SOCIETY FOR SCIENCE AND EDUCATION UNITED KINGDOM

JBEMi JOURNAL OF BIOMEDICAL ENGINEERING AND MEDICAL IMAGING VOLUME 1, ISSUE 1

Nuclear Magnetic Resonance Spectroscopy Studies Of Human Immunoglobulin 'G' In Duchene Muscular Dystrophy

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ABSTRACT

In the present paper, we have studied human Immunoglobulin G (IgG) in Duchene muscular dystrophy using nuclear magnetic resonance spectroscopy. A comparison with normal controls is also made. Some of the groups like phenylalanine (-CH) , cysteine , serine are not found in DMD patients . These groups are found in healthy controls. A group Leuicine (-CH) was appeared in cases only . NMR spectroscopy is a powerful tool to detect the chemical groups of amino acids in serum.

Keywords : Chemical shift; Gyromagnetic ratio , Nuclear Magnetic Resonance; Immunoglobulin G ; Duchene muscular dystrophy .

1. INTRODUCTION

In the present work we intend to report our studies on macromolecule involved in Duchene muscular dystrophy using nuclear magnetic resonance (NMR) technique. Our main aim is throw light on the crucial mechanisms, which are responsible for transition from normal state to dystrophic state. Nuclear magnetic resonance (NMR) technique of spectroscopy branch is a next step of X-ray crystallographic studies. This method can provide high resolution structure of biological molecules. These macro molecules are proteins and nucleic acids and their complexes at atomic resolution. When a molecule is placed in a magnetic field its electrons are made to circulate and while circulating they generate secondary magnetic field. Circulation of electrons about the proton itself generate a field aligned in such a way that act at the proton it opposes the applied field. The field experienced by the proton is diminished and the proton is said to be shielded. Circulation of electrons about nearby nuclei generates a field that can either oppose or reinforce the applied field at the proton depending on the proton location. If the induced field opposes the applied field, the proton is shielded. If the induced field reinforces the applied field, then the field felt by the proton is de-shielded. Compared with a naked proton, a shielded proton requires a higher applied field strength to provide the particular effective field strength at which absorption occurs.

DOI: 10.14738/jbemi.11.99
Publication Date:15th February 2014
URL: http://dx.doi.org/10.14738/jbemi.11.99

Shielding thus shifts the absorption up field and de shielding shifts the absorption downfield. Such shifts in the NMR absorption, arising from shielding and de shielding by electrons are commonly called chemical shift ((δ)) and are measured in parts per million (ppm). The ^{1}H atom is the atom present in proteins that can be observed by NMR. Low natural abundance of ^{13}C atom can be used to some extent, and ^{13}C and ^{15}N atoms can be incorporate into the protein during biosynthesis. All the ^{1}H atoms of a protein can be observed, except those labile hydrogen atoms of –NH–, –NH2, –OH, and –SH– groups that are exchanging with hydrogen atoms in the aqueous solvent at rapid rates.

An NMR spectrum is a graph of the intensity of absorption (or emission) between frequencies. The spectrum of NMR generally appears in the low range of frequency, i.e., 10 to 800 MHz. Positions of the lines can be measured in ppm. This shift scale tells about the frequencies. It has given that frequency of NMR line is directly proportional to the magnetic field strength [1]. NMR spectroscopy is a powerful tool for both qualitative and quantitative analysis of organic compounds. There is a little use in clinical laboratory. The cost and complexity of the instrumentation is that NMR has not had sufficient quantitative sensitivity for most compounds of clinical interest. NMR spectra provide so many informations related to clinical aspects of the human system. The position of peaks determined by chemical shifts and spins – spin coupling constants, are characteristic of particular compounds and useful for quantitative analysis. The peak areas under some appropriate conditions are proportional to the number of resonating nuclei. This provides accurate quantitative analysis. Relaxation times and peak widths give informations about molecular dynamics and chemical kinetics .A single spectrum discloses the presence of any detectable compound. This technique may be used as a screening technique in selected situations NMR may also be used to screen for ingested toxins at high concentrations If a patient is alcoholic serum of the patient has high osmolality. We can eliminate problems of extractions recovers, contamination, or other artifacts by performing measurements directly on human serum.

2. BASIC THEORY OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The nuclear magnetic moment is a quantum mechanical feature of a nucleus. NMR is based on the following principle. If a nucleus is well placed in a magnetic field which is static in nature, the nuclear spin will start to process around this applied field. This happens due to the fact that magnetic moment ( ) of the nucleus is related to the nuclear spin by the following relation.

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that magnetic moment ($\vec{\mu}$) of the nucleus is related to the nuclear spin $\vec{I}$ by the following relation.

$$\vec{\mu} = \gamma\hbar\vec{I} \tag{1}$$

We may see a precession called Larmor precession in the Figure 1.

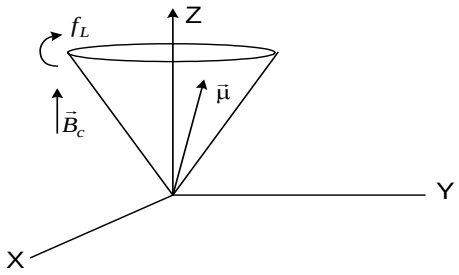

Figure 1 : Larmor precession

γ is called gyromagnetic ratio of the nucleus.

The frequency of this precession is called Larmor frequency and is given by the following relation

$$f_L = \frac{\nu}{2\pi} B_0 \tag{2}$$

B_0 is magnitude of the magnetic field

This field is aligned with Z- axis and f_L is termed as Larmor frequency.

Each isotope has a well-known gyromagnetic ratio. $\gamma = 2\pi \times 42.58$ MHz /T (for protons)

Some informations have supplied regarding adiabatic change in magnetic field by [2] . If the change of direction of the magnetic field is sufficiently very slow, the axis of the precession cone of the magnetization vector follows the direction of the field. The angle of the cone will be unchanged.

If we consider the momentary rate of change of $\vec{B}$ as

$$\frac{d}{dt}\vec{B} = \vec{\Omega} \cap \vec{B} + \Omega_1 \times B \tag{3}$$

The rate of change of $\vec{B}$ is expressed as the sum of the two components, perpendicular and parallel to $\vec{B}$. The quantities Ω and Ω_1 are dimensions of S^{-1} can be interpreted as Ω determines the angular velocity with which $\vec{B}$ changes its direction and Ω_1 determines the speed of change of $\vec{B}$. If the coordinate system is Cartesian in which z-axis follows $\vec{B}$ and maintains the direction. The motion of the magnetization in the rotating frame of reference is given below

$$\frac{d}{dt}M = \gamma M \times \left(B + \frac{\Omega}{\gamma}\right) \tag{4}$$

If we have no quadrupole interaction, the motion of the quantum mechanical spin moment $\vec{\mu}$ can be explained classically. We may add all these spin moment of an given ensemble of nuclei add up to a magnetization vector which is given here as

$$\vec{M} = \sum_k \vec{\mu}_k \tag{5}$$

$\vec{M}$ is called macroscopic magnetization. We can manipulate this magnetization vector in NMR experiment. Bloch [3] was the first scientist who described the equation of motion of this magnetization vector. Because it is composed of magnetic moment, it will experience a torque $\vec{M} \times \vec{B}$ on putting in a magnetic field $\vec{B}$.On the basis of this act we may conclude that as a result of this any magnetization which is de aligned with the magnetic field will presses around this field. The change in magnetization together with an extra relaxation contribution gives a Bloch equations which is given below

$$\frac{dM(t)}{dt} = \vec{M}(t) \times \vec{B}(t) - R\left(\vec{M}(t) - \vec{M}(0)\right) \tag{6}$$

Here R is tensor represents the relaxation mechanism.

