1H NMR Spectroscopic Studies on Human Seminal Plasma: A Probative Discriminate Function Analysis Classification Model

A. gupta[1], A. A. Mahdi[1], K. Ahmad[1], K. K. Shukla[1], S. P. Jaiswar[2], and S. N. Shankhwar[3]
[1]Department of Biochemistry, King George's Medical University, Lucknow, UP, India, [2]Department of Obstetric & Gynecology, King George's Medical University, Lucknow, UP, India, [3]Department of Urology, King George's Medical University, Lucknow, UP, India

SYNOPSIS: Quantitative analysis of seminal plasma (n=175) profile was carried out using ^{1}H NMR spectroscopy and clinical symptoms were also observed in same samples with standard laboratory method. Multivariate discriminant function analysis (DFA) was carried out for the NMR observed metabolites and clinical symptoms data of the infertile and control cases, to find out important signature descriptors for classification. A new "INFERTIX" classification model was developed and proposed which is based on the results obtained from DFA for the different classes of infertile patients, with very high sensitivity and specificity values.

INTRODUCTION: Male Infertility is a global health issue. The assessment of male infertility is traditionally based on sperm morphology, motility and concentration of seminal plasma (SP) constituents, even though these are inefficient to serve the purpose of complete differential diagnosis of infertility[1-3]. Therefore, there is a need to develop signature biomarkers which offers, a rapid, noninvasive, sensitive, precise and unambiguous prognostic method for the differential diagnosis of infertility. The NMR technique has been used to differentiate between spermatogenic failure and obstructive azoospermia as well as different forms of spermatogenic failure on the basis of GPC/choline ratio[4]. However, these ratios do not provide a complete picture of metabolic profile of infertility. The potential advantages of NMR spectroscopy and the persistent problem regarding the accurate prediction of subgroup classification of infertility have pushed us to explore ^{1}H NMR spectroscopy as a diagnostic utility based on the analysis of SP from 175 cases to bring out a combined approach involving the absolute quantitative NMR metabolite information and the routine clinical symptoms in the form of a new classification model.

MATERIALS AND METHODS: The SP samples were collected, from subjects (22-45 years of age) comprising control group (CZ, n=30), normozoospermic (NZ, n=55) oligozoospermic (OZ, n=30), asthenozoospermic (ASZ, n=30), and azoospermic (AZ, n=30) patients, following WHO guidelines. The snap-frozen seminal plasma samples were thawed and 500 µL of each sample was taken separately in 5mm NMR tube containing 25µL of 0.375% sodium salt of tri-methylsilylpropionic acid (TSP) in D_2O for field-frequency-lock and quantitative measurements of metabolites. One-dimensional ^{1}H NMR experiments were performed on a Bruker Avance 400 MHz spectrometer at 22^o C by suppression of the water resonance by pre-saturation. Acquisition parameters used were: spectral width: 8000 Hz; time domain points: 32K; relaxation delay: 3s; pulse angle: 45^o, number of scans: 128; spectrum size: 32K and spectra were processed with line broadening: 0.3 Hz. Quantification of metabolites in all 175 SP samples were determined with in-house custom program through the area of reference signal from TSP. The clinical descriptors *viz*. sperm count, motility, lipid persoxidation (LPO) and total protein were also evaluated with standard laboratory methods in same samples. The statistical significance for the NMR derived quantified metabolites (n=12) and clinical variables (n=4) were determined by univariate analysis (one-way ANOVA) followed by a *post hoc* Student-Newman-Keuls multiple comparisons test, where a *p*-value of less than 0.05 indicated statistical significance in all. The data were subjected for multivariate discriminant function analysis (DFA) with a stepwise-forward variable selection procedure in order to define important variables for differentiation of infertile group of patients from controls, followed by discrimination of four types of infertility based on the discriminant function based Z-cut-off values. On the basis of DFA we have constructed a classification model "INFERTIX" comprising four separate sets of phases; (1) CZ vs NZ + OZ + ASZ + AZ, (2) AZ vs NZ + OZ + ASZ, (3) NZ vs OZ + ASZ and (4) OZ vs ASZ.

