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Original Research Article

Biochemical Variations and FTIR-ATR Spectral Studies on Renal Disease Induced By Gentamicin in Male Wistar Rats

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ABSTRACT

Renal Disease (RD) is a major health burden, which has received increased attention in recent times and has thus become one major focus of intensive research. Commonly used animal models for RD are bedeviled by methodologically induced complexities, which make the procedures not only laborious but also make interpretation of results less explicit. We have therefore characterized a simple and reproducible method for inducing renal failure in rats; in which the pathological parameters better reflect the usual findings in clinical situations. This approach has methodological and experimental advantages with respect to commonly used procedures for inducing RD and studying biochemical variations experimental rats. FTIR-ATR spectroscopy has significant advantages composed to many other methods for the characterization of biochemical molecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds such as carbohydrates, ester, albumin, proteins, as well as inter atom chemical bonds. This study attempts to evaluate the spectral difference between healthy and rat induced renal disease To achieve this male wistar rat weighing 180 $\pm$15 gms were chosen for control and the experimental rats followed by injection of gentamicin subcutaneously for 21 days as per standardized protocol. The renal failure was assessed by elevated blood urea and serum creatinine and results showed significant biochemical variations in blood serum. At the end, control and experimental animals' blood serum were analysed for FTIR-ATR spectral evaluation and quantification of biomolecules. The simplicity and reproducibility of this model, coupled with a better correlation with the known features of RD makes it a useful rat model not only for research purposes but also for testing of therapeutic maneuvers commonly used in the clinical Diagnosis. The significance of FTIR -ATR spectroscopy in analyzing biochemical variations in blood serum in experimental animals discussed statistically.

Key words: FTIR-ATR, Gentamicin Renal Disease, Spectral Analysis.

INTRODUCTION

The kidney is vital organ in health and disease. Many environmental contaminants and chemical variables including drugs alter the functions of the kidney. As the increasing prevalence of kidney diseases has been observed in the developed countries of the world, the new methodology for detecting kidney disease in its early stages as well as its severity is becoming an important issue concerned by nephrologists. Current protocols used widely in clinics are usually evaluating renal functions tests like urea, creatinine, uric

acid, total proteins, albumin, sodium, potassium, complete blood count etc., are the most common indicator of renal function. The glomerular filtration rate estimation for diagnosing renal function typically measures the variance of the many molecules involved with the system disorder, which are usually associated with renal function. Several molecular studies also contributed to the finding of potential biomarkers related to kidney disease. A majority of patients with renal disease suffer from hypertension, irrespective of the cause of their renal disease. In these groups of patients, uncontrolled hypertension, as a result of increased cardiovascular risk factors, contributes to the high morbidity and mortality associated with the disease. Schmidtand Baylis. [1]

Diagnostic tests are an important part of medical care and tests performed on samples taken on and from the body, and used in a broad range of applications. Test results can be used to aid the patient, physician, and caregiver in reaching decisions. The increasing interest in extending metabolomics applications has coincided with a concomitant interest in pursuing alternative measurement technologies as complementary options to NMR, mass spectrometry (MS), liquid chromatography has attracted increasing attention. [2,3] The current studies interested in the application of Fourier- transform infrared spectroscopy (FT-IR) in metabolomics. Moreover the technique is low cost with easy handling without skilled persons and reagents and results obtained could extend the at the level of different bond stretching in bio molecules. The ATR- FTIR spectroscopy is based on the phenomenon known as Total Internal Reflection (TIR). [4] This radiation strikes the interface between the IRE and the serum and tissue sample composed of a lower refractive index. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the serum sample held in contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns (0.5µ-5µ) beyond the crystal surface and into the sample. The depth of penetration of infrared radiation from denser IRE into the test material depends on refractive indices of the materials to be investigated and the wave number of the infrared radiation. As the sample absorbs IR radiation at certain frequencies, the resultant totally reflected radiation (or) evanescent wave will be attenuated (altered) in regions of the infrared spectrum where the sample absorbs energy. This attenuated IR radiation of evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and it is detected by the detector in IR spectrometer and system generates an infrared spectrum.