We may write Bloch equation in three Cartesian coordinate along all the three coordinate axes respectively.

$$\frac{dM_z(t)}{dt} = \gamma\left[M_x(t)B_y(t) - M_y(t)B_x(t)\right] - R_1\left[M_z(t) - M_0\right], \tag{7}$$

$$\frac{dM_x(t)}{dt} = \gamma\left[M_y(t)B_z(t) - M_z(t)B_y(t)\right] - R_2\left[M_x(t)\right], \tag{8}$$

$$\frac{dM_y(t)}{dt} = \gamma\left[M_z(t)B_x(t) - M_x(t)B_z(t)\right] - R_2\left[M_y(t)\right] \tag{9}$$

If we are dealing with pulsed NMR spectroscopy under the rotating frame for short pulse duration $\tau < 1/R_1$ or $1/R_2$ and if it is time independent B_1 and φ then Bloch equations became.

$$\frac{dM_z(t)}{dt} = \gamma\left[M_x(t)B_y^{\,r} - M_y(t)B_x^{\,r}\right] \tag{10}$$

$$\frac{dM_x(t)}{dt} = -\Omega M_y(t) - \gamma M_z(t)B_y \tag{11}$$

$$\frac{dM_y(t)}{dt} = \Omega M_x(t) + \gamma M_z(t)B_x^{\,r} \tag{12}$$

These equations can be written in matrix form

$$\frac{dM(t)}{dt}=\frac{d}{dt}\begin{bmatrix}M_x(t)\\M_y(t)\\M_z(t)\end{bmatrix}=\begin{bmatrix}0 & -\Omega & -\gamma B_y^{\,r}\\ \Omega & 0 & \gamma B_x^{\,r}\\ \gamma B_y^{\,r} & -\gamma B_x^{\,r} & 0\end{bmatrix} \tag{13}$$

and

$$M(\tau_p)=R_x(\phi)R_y(\theta)R_z(\alpha)M(0) \tag{14}$$

If we apply a radio frequency (RF) field. The total magnetic field is the sum of the static magnetic field $\vec{B}_0$ and the time varying magnetic field produced by the RF field $\vec{B}_{RF}(t)$:

$$\vec{B}(t)=\vec{B}_0+\vec{B}_{RF}(t) \tag{15}$$

If RF is relatively small then

$$\vec{B}_{RF}(t)=\begin{bmatrix}B_1\cos(\omega_{RF}t+\varphi)\\ B_1\sin(\omega_{RF}t+\varphi)\\ 0\end{bmatrix} \tag{16}$$

B_1 is the strength of the RF magnetic field and is directed perpendicular to the Z –axis, ω_{RF} is the frequency, φ is a phase offset of the field.

Bloch equation can be solved easily on the basis of this fact if we consider a coordinate frame rotating with the RF frequency, i.e., fixed to $\vec{B}_1$. We have already known about this static coordinate frame which is called laboratory frame and this rotating frame is called rotating frame of reference.

Under the influence of this rotating frame, the precession of the magnetic moment $\vec{\mu}$ of a nucleus is described by the following equation

$$\frac{\partial\vec{\mu}}{\partial t}=\gamma\vec{\mu}\times\left(\vec{B}_0+\frac{\vec{\omega}_{RF}}{\gamma}\right) \tag{17}$$

This equation (17) shows that the effective magnetic field in the rotating frame of reference along the Z-axis is given by the following relation

$$\vec{B}_{eff}=B_0+\frac{\vec{\omega}_{RF}}{\gamma} \tag{18}$$

The total magnetic field within this frame can be written as

$$\vec{B}(t)=\begin{bmatrix}B_1\cos(\varphi)\\ B_1\sin(\varphi)\\ B_0-\omega_{RF/\gamma}\end{bmatrix} \tag{19}$$

If the RF frequency ω_{RF} is exactly equal to Larmor frequency ω_L, the Z-component of this total magnetic field in the rotating coordinate frame is equal to zero due to resonance

condition given by equation (1) Now magnetization will process within the rotating frame around the direction of $\vec{B}_1$ with a frequency

$$f_1 = \frac{\gamma}{2\pi} B_1 \tag{20}$$

The magnitude of RF field is much smaller than the main magnetic field, f_1 is also the Larmor frequency. If we have applied B_0 is 1 T then f_1 will be equal to 42.58 MHz. Radio frequency (RF) is to be kept at same frequency. The amplitude of this field 1 mT. For a rotation of magnetization by a quarter of a full cycle (90^0 pulse), RF field needs to be 6 μs. We may say not only 90^0 pulse can be applied by the RF field, but more general value of angle of rotation β is given by the relation

$$\beta = 2\pi f_1 t_{RF} \tag{21}$$

This angle is called flip angle t_{RF} is the duration of the RF pulse. The phase offset φ between the rotating frame and the RF signal in equation (21) determine the axis around the rotation of $\vec{M}$ will occur. If a β pulse is giving a rotation around the x' -axis is denoted by $\beta_{x'}$ (flip angle of pulse). We shall consider here the action of a RF pulse on this magnetization as viewed in the rotating frame is presented in Fig. 2.

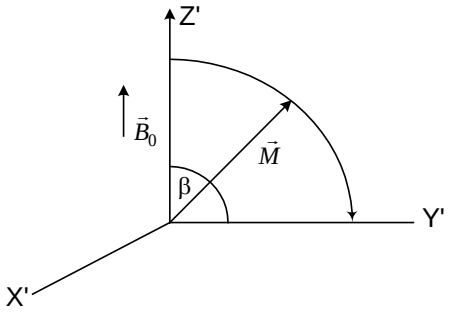

Figure 2: Rotation of magnetization with in the rotating frame of reference induced by a $\beta_{x'}$ pulse.

The magnetization, which was originally aligned with the z' axis of the rotating frame, now has changed into

$$\vec{M}(t) = M_0 \begin{bmatrix} 0 \\ \sin\beta \\ \cos\beta \end{bmatrix} \tag{22}$$

M_0 is original amplitude of this magnetization. This magnetization is stationary in the rotating coordinate frame, but in the laboratory frame, it is still processing with Larmor frequency.

