ≦ r) and negative (r ≦ -0.1500) regression among the three sperm parameters

As indicated in Table 1, the amino acids involved in NH_3 metabolism (Asp, Asn, and Gln) showed strong correlations with sperm parameters in regression analyses. In contrast, Arg and Car, which are well-known as amino acids for increasing spermatogenesis, showed a negative correlation with correlation with two parameters, respectively.

PCA of amino acids and NCs in human seminal plasma

PCA extracted the main contributing factors to the amino acid, NC, and sperm parameter data. Figure 3 summarizes the eigenvalues and contains biplots for amino acids and NCs with sperm concentration and motility/morphology. Over principal components 6 (PC6), the accumulated eigenvalues exceeded 86% of sperm concentration, motility, and morphology. In the plots assessing sperm concentration, the distance between Orn and NH_3 were close to the sample sperm concentration in the PC2–3 and PC2–4 plots (blue ring). Gln, in contrast, showed a close distance with the sample sperm concentration in the PC4–6 plot (red ring). In plots assessing motility and morphology, Gln and Asn showed close distances to the sample

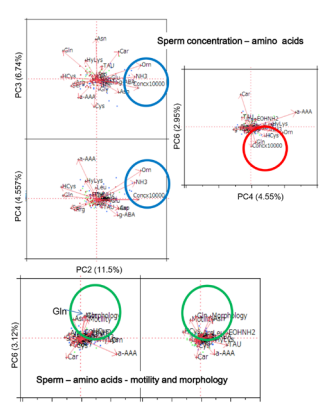
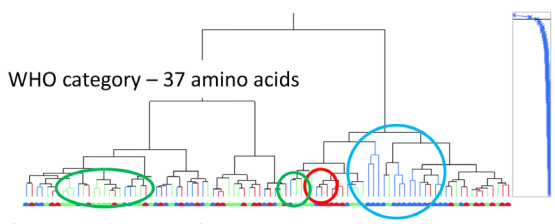


Figure 3: Biplot among sperm parameters and amino acids

Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

motility and morphology in the PC4–6 and PC5–6 plots (green ring). In the preceding regression analysis, Orn and NH_3 were also found to be correlated with sperm concentration. As such, it was clear that Asn and Gln were negatively correlated with all three sperm parameters. Using data derived from PCA, we were able to characterize that Gln levels reflected sperm concentration characteristics, and that Gln and Asn reflected sperm motility and morphology characteristics. Amino acids Arg, Ser, and threonine (Thr) also showed high correlation with PC1; however, they did not correlate with any of the sperm parameters. PCA also suggested a relationship between the sperm parameters and amino acids involved in NH_3 metabolism. The biplots suggested that Asp, γ-ABA, Asn, taurine (Tau), and Gln might also be notable correlates with sperm parameters; however, we did not find correlations in regards to Arg and Car, which are traditionally implicated in spermiogenesis.

Cluster analysis of amino acids in human seminal plasma



Cluster analysis results are summarized in Figure 4. Each sample was labeled with one of three colors. Figure 4: Cluster analysis of sperm concentrations - amino acid relationships. Groups include normozoospermia (blue), oligozoospermia (green), and azoospermia (red)

The results from the cluster analysis revealed that about 40% of the samples were clearly distributed into normozoospermia, oligozoospermia, and azoospermia. The amino acids and NCs were found to be different among the WHO-categorized groups when analyzed by sperm concentration.

Relationships between sperm parameters and asparagine, glutamine, and ammonia

Overall, the results supported the relevance of NH_3 metabolism to the sperm parameters assessed. Figure 5 depicts the regression analyses between NH_3 and related amino acids (Asp, Asn, Glu, and Gln). Strong regression values were observed for Asp and Glu (r = 0.6975 and 0.7649, respectively), a moderate regression value was observed for Asn (r = 0.2566), and a very low regression value was observed for Gln (r=0.0549).

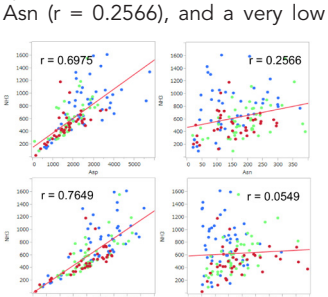


Figure 5: Regression of NH_3 –Asp, Asn, Glu, and Gln

We next compared the amino acids participating in the aminotransferase pathways, specifically glutaminase and aspartate aminotransferase (AST) mediated conversion of Glu to Asp and alanine aminotransferase (ALT) mediated conversion of Ala to Glu (Fig. 6).