RESULTS: The quantification of unequivocally present metabolites *viz*. lactate, alanine, citrate, choline, GPC, glycine, tyrosine, histidine, phenylalanine and uridine in all samples were estimated from their respective resonances (Fig.1). Subjecting the data on DFA involving the control (CZ) and disease groups (NZ + OZ + ASZ + AZ), a combination of the NMR-derived variables (lactate, GPC, uridine) and the clinical symptom variables (sperm count, motility, LPO and protein) could classify overall 100% cases into the respective groups (*F* ratio: 3.24, *p*-value: <0.0001). When AZ and NZ + OZ + ASZ cases were subjected to DFA, NMR derived metabolites (lactate, citrate, choline, glycine) and the clinical symptom variables (sperm motility, LPO, protein) could classify overall 100% cases into the respective groups (*F* ratio: 11.79, *p*-value: <0.0001). Subjecting the NZ and OZ + ASZ cases for DFA with clinical variables (sperm motility, LPO) and NMR derived metabolites (histidine, phenylalanine), overall 100% cases could be classified into the respective groups (*F* ratio: 2.22, *p*-value: <0.038). Eventually, when OZ and ASZ cases were subjected to DFA, NMR derived metabolites (only citrate, tyrosine) and clinical symptom (sperm count, motility, LPO) could classify overall 100% cases into the respective groups (*F* ratio: 3.11, *p*-value: <0.005). The prediction possibility of this DFA classification model was further checked with a Jack-Knife (leave-one-out) method of Lachendruch; which correctly identified 98, 100, 100, 95.2 and 95.2% of CZ, AZ, NZ, OZ, and ASZ cases, respectively, thereby revealing an overall improvement in the differential diagnosis of infertility when important clinical and NMR descriptors where combined together and taken for the analysis.

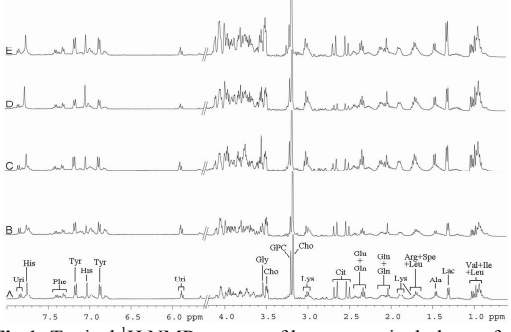

Fig.1: Typical ^{1}H NMR spectra of human seminal plasma from different groups viz: **A:** CZ, **B:** NZ, **C:** OZ, **D:** ASZ, **E:** AZ

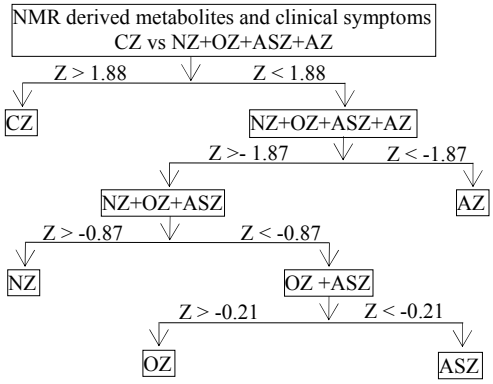

Fig. 2: "INFERTIX" classification model for the different classes of infertility based on Z score cut off values obtained through DFA.

DISCUSSION: This study has involved a combination of clinical symptoms with NMR observed metabolite concentrations as a 'method of choice' for a quick differential diagnosis of male infertility. The "INFERTIX" classification model (Fig. 2) based on DFA analysis defined that sperm count, motility, LPO, protein (clinical variable), and lactate, GPC and uridine (NMR derived metabolites) were necessary to separate out the disease cases from the control group with 100% sensitivity and specificity. The citrate, glycine, lactate, and choline (NMR derived variables), sperm motility, LPO, and protein (clinical variables) were the vital descriptors for the segregation of AZ from the NZ + OZ + ASZ case, unambiguously. Moreover, the phenylalanine, histidine, sperm motility and LPO are the important variables for segregation of NZ cases to remaining OZ + ASZ cases, explicitly. Eventually, citrate, tyrosine, sperm count, motility and LPO were the key descriptors for classification of OZ to ASZ cases, overtly. This classification model will be helpful for better treatment of male infertility.

REFERENCES: (1) N Engl J Med 2001; 345: 1388-93 (2) Hum Reprod 2001; 16:1165–71 (3) Fertil Steril 2006; 86: S202-209. (4) Hum Reprod 1998; 13:132-35.