Recent literature reveals the use of FT-IR in biomedical research include the analyses of body fluids from diabetes [5] and arthritis patients [6] jaundice [7] thyroid [8] brain material infected with transmissible spongiform encephalopathies [9] and follicular fluid for investigating oocyte development. [10] Reports on applications to toxicology remain few but include evaluations of the effects of toxic agents such as carrageen an [11] and carbon tetrachloride [12] on internal organs. FT-IR analyses of urine have focused primarily on multianalyte measurements of specific urinary components [13] although there is one report on the use of FT-IR-based urine analysis to distinguish normal from rejecting renal allograft. [14] We report the suitability of FT-IR as a technology that could be employed in investigations in renal diseases and on its contribution to characterizing a new model for idiosyncratic susceptibility. Gentamicin is an effective widely used antibiotic and long term use leads to risk of renal complications. Gentamicin is known to generate reactive oxygen species associated with an increase in lipid peroxidation and decrease in antioxidant enzyme activity in the kidney additionally; it acts as an iron

chelator by forming an iron-gentamicin complex that is a potent catalyst of radical generation. [15]

FTIR spectroscopic imaging has significant advantages composed to many other imaging methods for the characterization of bio molecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds such as carbohydrates, ester, albumin, proteins, as well as inter atom chemical bonds. Current study involves in ATR-FTIR spectroscopic method where the spectrum obtained due different bond stretching and functional groups exist in serum might be the additional information to support the earlier methods. In the present study we artificially induced renal disease in otherwise healthy and metabolically stable rats in order to elucidate the existing controversies related to effects of experimentally induced renal in rats.

Apart from general metabolic disturbance, impairment of kidney causes serious problems that may affect patient's daily functioning and result in additional stress and depression. Therefore, the research on the renal disease has not only significant medical but also social implication.

MATERIALS AND METHODS

Experimental design and Induction of Renal Disease

Rats were housed in the animal house of Research and Development, Saveetha Medical College and Hospital, Thandalam Chennai, India. All experiments were carried out according to the guidelines for care and use of experimental animals, and are approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Study proposal was approved by the Institutional Animal Ethical Committee. Six rats per cage were housed in polypropylene cages (32.5 ×21×14) cm lined with raw husk which was renewed every 48 hours. The animal house was maintained at an average temperature (24.00C± 20C) and 30-70% RH, with 12hr. light-dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). These experimental rats received humane care and were fed with commercial pellet diet and the animals were acclimatized for one week before the start of the experiment. The rats were divided into two groups: animals from the first group were made renal failure by injecting Gentamicin (Ranbaxy) sub cutaneously (100mg/kg body weight) for three weeks following standard procedure [16] and animals in the other group were untreated control. On day 22 of the experiment animals were weighed and killed by decapitation.

Sample preparations for Biochemical studies

At the end of experiment (21 days) the rats were fasted overnight. Blood samples of the rats were withdrawn on from the heart, under mild anaesthesia before killing and collected in plain and EDTA tubes for further analysis. Plasma and serum were separated by centrifugation was obtained by centrifugation at 3000 rpm for 15 minutes. The blood and serum properly stored for hematology and biochemical studies. The biochemical analyzer (C311, Roche Co., Germany) was employed for this purpose using the specified analysis kits supplied from analyzer's manufacturer in S.S. Diagnostic Centre a reputed clinical laboratory in Chennai, India.

FTIR-ATR Spectral Measurements

FTIR-ATR Spectral Analysis the serum samples were properly preserved in ice bags and immediately transported to the wet lab for spectral studies. FTIR-ATR spectral measurements of serum samples and lyophilized tissues of different organs of rat induced hypothyroidism were carried out at Sophisticated Analytical Instrumentation facility (SAIF-SPU), St. Peter's University, Avadi, and Chennai-600 054, using PerkinElmer Spectrum-Two FTIR Spectrophotometer with attenuated Total Reflectance accessory having highly reliable and single bounce diamond as its

Internal Reflectance Element (IRE). Experimental serum samples were analyzed immediately for spectral recordings in the MidIR region of 4000-450 cm^{-1}. As water is a good absorbent of infrared radiation, it affects the actual spectral response of the test material and dominated in the FTIR spectrum of serum sample. Serum sample was placed on the IRE crystal and the water content on the serum sample is removed by air drier. FTIR spectral measurements were carried at room temperature and each measurement was repeated to ensure the reproducibility of the spectra. These spectra were subtracted against the background of air spectrum. After every scan, the crystal is cleaned with isopropyl alcohol or methanol soaked tissue and a background of new reference air was taken to ensure the crystal cleanliness.