Relaxation speed may be defined as linearly dependent on the difference of the actual magnetization and the equilibrium magnetization $\vec{M}(0)$. This will give exponentially decaying magnetization components.

If $\vec{B}_1$ is aligned with the z-axis, the relaxation tensor can be written as in the laboratory frame

$$R = M_0 \begin{bmatrix} 1/T_2 & 0 & 0 \\ 0 & 1/T_2 & 0 \\ 0 & 0 & 1/T_1 \end{bmatrix} \tag{23}$$

T_1 and T_2 are called longitudinal and transverse relaxation times respectively.

The physical background of the two relaxaton mechanisms is different. The longitudinal relaxation time is the spins need to exchange energy with the surroundings thermal reservoir or lattice. We may termed as spin –lattice relaxation time. The transverse relaxation time is the time in which spin dephase due to interactions with their neighbours and the presence of fast changing molecular fields. It is called spin – spin relaxation time.

Spin lattice relaxation time is calculated with the help of an equation which is given here as

$$\frac{dM_z(t)}{dt} = R_1\left[M_0 - M_z(t)\right] \tag{24}$$

Transverse relaxation time may be calculated by the help of eq. (a) and (b) of eq. (25) as

$$\frac{dM_x(t)}{dt} = R_2\left[M_x(t)\right] \tag{25a}$$

$$\frac{dM_y(t)}{dt} = R_2\left[M_y(t)\right] \tag{25b}$$

NMR spectroscopy can be used in protein dynamics and some of the calculations are shown here. The spectral density function $J(\omega)$ may be related to the relaxation rates.

The probability function of finding motions at a given angular frequency ω can be described by the special density function as

$$J(\omega) = \frac{2\tau_c}{1+(\omega\tau_c)^2} \tag{26}$$

The relaxation rates which are associated with the protein dynamics given below.

$$R_1 = 3AJ(\omega_N) + AJ(\omega_H - \omega_N) + 6AJ(\omega_H + \omega_N) + BJ(\omega_N) \tag{27}$$

$$R_2 = 2AJ(0) + \frac{3A}{2}J(\omega_N) + \frac{A}{2}J(\omega_H - \omega_N) + 3AJ(\omega_H) + 3AJ(\omega_H - \omega_N) + \frac{2B}{3}J(0) + \frac{B}{2}J(\omega_N) \tag{28}$$

$$\sigma = -AJ(\omega_H - \omega_N) + 6AJ(\omega_H + \omega_N) \tag{29}$$

Coefficient A and B are

$$A = \left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N{}^2 \gamma_H{}^2 \hbar^2}{4r^6{}_{NH}}, \tag{30}$$

$$B = \frac{\Delta\sigma^2{}_N B^2{}_0 \gamma^2{}_N}{3} \tag{31}$$

Nuclear Overhauser effect (NOE) may be calculated and it is given here

$$NOE = 1 + \frac{\sigma}{R_1}.\frac{\gamma_H}{\gamma_N} \tag{32}$$

μ_0 is the permeability of the vacuum, γ_N is gyromagnetic ratio of the ^{15}N nucleus, γ_H is gyromagnetic ratio of the ^{1}H nucleus, $\hbar$ is plank's constant , r_{NH} is $^{15}N - {}^{1}H$ inter-nuclear distance, $\Delta\sigma_N$ is chemical shift of anisotropy of the ^{15}N nucleus, B_0 is static magnetic field strength

It has been well established that the use of NMR spectroscopy in the field of biology is vast and very much fruitful in studying the structure of protein in detail. Small amount of the sample is needed for study. Due to this fact we have used NMR technique and applied it to study the structure of human immunoglobulin G (IgG). Immunoglobulins are called as globular proteins.

The proton is most widely used nucleus for applications of NMR spectroscopy in clinical investigations. The wide spread biological applications of NMR can not be covered in small detailed work. We may use NMR to detect the correct diagnosis of diseases.

The nuclear spin is associated with a magnetic moment which is required for obtaining nuclear magnetic resonance. This also defines the basic resonance frequency. It has been seen that at 14.1 Tesla there is a resonance frequency of 600 MHz for ^{1}H and 150 MHz for ^{13}C . Every spin in a given molecule produces a nuclear magnetic resonance line. The resonant frequency at exact position depends on the chemical environment of each spin. The NMR spectrum of protein shows NMR signals with slightly different frequencies. The difference in frequencies is called chemical shift. The assigning of these chemical shifts of all the atoms of the molecule is a step in determination of structure of a molecule by NMR. Experimental parameters may be measured after the assigning of NMR signals. Structural informations derived from NMR spectra are completely based on nuclear Over hauser effect (NOE). This effect is due to dipolar interactions between different nuclei. The intensity of NOE is given by the following relation

$$NOE \propto \frac{1}{(r)^6} f(\tau_c) \tag{33}$$

r is called the inter nuclear distance

$f(\tau_c)$ is called the correlation functions.

This function describes the modulation of the dipole – dipole coupling by stochastic rate process, with an effective correlation time τ_c.

NOEs may be observed between protons which are separated by less than 5 -6 Å. A new factor which is called J coupling constant are mediated through chemical bonds may provide some information about dihedral angles. These can define peptide backbone and side chain conformations. Residual dipolar coupling (RDC) and Cross correlation relaxation effect (CCR) have been shown to provide distance independent projection angles for bond vectors. These are N-H and C^{α} & H^{α} bonds in proteins. The residual dipolar couplings may be measured in anisotropic solution also.

It has been reported in the literature the 3-D structures can be obtained for proteins up to 50 kDa molecular weight. Pelton et.al. [4] have used this technique and NMR spectra can be recorded for molecules well above 100 kDa. The use and application of biomolecular NMR in structural biology are introduced. Biomolecular NMR may provide very important information in solution for structure determination . This sophisticated technique of spectroscopy can provide information about the conformational or chemical exchange, internal mobility and dynamics at time scales. The averaging of side chain conformation may be estimated and the populations of different conformations can be determined. NMR may provide information on the location of secondary structural elements within the protein sequence.

3. REVIEW OF THE LITERATURE

The application of NMR in the study of biological molecules has become an important ingredient. This technique was first applied to investigate the bulk material by Purcell [5] and Bloch et. al. [3]. Pioneering work was carried out by Jardetzky et.al . [6], Kowalsky et.al. [7] and McDonald. et.al. [8] in the field of relevant biomolecules. Proteins are the most studied by biopolymers. The protein spectra are very complex and simulation by mixing of amino acids does not produce the replica of the observed spectra in them as the intricate folding at the polypeptide chain makes major changes in both chemical shift as well as relaxation effect (line broadening).