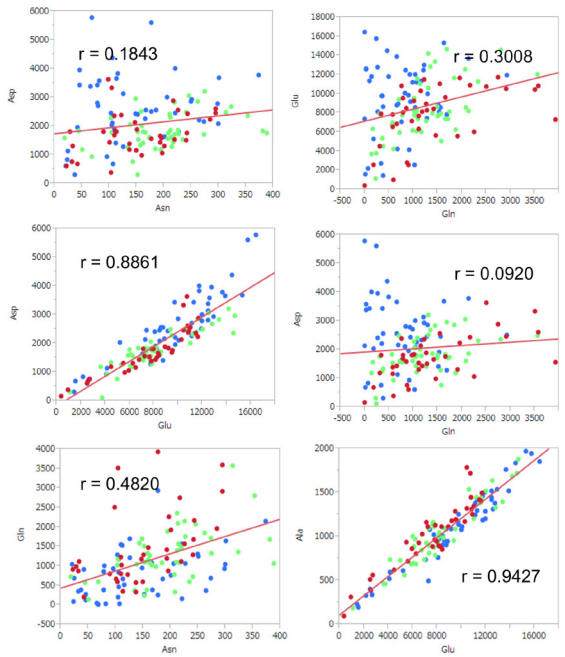


Figure 6: Regression of Asp, Asn, Glu, Gln, and Ala

Highly positive correlations were observed between Glu and Asp (r = 0.8861) and between Ala and Glu (r = 0.9427). Positive correlations were also observed between Glu and Gln (r = 0.3008) and between Asp and Asn (r = 0.1843), albeit weaker.

Discussion

Previous studies have typically only analyzed the 20 proteogenic amino acids in human SP in relation to male reproductive functions (Morgante *et al.*, 2012; Srivastava *et al.*, 2006). While a subset of authors have drawn attention to particular amino acids such as Arg, Orn, and Car (Aniello *et al.*, 2012; Papp *et al.*, 1983; Paulsen *et al.*, 1980; Srivastava *et al.*, 2006;), in the present study, these amino acids were found to have no characteristic relationship with the sperm parameters examined. In the present study, we increased the number of items analyzed by examining an additional 17 amino acids and 3 NCs, and conducted multivariate statistical analyses in addition to ANOVA. From this, we constructed a novel metabolomics concept, the most notable characteristic being that the NH_3 concentration in human SP was approximately 400 times higher than the levels found in BP. Further, we observed that the abundance ratios of Asn/Asp and Gln/Glu were substantially reversed between BP and SP. Results from ANOVA suggested that the sperm parameters were correlated with Asp, NH_3, γ-ABA, Glu, Orn, Gln, Asn, Car, and HCys. These correlations were confirmed for Asp, NH_3, γ-ABA, and Orn by regression analyses and results from PCA identified Asn and Gln as important factors, revealed Asp, Glu, and NH_3 to be present at high concentrations in SP and conversely, Asn and Gln to be present at levels were low. It is well-known that glutaminase and AST deamidates Gln and Asn to generate NH_3, while ALT carries out the transamination of Ala to create Glu. AST converts Glu into Asp, and Asp is then converted to urea and NH_3 by way of Arg. The concentrations of AST and ALT found in human semen range from 90–720 U/ml (Eliasson *et al.*, 1965). They are

Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

more than 10 times higher than the concentrations found in human BP, which ranges from 15–48 U/ml (AST) and 8–42 U/ml (ALT) (Tietz *et al.,* 1992; Nelson *et al.,* 1997).

Glutaminase is also present in human semen at levels that are more than 10 times higher than in BP (Eliasson *et al.,* 1965). Overall, findings from the present study demonstrated that the sperm parameters assessed were positively and negatively correlated with NH_3 and the amino acids involved in de- and trans-amidation in NH_3 metabolism.

As shown in Table 1, the NH_3 concentration in SP was uncommonly high in all three sperm groups, with the highest levels being found in normozoospermia. In contrast, Gln and Asn were found significantly lower in normozoospermia than in the other two groups. In regards to Figures 5 and 6, while it is known that NH_3 translates amino acids, positive correlations were only observed between NH_3-Asp, NH_3-Asn, NH_3-Glu, Asp-Glu, and Ala-Glu. No correlations were observed for NH_3-Gln, Asp-Asn, and Asp-Gln. These facts indicated the possibility of NH_3 neogenesis due to catabolism of Gln and Asn. To verify the accuracy of our analytical approaches, it will be essential to increase the sample size of the specimens collected as well as examine as many other sperm parameters as possible. We believe that it will be primarily important to clarify whether the origin of NH_3 was due to the deamidation of Gln and Asn by the sperm, due to extracellular processes, or due to bilateral processes.