Statistical analysis

All statistical analysis was performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows. The data were not normally distributed.

And therefore Non - parametric tests were performed. Descriptive statistics were presented as numbers and percentages. The data were expressed as Mean and SD.

A one way analysis of variance (ANOVA) /Kruskal-Wallis test with a post hoc Tukey HSD was used. Independent sample student t test / Mann-Whitney test were used to compare continuous variables between two groups. A two sided p value <0.05 was considered statistically significant.

RESULTS

Biochemical Analysis

At the end of experiments, blood samples analysed for biochemical and hematological parameters. The renal markers assayed include like creatinine, urea, uric acid, calcium, phosphorous, total protein, albumin etc., to assess the renal failure status of the animals. The other biochemical parameters such as cholesterol, Triglycerides, HDL cholesterol, T3, T4, TSH, etc., were also analysed for clinical correlations. Table 1 shows Various blood chemical parameters in blood and these involved the enzymes employed for evaluation of the organ functions. Subcutaneous Injection of Gentamicin on rats with body weight of 230.4 ± 2.3 g led to a rapid progression of renal failure resulting in CRF. A significant increase in urea and creatinine, uric acid, total protein on rats developed a renal failure (Table 1). A significant elevation in total protein, albumin, uric acid etc., was observed. There was no significant difference in the other biochemical parameters of controls compared to the renal rats.

Table 1: Changes in biochemical composition levels in Blood serum before and after Gentamicin injection in wistar rat

Biochemical Composition in blood serum	Control	Gentamicin injected	Statistics
Urea (mgs/dl)	40±7.10	53±7.20*	P<0.05
Creatinine (mgs/dl)	0.88±0.4	2.56±0.5*	P<0.05
Total Protein (gm/dl)	7.1±2.89	8.6±2.96*	P<0.05
Albumin(gm/dl)	4.3±1.11	5.9±1.56	P<0.05
Globulin (gm/dl)	2.8±0.77	4.1±0.98	P<0.05
Plasma Glucose mg /dl	112±5.42	139±4.48*	P<0.05
Uric acid (mg /dl)	4.8 ±1.12	6.9±1.33	P<0.05
Calcium(mg /dl)	7.9±2.43	9.0±1.72	P<0.05
Total Cholesterol(mg /dl)	167±31.10	179±3.82	NS
Triglyceride (mg /dl)	120±30.89	134±4.07	NS
HDL Cholesterol (mg /dl)	47±9.80	41±4.09	NS
T3(ng/dl)	161±15.87	178+8.19	NS
T4(µ/dl)	5.9±1.20	5.7+1.09	NS
TSH (mIU/dl)	4.8±1.33	5.2±3.11	NS

FTIR-ATR band assignment for the control and renal failure caused by gentamicin Induction

A vibration band assignment is done with the idea of the group frequencies of the various analytes present in the sample. The spectral pattern for control rat and renal disease induced by gentamicin given in figure1

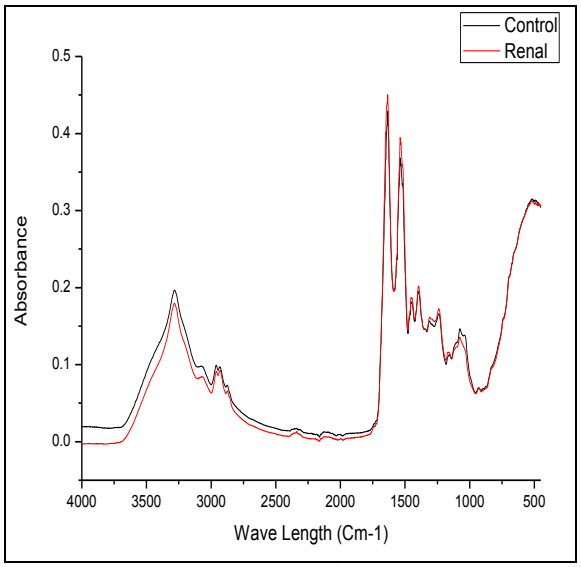

Fig.1 FTIR-ATR Spectral pattern of Wistar male rat (control and renal disease induced by gentamicin)