Important structural information are revealed by NMR spectra, which are characterized by parameters like peak position, width, intensity and multiplicity of its line. One can find the different proton groups present in the molecule by the number of lines present in NMR of a particular molecule. The integrated area of a line gives the relative number of protons in each groups and also information about the environment of the molecule. The hyperfine splitting reveals the interaction between a particular nucleus and its neighbors. The line width increases with the size and rigidity of the molecule. However in a large molecule there is an overlapping of peaks which can be overcome by increasing the frequency of the spectrometer. The NMR spectra arise due to the absorption of energy during transition from low (more populous) to the higher (less populous) energy state in a nucleus. The detectable absorption is characteristics of the environment of the nucleus. With the variation of frequency the resonance will be the

function of the local molecular environment and of the magnetic field in which the nucleus find itself.

Improvement in NMR technology is one of the most recent method, applied to the diagnosis of human diseases [9-12]. Chalovich et.al. have given a statement that the nuclei common in tissues ^{1}H was used for NMR imaging of the organs of human subjects and to analysis the composition of human serum of the various applications of NMR for medical purpose, the method is most sensitive for analysis of serum. Bradbury et.al. [13] has also applied NMR in the investigation of structure of histones Leeet.al. [14] and Chapman et.al.[15] have studied molecular basis of interaction of histones with nucleic acid, analysis of flexibility gradient in phospholipids membranes and in understanding the biological importance of bound water.

Wüthrich [16] has supplied informations related to NMR studies of structure and function of biological macromolecule. NMR spectroscopy is unique among the techniques available in science for 3-dimentional structure determination of proteins and nucleic acids. NMR can be done in solution. Body fluids such as blood, stomach liquid and saliva and protein solutions where these molecules perform their physiological functions. Study of the molecular structures in solution is to much relevant and desirable. Solution conditions such as the temperature, pH and salt concentration can be adjusted so as to closely mimic a given fluid (physiological fluid). Solutions can be changed into non-physiological conditions such as protein denaturation. NMR investigates the dynamic features of the molecular structure, as well as studies of structural, thermodynamic and kinetic features of interactions between proteins other solution components. These may be other macromolecules or low molecular weight ligands.

Some of the major improvements in NMR hardware and methodology have been made by the scientists. Due to these improvements the use of NMR for the characterization of structure and dynamics of biological molecules in solution has become very important. These improvements are still into consideration and on going and are designed to overcome the main problem with NMR of biomolecules, namely signal to noise ratio and spectral overlap. Biomolecular NMR spectroscopy may give information about conformational dynamics and exchange processes of biomolecule at time scales ranging from 10^{-8} seconds (picoseconds to seconds). This technique is also effective in determining ligand binding and mapping interaction surfaces of protein /ligand complexes. Proteins are orders of magnitudes larger than the small organic molecule. NMR spectroscopy is applied to study of proteins because increased number of each element present in the molecule.

Kurosu et al. [17] have studied synthetic macromolecules with the help of NMR. NMR has been the most suitable technique to characterize and to investigate the correlation between the structure and physical properties. Prior [18] has studied NMR in living systems such as eye, tissue of heart muscle, reproductive tissue, brain, liver, bone marrow, etc. The author has reported that the linewidth of lipid and water differed significantly between groups.

Jardetzky [19] has reported the finding on determination of macronuclear structure and dynamics by using NMR. He pointed out the basic problem of interpreting spectroscopic data in structural terms from the fact that measured parameters represent motional as well as ensemble averages. If we have a non-rigid system, a unique correlation between measured spectroscopic parameters and structural parameters such as interatomic distances and coordinates does not exist. A straightforward calculation of the structure is not possible. NMR parameters are the function of distances, motions, frequencies and amplitudes. These parameters can supply the well-defined relationship regarding the interatomic distances. Hounsell [20] has studied carbohydrates, lipids and membranes with the help of NMR. There is a study on cerebrospinal fluid of different diseases and a clear cut differentiation was found in the atomic and molecular level in terms of chemical shifts. Simpson [21] has studied and reported the findings on proteins and nucleic acids. The assignment of small proteins is regular system nowadays. The specific amino acid labeling can still be of use for assisting peak identification. Consanni. et al. [22] have studied nuclear magnetic resonance and chemometrics to access geographic origin and quality of traditional food products. They have reported their view for NMR and chemometrics, which were reviewed and applied to food quality and geographical origin determination.

Adam. et al. [23] have applied carbon-13 NMR to study the proteins and glycoproteins. They have also studied globular proteins and recorded NMR spectra at 15.18 MHz. Some overlap between the aliphatic region and amino acid residues has been obtained. NMR can be used as a tool to study the bio-molecules in the field of biochemistry. Talebpouret al. [24] have applied this technique to identify and determine the caffeine and theophylline in human serum. They have reported their findings for the caffeine peaks which were obtained at 2.75, 2.93 and 3.40 ppm. Theophylline peaks were found at 2.77 and 2.97 ppm. Wishrat [25] has studied NMR technique for the determination of proteins with the development of drug discovery. It has been noticed that the first crude protein structure was determined in the year 1980. NMR can play a role in the area of protein based drug discovery. It gives the structural information along with the dynamics of the protein. Newman et.al.[26] have applied nuclear magnetic resonance spectroscopy to the forearm muscle in DMD and found that the phosphorous spectrum was abnormal in the ratios of phospho-creatine to adenosine triphosphate and to inorganic phosphorus were reduced .They have also reported that the concentration of phosphocreatine in muscles was appreciable reduced . Sharma et.al.[27] have studied skeletal muscle metabolism in Duchenne muscular dystrophy and reported that decrease in levels of glucose may be attributed to the reduction in the concentrations of gluconeogenic substances or membrane abnormalities . A decrease in the concentration of lactate in the muscle of DMD patients may be due to the reduction in anaerobic glycolytic activity or lower substrate concentration.

Matsumura et.al.[28]have studied DMD carrires with the help of magnetic resonance imaging and p ersumed that degenerative muscular changes accompanied by interstitial edema

responsible for this disease . They have given a conclusion that NMR is useful for study the dynamic state of water in normal and pathological skeletal muscles . Zochodne et.al .[29] have studied fore arm P-31 nuclear magnetic resonance spectroscopy in oculopharyngeal muscular dystrophy and found that this dystrophy is a more wide spread disorder of striated muscle than clinically appreciated . Sharma et.al.[30] have studied biochemical characterization of muscle tissue of girdle muscular dystrophy with 1H and 13C NMR spectroscopy . They have found that a significant reduction in the concentration of choline in the patients and healthy controls .Lower concentration of choline may be out come of decreased rate of membrane turn over in the patients .Barbiroli et.al [31] have studied 31P NMR spectroscopy of skeletal muscle in Becker dystrophy and DMD/BMD carriers . They have found that in the working muscle of BMD patients and female DMD/BMD carriers a defect of phosphate metabolism. It reflects a deficit of energy metabolism.