Conclusion

The concentrations of Asn and Gln in SP were found to be one-tenth the concentrations of Asp and Glu, a finding that is reciprocal to the concentrations of the same amino acids found in BP. ANOVA and the multivariate statistical methods suggested the following relationships: first, Asp, Glu, Gln, γ-ABA, NH_3, Car and HCys showed significant differences among the WHO-categorized groups by ANOVA. Second, by regression analyses, Asp, NH_3, γ-ABA, and Orn showed high correlations with sperm properties while Ser, Asn, Gln and HCys were negatively correlated with sperm properties. Third, by PCA, Asn, Gln, NH_3, and Orn were identified as key amino acids related to at least one sperm parameter. Finally, cluster analysis confirmed a clear separation of groups matching the WHO group of sperm categorization.

Conflict of interest

The authors declare that they have no conflict of interest.

TABLE -1
RESULTS OF AMINO ACIDS CONCENTRATIONS (μg/ml) IN HUMAN SEMINAL PLASMA
ND* ND means amino acids concentration under 0.2 μg/ml.

	Total specimens (n=132)	Normozoospermia (n=48)	Oligozoozpermia (n=47)	Azozoospermia (n=37)
TAU	65.4±28.73	70.56±32.02	62.84±23.56	62.24±29.84
PEA	ND	ND	ND	ND
Urea	218.1±104.9	229.2±129.5	199.8±86.56	224.6±88.12
Asp	819.2±395.6	1019±476.4	704.0±274.3	428.4±307.8
Thr	802.1±309.9	898.4±332.9	769.6±283.7	768.4±307.7
Ser	1861±837.6	1796±974.8	1870±698.0	1950±791.2
Asn	66.12±33.99 (n=128)	57.32±34.98 (n=47)	78.48±32.37 (n=45)	62.04±30.72 (n=36)
Glu	3435±1353	3820±1498	3310±1210	3125±1215
Gln	453.6±318.8	322.0±246.4	497.6±280.1	572.0±390.3
Sar	37.14±15.05(n=9)	40.24±12.66(n=8)	ND	12.36(n=1)
α-AAA	21.2±24.38(n=108)	26.32±28.69(n=39)	20.22±25.20(n=36)	14.42±15.24(n=33)
Gly	874.4±339.4	938.8±387.9	838.4±285.7	807.6±330.2
Ala	421.6±158.2	443.6±178.4	409.2±142.7	411.2±148.8
Cit	43.60±28.96(n=26)	54.48±33.82(n=14)	30.10±10.01(n=7)	32.01±21.71(n=5)
α-ABA	13.97±6.69(n=6)	13.97±6.69(n=6)	ND	ND
Val	783.6±305.8	806.4±329.1	758±270.5	740.0±283.2
Cys	50.9±22.66	57.04±29.92	46.32±14.77	49.04±19.6
Met	13.08±9.15(n=29)	16.89±12.29(n=11)	11.52±6.36(n=10)	9.04±3.86(n=8)
Cysthi	1050±406.8	1136±462.8	1009±355.3	994.4±377.5
Ile	1096±427.2(n=130)	1194±473.2	1041±376.2(n=46)	1042±406.8(n=36)
Leu	595.2±215.6	643.6±253.0	582.4±190.9	554.4±184.6
Tyr	ND	ND	ND	ND
Phe	379.2±139.8	400.8±155.1	368.4±127.5	367.0±132.7
β-Ala	9.08±6.54(n=12)	3.52±1.51(n=2)	9.47±6.92(n=8)	13.11±6.20(n=2)
β-AiBA	9.47±6.02(n=41)	8.07±6.53(n=8)	10.89±6.11(n=21)	8.44±5.54(n=12)
γ-ABA	10.25±4.40 (n=128)	11.88±5.12(n=44)	9.77±3.42(n=47)	8.72±3.47(n=37)
HCys	30.02±16.91	25.22±14.32	34.19±19.02	30.92±15.94