The prominent absorption peak 3283 cm-¹ is due to the N-H stretching mode (amide A) of proteins. The spectral region 3072 cm-¹ comprises of C-H and O-H stretch of lipids of unsaturated fatty acids and N-H stretching vibrations of the Amide B band due to overtone of amide I band. The symmetric /asymmetric stretching vibrations of methyl group of protein and C-H lipids (fatty acids and triglycerides) are fond to be present around 2930-2875 cm-¹.The absorption peaks at 1743 cm-¹. Corresponds to C=O group of cholesterol ester (HDL). The strong absorption band at 1634 cm^1 corresponds to aryl substituted C=C amide I band mainly due to C=O ,C=N and N-H stretch, where as the vibration at 1538 cm-¹ is attributed as amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein. The absorption peaks in the region (1400-1300) cm-¹ arise due to the C-H deformation of methyl and methylene group of the proteins, lipids. The asymmetric and symmetric P-O stretching vibrations are found to be around 1245 cm-¹ and symmetric P-O stretching of nucleic acid vibrations and ring vibration mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric cholesterol ester, Phosphoric acid are found to be around 1245 cm-¹ and 1165 cm-¹ respectively. The spectral region 1115-1040 predominantly occupied by C-O characterization and stretching of glucose and glycogen .The ribose and Phospholipids and polysulfidic S-S stretch in cystic acid vibrations are found to be at 934 cm-¹ and 517 cm-¹ respectively (Table2).

Table 2

Sr. No	Wave Number (cm^{-1})	Vibration Band assignment
1	3283	N-H stretch due to protein and Urea
2	3072	Amide B band due to overtone of Amide I band and olefinic group C-H stretch Lipids of Unsaturated fatty acid
3	2961	C-O-C Asymmetric / Symmetric stretch vibrations of Methyl group of Protein and C-H Lipids (Fatty acids and TGL)
4	2932	Asymmetric stretching vibrations of Methylene group of protein and lipids
5	2879	Symmetric stretching vibrations of Methylene group of protein and lipids
6	1743	C=O group of cholesterol ester (HDL)
7	1634	Aryl substituted C=C Amide I band mainly due to C=O ,C=N and N-H stretching
8	1538	Amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein.
9	1453	Asymmetric bending Vibrations of lipids, proteins of CH3 groups.
10	1394	Free Amino Acid and Fatty Acids
11	1313	Amide III erythrocyte
12	1238	Amide III and Asymmetric PO2 stretching vibration mode of Nucleic acid
13	1165	Ring vibration mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric Cholesterol ester, Phosphoric acid
14	1115	Stretching vibration of glycogen
15	1075	C-O chacterization stretching of glucose
16	1040	Primary alcohol C-O stretch glucose-Muco Poly saccharide
17	934	Ribose , Phospholipids
18	517	Poly sulfidic S-S stretch in cystic acid

Internal standard parameter ratio

These spectra were used in Internal ratio Parameter calculation and analysis requires spectra with change in sensitive peaks and no change in sensitive peaks for control and experimental. The internal ratio parameter of protein, lipid and glycogen of control and hypothyroid experimental animals given in the Table 3 Internal ratio parameter is calculated to fortify the results obtained from the FTIR intensity of absorptions. Internal ratio Parameter ignores the difference in the amount of sample analyzed; it nullifies the contradiction in the quantity of the sample ($I_{1634}/_{1075}$ $I_{1538}/_{934,}$ $I_{1453}/_{1040}$ $I_{1312}/_{516}$ and $I_{1239}/_{1115}$). The results show that peak ratio for Amide I / Glucose as well as Amide II /Phospholipids are significantly increased. The small changes in the absorption is also appreciable in the FTIR-ATR spectra as it depends on the short existing, effective evanescent wave with 0.5 µ-5 µ depth of penetrations. The absorption ratio of symmetric the ratio of (VibLipoprotein-CH3group) asym / Mucopoly saccharide, Amide III Erythrocyte/Cystic Acid and Amide II of Po2/ Glycogen renal and control rat are not significant