Donaldet.al.[32] have studied 31P NMR in DMD to measure high energy phosphate compounds and phosphorylated diesters in resting gastrocemius muscle . They have shown a progressive metabolism deterioration in this disease. Griffin et.al. [33] have studied metabolic profiling of genetic disorders in dystrophic tissue with the help of NMR spectroscopy . They have reported that many metabolic pathways are perturbed in dystrophic tissue . Sharma et.al [32] have made analysis on the basis of NMR spectroscopy and given a statement related to ex vivo and in vivo NMR spectral peak observations in different diseases .This can support the reliability of clinical applications using in vivo localized 1H NMR spectroscopy peaks to determine the biochemical cause of disease .Šuput et.al.[35] have used magnetic resonance imaging technique to muscular dystrophies and neuropathies . They have reported that musculature may be replaced gradually by adipose tissue. Authors disclose clear differences in the degeneration of particular groups of muscles. Cady et.al [36] have studied this spectroscopy to muscle metabolism and reported that the clinical use of NMR is very useful in the study of muscular dystrophy . They have mentioned in the article as the mean resting metabolic levels and pH determined by 31P NMR and indicates significant differences between values for PCr, Pi, PCr/Pi and PCr/ATP compared with normals. Chance et. al. [37] has applied this technique to normal and diseased muscle. They have used phosphorous magnetic resonance spectroscopy ,which affords and innovative approach to study of the oxidative enzyme content of normal and diseased muscles .Michael et.al.[38] have used Carbon13 NMR spectroscopy to study normal and abnormal muscles and reported that13C NMR of isopentane – extracted muscles have shown a clear cut differentiation of normal from diseased muscles and within diseased muscle, grading of the severity of the disease .Schreiber et.al [39] have used magnetic resonance imaging to muscular dystrophy in children .They have given a statement on the basis of scans of the five muscles groups, i.e. neck , shoulder gridle, pelvis gridle , thigh and calf . They demarcated the involved muscles. The severity of the disease estimated by the degree of muscle involvement. Kaisr et. al [40] have used 31P NMR to study normal and diseased muscle. They have pointed out that abnormal muscles showed characteristic changes in

phosphorous spectrum , when compared with normal muscles .Bottomley et.al .[41] have used proton magnetic resonance spectroscopy and given a statement that this spectroscopy can be used to image and noninvasively quantify total creatine in human muscle. Role of altered creatine metabolism in muscle disease can be studied with this spectroscopy .Garrood et.al. [42] have used NMR imaging and reported that referenced signal intensity measurement may be used to quantify difference between dystrophic and normal muscle without T(1) mapping.Ogino et.al. [43] have studied serial water changes in human skeletal muscles .

They have measured the changes in signal intensity in both calf muscles after walking race exercise .The time intensity curves were also used to draw a clearance curve for each muscle group after exercise. Srivastava. et.al.[44] have studied high resolution NMR based analysis of serum lipids in Duchenne muscular dystrophy patients and its possible diagnostic significance and reported that concentration of triglycerides, phospholipids , free cholesterol esters and and total cholesterol was significantly higher in DMD patients as compared to healthy patients . They have also given a statement that no significant quantitative difference was observed in the serum lipid constituents of positive and negative gene detection in cases of DMD. It may be useful to provide the possibility of the diagnostic importance for DMD, especially in cases where genetic analysis failed to give the correct diagnosis. Sarpel et.al.[45] have studied erythrocytes in muscular dystrophy with the help of 31P NMR and measured the inorganic –phosphate fraction contained the highest average phosphate concentration over sixteen hour period .This result contributed to the difference in the total phosphate between two groups. Bock, J.L [46] have studied serum by high field proton magnetic resonance spectroscopy and suggested that the nondestructive nature of NMR, which is the basis of its in vivo applicability .We can also use this facility in the vitro analysis. We can eliminate problems of extraction recovery, contamination, or other artifacts .Forbes .et .al. [47] have used this technique to study skeletal muscle of ambulant children with Duchenne muscular dystrophy and stated that the MR protocol implemented in the study achieved highly reproducible measures of lower extremity muscles in ambulant boys of DMD.

4. MATERIALS AND METHODS

The blood samples of DMD patients were collected from the Department of Neurology, Safdarjang Hospital, New Delhi. Twenty milliliters freshly drawn blood from each patient was collected in clean and dry test tube without any anti- coagulant. The test tube was kept for 45 minutes at room temperature ($22 \pm 2^{0}C$) for the formation of clot. Sera of different patients were separated by centrifugation at 1500 r.p.m. upto 15 minutes and were collected in screw capped test tubes. IgG sample were prepared on protein A –Sepharose [48]. The IgG binding properties of protein A, make affinity chromatography with protein A -- sepharose CL- 4B a very simple method for preparing IgG. 1.5 g protein -A sepharose CL-4B was swollen in 10 ml phosphate buffered saline (PBS) for 1 hour at room temperature and then packed into a small chromatography column. 10 ml human serum was diluted with an equal volume of PBS. The serum was filtered through the column at a flow rate of 30 ml/h. Washing was done through

unbound protein with PBS. Until no more protein left the column (the protein was monitored with a UV flow cell). The bound IgG was eluted with glycine- HCL buffer having a pH value of 2.8. The pH of the purified IgG solution was titrated to near neutrality with NaOH.and dialysed against PBS. The column was regenerated by washing with 2 column bed volume of PBS. The column was stored at 4°C. The protein A content of the swollen gel is 2 mg /ml and the binding capacity for human IgG is approximately 25 mg /ml of packed gel.As the binding of protein A to IgG involves tyrosine residues on the protein A glycosyl tyrosine (0.1M in 2 % (NaCl) can be used to elute the IgG rather than the the glysine – HCl buffer. The NMR spectra of the IgG samples extracted from normal person and Duchenne muscular dystrophic patients were recorded on Av Bruker 500 MHz NMR Spectrophotometer (Fig. 3) in central NMR facilities I.I.T. New Delhi, India.