Volume : 5 | Issue : 9 | September 2015 | ISSN - 2249-555X

Trp	ND	ND	ND	ND
$EOHNH_2$	39.57±19.46	39.50±21.54	37.38±16.75	42.52±19.93
NH_3	250.9±130.1	310.2±150.6	231.1±109.8	197.6±90.72
HyLys	22.40±7.33	22.66±7.84	21.88±7.39	22.75±6.58
Orn	96.28±140.0	139.4±186.1	75.76±94.28	64.32±96.08
Lys	1854±731.6	1909±840.4	1842±645.2	1804±690.4
1MeHis	7.87±6.10(n=4)	ND	16.90(n=1)	4.86±4.51(n=3)
His	1372±541.2	1490±610.1	1311±462.8	1304±522.4
3MeHis	6.72±3.49(n=17)	8.94±5.82(n=4)	5.74±1.09(n=3)	6.13±2.68(n=10)
Car	171.0±141.1(n=127)	150.4±130.2(n=46)	221.9±161.5(n=45)	133.3±105.7(n=36)
Arg	1084±464.8 (n=130)	1071±527.6 (n=46)	688.4±384.7 (n=47)	1030±482.4(n=37)
HyPro	ND	ND	ND	ND
Pro	353.8±137.3	363.1±154.9	352.6±120.0	344.4±135.0

REFERENCE Aniello GD, Ronsini S, Notari T, Natascia, Fiore MD, Fisher G, Antimo Aniello AD. D-Aspartate, a key element for the improvement of sperm quality. Advances Sexual Med 2012;2:47-53. | Calogero AE, Hall J, Fishel S, Green S, Hunter S, D'Agata RA. Effects of gamma-aminobutyric acid on human sperm motility and hyperactivation. Mol Hum Reprod 1996;2:733-738. | Eliasson R. Origin of transaminase in human semen. J Reprod Fertil 1965;11: 281-282. | Hernvann A, Gonzales A, Troupela S. Amino acid content of human semen in normal and infertility cases. Andrologia 2009;18:461-469. | Katayama M, Kaneko S, Takamatsu K, Tsukimura T, Togawa T. Determination of trace metals in human SP using inductively coupled plasma mass spectrometry and multivariate statisticalanalyses for sperm parameters. J Mol Biomark Diagn 2013;4: 1000147. | Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. | Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Laboratory reference values. N Engl J Med 2004;351:1548–63. | Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S Predictive value of abnormal sperm morphology in vitro fertilization. Fertil Steril 1988; 49: 112–117 | Morgante G, Scolaro V, Tosti C, Di Sabatino A, Piomboni P, De Leo V Treatment with carnitine, acetyl carnitine, L-arginine and ginseng improves sperm motility and sexual health in men with asthenopermia. Minerva Urol Nefrol 2012; 62:213-218. | Nelson textbook of pediatrics. Nelson, Waldo E (Waldo Emerson), 1898-1997; Behrman, Richard E, 1931- .14th ed. | Papp GY, Grof J, Menyhart J. The role of basic amino acids of the seminal plasma in fertility. Int Urol and Nephrol 1983;15:195-203. | Paulsen H, Nissen HP, Heinze I, Schirren C, Kreysel HW. Protein composition of human sperm from oligozoo-, teratozoo-, asthenozoo- and azoospermia. Andrologia 1980;12:61-65. | Scriver CR, Beaudet AL, Valle D, Sly WS, Childs B, Kinzler KW, Vogelstein B, eds. The Metabolic and Molecular Bases of Inherited Disease. 8th ed. New York, NY: McGraw-Hill, Inc; 2001;1665-2105. | Siegel GJ, Agranoff BW, Albers RW, Fisher SK, Uhler MD. Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th ed. Philadelphia: Lippincott-Raven; 1999. | Silvestroni L, Morisi G, Malandrino F. Free amino acids in semen: measurement and significance in normal and oligozoospermic men. Arch Androl 1979;2:257-261. | Srivastava S, Desai P, Coutinho E, Govil G. Mechanism of Action of L-arginine on the Vitality of spermatozoa is primarily through increased biosynthesis of nitric oxide. Biol Reprod 2006; 74:954–958 | Tietz NW, Shuey DF, Wekstein DR. Laboratory values in fit aging individuals-sexagenarians through centenarians. Clin Chem 1992; 38: 1167 -1185 . | Venta R. Year-long validation study and reference values for urinary amino acids using a reversed-phase HPLC method. Clin Chem 2001; 47:575–583. | WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus | Interaction 4th Edition (1999). |