Table3: Internal Standard ratio Parameters calculation for bio molecules between control and Hypothyroid blood serum of male wistar rat

Peak ratio	Wave Number (cm^{-1})	Absorbance	
		Control	Renal
Amide I / Glucose	$I_{1634}/_{1075}$	2.9242	3.3190
AmideII /Phospholipids	$I_{1538}/_{934}$	5.2902	5.7314
(Vib.Lipoprotein –CH3 group)$_{asym}$ /Mucopoly saccharide	$I_{1453}/_{1040}$	1.3157	1.5522
Amide III Erythrocyte/Cystic Acid	$I_{1312}/_{516}$	0.4974	0.5102
Amide II of Po2/ Glycogen	$I_{1239}/_{1115}$	1.3227	1.4275

DISCUSSION

In the present study there was significant ($P<0.05$) increase in the blood levels of urea, creatinine, uric acid, total protein, albumin, calcium and phosphorous are indicative of the functional efficiency of kidney. The levels of these enzymes are very sensitive to any disease conditions of such organs. [17] Blood level of creatinine and urea are of special significance to evaluate renal function [18,19] which support this study. Creatinine is considered as one of the most reliable indicators of the efficiency of renal function. [20,21] Increased blood creatinine is strongly related with renal damage [22] and observations recorded in this study associated with distinct renal structural damage in the earlier studies. [23,24] Further Nephrotoxicity demonstrated in the present case, is also associated with increased urea in blood. [25-27]

The spectral region 1234-934 cm^{-1} is predominantly occupied by C-O-C asymmetric and symmetric vibrations of phospholipids of proteins and carbohydrates (glycogen and glucose) and similar findings was noticed in earlier study where the author was described about the C-O-C asymmetric and symmetric vibrations of phospholipids of proteins rather than carbohydrates. The current study concluded a systematic approach has been made using FTIR spectroscopic technique to study the spectral difference between healthy and renal failure blood samples and the spectral results are well supported by the clinical values of various proteins in plasma on the basis of their most characteristic IR absorption peaks. [28] For albumin, the best correlation with results obtained by a comparison method was found using the N-H absorption region (1600-1480) cm^{-1} common to all plasma proteins and concluded that the FT-IR spectrometry is a useful tool for determining concentrations of multiple bio molecules in micro samples of plasma.

Nucleoprotein (Symmetric Methylene group of protein and lipids) to asymmetric nucleoprotein (Asymmetric Methylene group of protein and lipids) among control and male wistar rat induced thyroid found to be slightly high. Further the

internal peak absorption ratio of asymmetric lipoprotein (Asymmetric methylene group of protein and lipids) to amino acid and Fatty acid (Free amino and fatty acid) and Amide II (NH vibrational stretch coupled with C-N stretch vibrations in protein) to Amide III were significantly elevated in hypothyroid male wistar rat than in control. The trends observed on absorptions of internal peak male wistar ratio of experimental animal was more than control which support earlier studies on different diseases like thyroid [8] atherosclerosis, [29] cancer [30,31,30] and Hepatitis. [31] The results of FTIR-ATR spectral analysis of variations in bio molecules is resembles to that of quantification of bio molecules in this study. Further FTIR-ATR analysis might be the additional tool in qualitative as quantitative analysis of bio molecules and standardization could be established.

CONCLUSION

The finding of this study indicate that exposure to gentamicin is capable of inducing adverse significant blood chemical changes and marked renal alterations in male wistar rats. The biochemical variations observed in FTIR-ATR spectral analysis is additionally supporting routine methods. The present result may contribute to better understanding of the gentamicin induced nephrotoxicity in male wistar rat. FTIR-ATR may be considered as an additional tool in evaluating the biochemical variation in the experimental animal sample and which is the base of the relevant human studies.

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