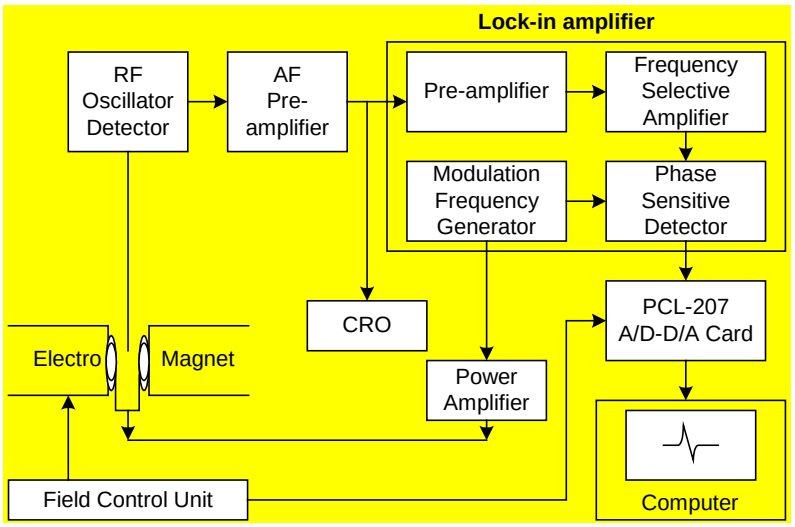

Figure 3: Block diagram of Nuclear Magnetic Resonance Spectrometer

5. RESULTS

We have applied NMR spectroscopy to IgG molecule of DMD children and findings are reported in the table form. We have compared our data with the normal healthy controls. Typical NMR spectra of normal and DMD patient are given in Fig.4 and Fig.5

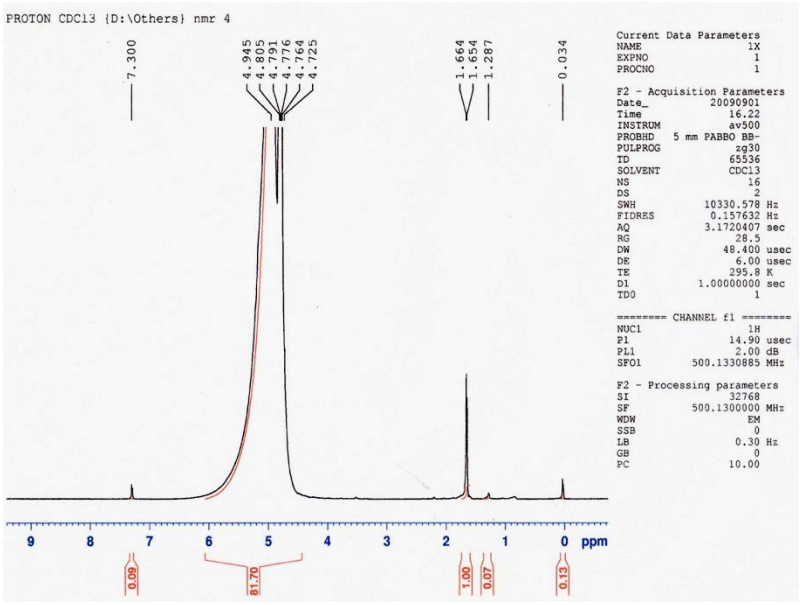

Figure 4: Typical NMR spectra of normal sample

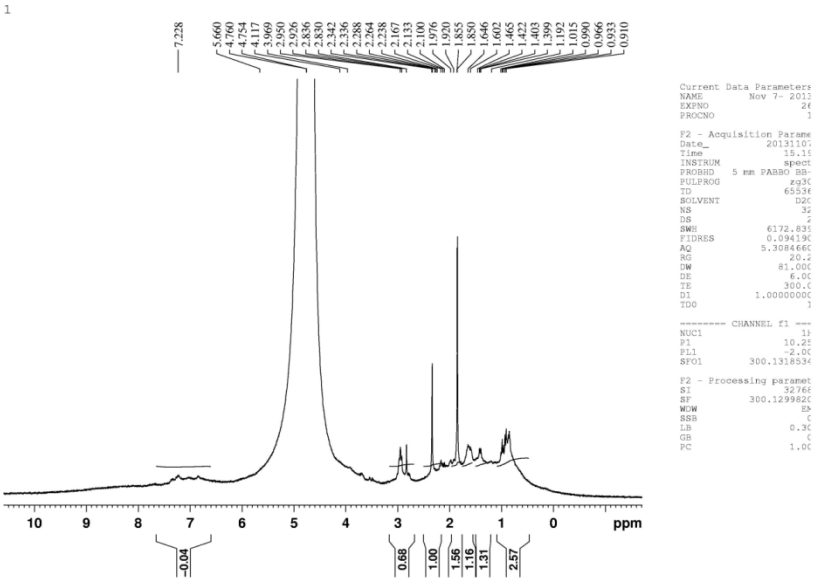

Figure 5: Typical NMR spectra of DMD sample

Table 1. Comparison between different probable groups of the amino acids of IgG with peak intensities in DMD and Controls

S. No.	Type of sample	Peak position	Chemical shift ☐ ppm	Probable group	Standard values	D_2O peak
1	N	1 2	0.87 1.34	Isoleucine, ☐H_3 Alanine, ☐H	0.89 1.36	4.73

		3 4 5 6	2.05 3.24 4.14 4.65	Glutamic acid, -CH Cystine, C Isoleucine -CH Phenylalanine -CH	2.09 3.28 4.14 4.65	
2	N	1 2 3 4 5 6	0.88 1.28 2.04 3.25 4.61 4.64	Isoleucine, -CH_3 Alanine, -CH Glutamic acid, -CH Histidine, -CH Tyrosine, -CH Phenylalanine, -CH	0.89 1.36 2.09 3.26 4.62 4.66	4.73
3	N	1 2 3 4 5 6	0.87 1.28 2.03 3.22 4.12 5.31	Isoleucine, -CH_3 Isoleucine, -CH_2 Glutamic acid, -CH Phenylalanine, -CH Valine, -CH	0.89 1.22 2.09 3.22 4.13	4.73
4	N	1 2 3 4 5 6	0.87 1.20 1.34 2.12 3.66 5.90	Isoleucine, -CH_3 Isoleucine, -CH_2 Alanine, -CH Gln, -CH Glycine, -CH	0.89 1.22 1.36 2.13 3.64	4.77
5	N	1 2 3 4 5 6	0.88 1.21 2.09 3.22 3.66 4.14	Isoleucine, -CH_3 Isoleucine, CH Glutamic acid, -CH Phenylalanine, -CH Glycine, -CH Glycine -CH	0.89 1.22 2.09 3.22 3.64 4.14	4.78
6	N	1 2 3 4 5 6	3.75 3.93 4.32 4.74 5.14 -	Serine, -CH Serine, -CH Methionine, -CH Cystine, -CH	3.79 3.95 4.32 4.74	4.70
7	N	1 2 3 4 5 6	0.87 1.22 1.39 3.24 4.33 4.62	Isoleucine-CH_3 Isoleucine-CH_2 Alanine Cystine Alanine Phenylalanine	0.89 1.22 1.39 3.28 4.35 4.66	4.70
8	N	1 2 3 4 5 6	0.87 1.22 1.39 3.24 4.33 4.62	Isoleucine Isoleucine Cystine Phenylalanine Tyrosine	0.95,0.77 1.48,1.22 3.28 4.58 4.62 ----	4.66
9		1 2	0.79 1.25	Isoleucine Isoleucine	0.89 1.48,1.22	4.67

	N	3 4 5 6	3.27 4.56 4.62 4.83	Alanine Cystine Glycine Tyrosine	1.36 3.28 4.14 4.62	
10	N	1 2 3 4 5 6	0.83 1.23 3.22 4.29 4.44 4.48	Isoleucine Isoleucine Cystine Serine Tyrocine Cystine	0.89 1.48, 1.22 3.27 4.52 4.61 4.67	4.73
12	DMD	1 2 3 4 5 6	0.68 1.00 1. 16 1.31 1.56 2.57	Isoleucine, -CH_3 Isoleucine, -CH_3 Isoleucine, -CH_2 Alanine, -CH Leucine, -CH Asp, -CH	0.89 0.95 1.19 1.36 1.54 2.62	4.75
13	DMD	1 2 3 4 5 6	0. 34 0.50 1.00 1.13 1.50 2.22	Isoleucine, -CH_3 Isoleucine, -CH_2 Leucine, -CH Proline, -CH	0.95 1.22 1.50 2.22	4.75
14	DMD	1 2 3 4 5 6	0.54 1.00 1.02 1.54 1.72 1.74 3.64	Isoleucine, β Valine , β Leucine, C Leucine, CH Leucine, CH Proline, β CH	0.95 0.97 1.54 1.75 1.75 3.65	4.76
15	DMD	1 2 3 4 5 6	0.14 0.16 0.29 1.00 1.60 1.37 2.09	----- --- --- Isoleucine β Leucine, CH Analine, CH Glu CH	0.95 1.65 1.39 2.09	4.70
16	DMD	1 2 3 4 5 6	0.66 0.91 1.00 1.36 1.50 1.50 1.68 3.06 4.42	- Valine , β Isoleucine, -CH_3 Alanine , -CH Leucine , -CH - - Leucine , -CH Tyrosine , -CH Proline, -CH	-- 0.91 0.95 1.39 1.50 - - 1.75 3.08 4.44	4.75

According to data available with the present study we are giving some of the findings related to DMD patients . A group phenylalanine (β-CH) is completely absent in all DMD cases. We did not find any trace of this group in normal healthy controls .We also did not find another group called Leucine (β-CH and γ- CH) in normal controls .This group is found in DMD patients .Proline (β-CH)is present in two cases of DMD only .This group is also absent in all normal controls . We have also not found Cystine and Serine groups in DMD patients.

6. DISCUSSION

A magnificent use of NMR lies in fact that, because of the chemical shift, amino acids can be identified and isolated in the spectra of protein. It is well known in NMR theory that the motion of any type such as rotatory and translatory reduces the width of the resonance line .Due to this property motional narrowing feature starts and high resolution NMR is required. Proton magnetic resonance spectra of twenty amino acids and some representative di and tri peptide were studied by Mandel [49]. Vitolis et.al[50] have studied NMR spectrum of a serum sample shows both sharp narrow peaks from small molecule metabolites and broad peaks from proteins and lipids .The analysis of of spectrum of NMR for blood serum requires dealing with the effects of proteins and other large molecules.

NMR spectroscopy is the technique of spectroscopy that can provide detailed structural information about macromolecules at atomic resolution. Many scientists have been characterized small molecules by using empirical rules associated with the study of chemical shift regarding the conformation of the structure of the molecule.It has been seen that most of the amino acid spectra can be understood on the basis of first order effects. The chemical shift is larger than the spin –spin coupling.In the study of amino acids while the chemical shift is not large compared to the spin –spin coupling. We can compare the chemical shift and spin –spin interaction in this situation.If we have a situation that lies between these two experiments there are many amino acids the spectrum may have first and second order both. If we apply a large field to the system and we find a situation for the chemical shift, which is proportional to the field strength and kept spin –spin coupling as constant. Calculations based on chemical shifts are very useful in the structure of determination of globular proteins such as IgG.

Burton [51] has studied the structure and function of immunoglobulin G and reported some of the interesting applications of NMR to study this molecule. An antibody is a protein synthesized by an organism in response to invasion of the organism by a foreign substance termed as antigen. IgG found as principal antibody in serum. It has a molecular weight of about 150,000 and a domain structure is shown in Figure 6.

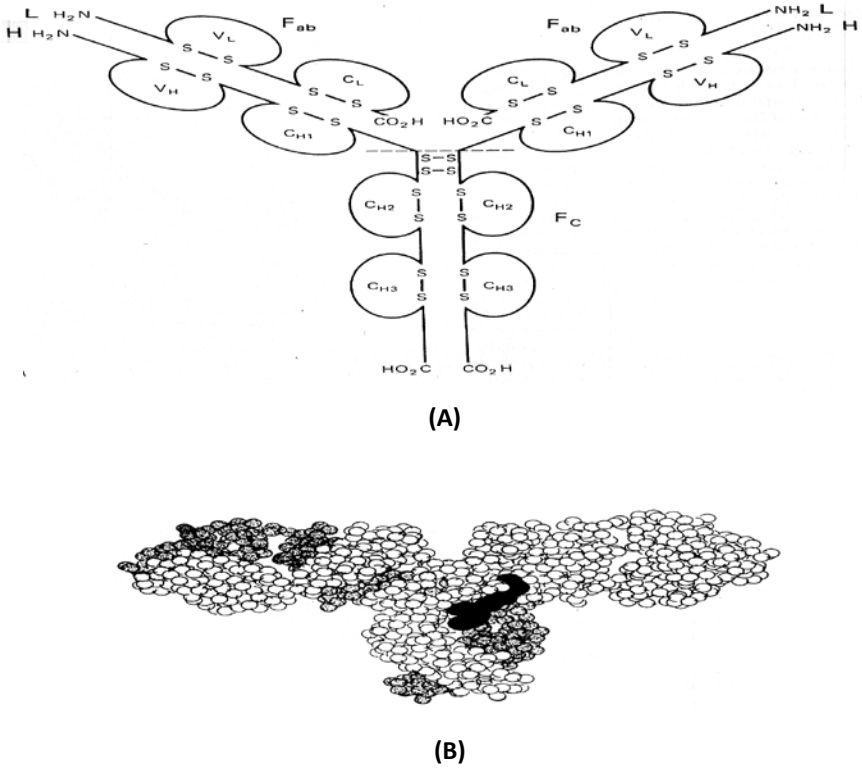

Figure 6: (A) Schematic diagram of a typical IgG structure; (B) Space filling model determined crystallographically

Mayer et al. [52] showed that IgG molecule can be cleaved into a number of proteolytic fragments. Antigen and complement bindings sites are also shown. If the antigen is not present, the complement site can be neglected. However, on the formation of a particular specifies antigen-antibody complexes, the first protein of the complement sequences binds to this site. This leads to activation of other proteins in the sequence and eventual destruction of the antigen.

It has been reported in the literature that IgG possess two equivalent tight Gd(III) sites located in the CH_3 domains of the Fc-region. Very much low affinity Gd(III) sites were also found on the FaS region. Experimental conditions may be adjusted such that IgG and the fragment Fc and pFc effects from the same two Gd(III) sites. They form a family of Gd(III) tight binding macromolecules of decreasing molecular weight. IgG has 150,000, Fc has 50,000 and pFc' has 25,000.

There is an internal motion found in the Fc portion of IgG. A considerable amount of motional freedom at Fc position was also found. A flexible rigid transition in the IgG molecule as the trigger for complement activation was also pointed out by Huber et al. [53].

Boyd et al. [54] have studied the mobility of protein on the basis of high resolution protein NMR of Fc and pFc' fragments. The spectra of such fragments appear resolved compared to the spectra of other proteins of similar molecular weight.

Cohn et al. [55] have studied the observations of amino acid side chains in proteins using this sophisticated technique of spectroscopy and reported in the research article regarding the side chain groups such as Glu, Asp, Lys, Arg, Met, Thr, Leu and others act as intrinsic non-disturbing probes of their local electronic microenvironment in a protein.Some of the authors [56-58] have studied the structure of proteins with the help of NMR spectroscopy in detail. They have found each proton resonance appears along the chemical shift axis in the expected fashion. The multiplets due to spin coupling appear only on the perpendicular axis.

If an amino acid residue in a protein is converted from a solvated state in a random coil polypeptide chain to buried inside the interior of a globular protein the chemical shift depends on the variation of magnetic susceptibility. Glick, et al. [59] have made their statements on amino acid residues and provide an interesting result such as chemical shift is directly proportional to the volume diamagnetic susceptibility K.

The NMR spectra of small globular proteins are very crowdy in nature. There is no evidence of unequivocal observations of Ser β-methylene proton resonance. Moore et al. [60] have studied and found that the assignments of the methyl-group resonance of Thr-47 and Thr-89 for ferricytochrome C and ferrocytochrome C.

Some of the authors Spero et.al. [61] and Kuszewski et.al. [62]have used chemical shift to obtain structural information regarding the correlation between chemical shifts and backbone torsion angles. It has been pointed out that different structures of proteins such as secondary, tertiary and quaternary have been successfully studied in solution and solid form and calculated with the help of chemical shift by various research scientists [63-70]. NMR chemical shifts are the best parameters and can be used in the structure elucidation of larger molecules. The assignment of backbone chemical shifts is necessary to determine the structure of protein.

Satoshi et al. [71] have studied proton nuclear magnetic resonance of human immunoglobulin G1 and its fragments. They have pointed out some information regarding the structure of hinge region and effects of a hinge region deletion on internal flexibility. Koichi et al. [72] have studied structural basis of the interaction between IgG and fcy receptors1. The studies on NMR spectroscopy show that Fcy RII binds to a negatively charged area of the CH_2 domain, corresponding to the lower hinge region. The binding of Fcy RIII onto one of the two related sites on the Fc induces a conformational change in the outer side.

Brab et al. [73] have studied NMR analysis on immunoglobulin G and glycenes and they have provided their fruitful results as glycan does not directly engage the cell surface receptors. The termini of both glycan brachs are highly dynamic and experience considerable motion in addition to tumbling of the Fc molecule.

The three dimensional structure of globular protein fluctuates incessantly and the fluctuation is closely related to the function of the protein. Nicholson et al. [74] have studied the dynamics of methyl groups in proteins.

Zilagyi et al. [75] have identified a correlation between αH chemical shifts and the helical and β-sheet structures. If the other effects are not present, helical conformation produces upfield shifts while β-structures shift the α proton downfield. Pasture et al. [76] have studied the secondary structure of proteins and Oldfield [77] studied three dimensional structure of proteins in terms of chemical shifts.

Jardetzky et.al. [78] have studied the protein spectra of amino acids and indicated these chemical shift are independent of concentration. Chemical shift mean a change in the chemical shift from that of the free amino acid. The real use high resolution NMR lies in the fact that because of the chemical shift specifies amino acids, which can be nicely and easily isolated in the spectra of protein. If we increase our understanding of the relationship between the chemical shifts and structure of the proteins or globular proteins such as immunoglobulin G molecule, we will be able to improve the accuracy of measurement and structure determination.

Proteins play a major role in the billions of process which occur in the body. It includes the development of muscles, skin, digestion of food, growth of cells and the germination of human emotions. These cells have a tendency to produce proteins continuously. We are not able to understand how these complex molecules exactly work.

Not only the chemical composition of the proteins but also the spatial structure of proteins is important for the performance of their functions. The way in which they fold and unfold in 3-D space help in determining the function of the molecules. It will be very difficult to understand the function of the molecules without detailed knowledge about their structure, spatial structure study is necessary now a days. NMR may help in the study of the determination of the structure of proteins. This technique can detect and quantify folding and conformation changes in proteins, while simultaneously providing detail structure information. If we use NMR spectroscopy in different diseases the NMR spectral peak observations can support the reliability of clinical applications. These peaks can determine the biochemical cause of the disease . However , peak identification ,peak intensities ,peak analysis of the given sample is a big support for the research scientists and clinicians.

7. CONCLUSION

The observed chemical shift in water peak in all the cases may be due to pathological conditions. The presence of the paramagnetic ions and the overlapping of protons from the changed line width. We have found peaks in all the spectra recorded on NMR due to the formation of some active centre's such as paramagnetic ions. The comparison of the diseased sample spectra with the normal's reveal some characteristics of the disease.

It is possible to identify the nature of the pathological disorder by looking at the NMR spectrum of patient's blood (immunoglobulin G molecule). It has been found that the peak intensities, line shapes and chemical shift were different. These basic properties suggest that there are perturbations present which vary from sample to sample. NMR spectra are interpretable only if the hydrogen atoms have unique chemical shifts . Considerable overlap of resonance occurs if there are too much resonances in any area of NMR spectrum . NMR also gives detailed information about the flexibility of the protein structure in solution[79] .The chemical shift suggests a transfer of electrons in enzymes and proteins in DMD. The dipolar anisotropy of unpaired electron causes a shift in line position. Sometimes delocalization is also coupled. We have found in the present study that the groups related to Phenylalanine, cysteine and serine were completely absent in all the DMD cases.

ACKNOWLEDGEMENT

The authors are thankful to Dr. P. K. Saxena, Principal, D.A.V. (P.G.) College, Muzaffarnagar for providing the facility of doing work. We are also thankful to Professor D. C. Jain, Head of the Department of Neurology, Safdarganj Hospital, New Delhi, for arranging the blood samples of the diseased and healthy controls. We are grateful to Dr. Manju Chauhan, Head, Department of Biosciences, D.A.V. (P.G.) College, Muzaffarnagar, for providing the facility of purification of IgG. Authors are thankful to Mr.Deepak Singh, Central NMR facilities, I.I.T. Roorkee for assistance in conducting experimental work.

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