CHAPTER 6

CHANGES IN ELECTROLYTE CONCENTRATIONS EFFECT THE IMPEDANCE DURING ISCHEMIA-REPERFUSION INJURY IN RAT BRAIN

Highlights of the chapter

- *The impedance of rat brain is increased during occlusion and reversed after reperfusion*
- *The in-vivo study demonstrated that the concentration of sodium ion is decreased and calcium ion is increased during occlusion in whole rat brain homogenate*
- *Measurement of impedance in aCSF solution revealed that an increase in concentration of calcium and potassium ions increased the impedance of solution whereas, an increment of sodium ion concentration lowers the same.*
- *Present study establish a relation between changes in impedance with change in electrolyte concentration in normal, occlusion and reperfusion conditions of rat brain.*

Abstract

Electrical impedance spectroscopy is an emerging tool to differentiate between normal and stroke conditions. In this study, the electrical impedance of rat brain was measured using two electrode impedance spectroscopy at different frequencies 100, 500, 1K, 5K, and 10K Hz in normal, occlusion and reperfusion conditions. Global cerebral ischemia was induced by BCCAO for 40 minutes followed by 40 minutes of reperfusion. The concentration of sodium, potassium, calcium and chloride ions of whole rat brain was determined by electrolyte analyzer. For the interpretation of *in-vivo* results, changes in electrical impedance with varying concentration of sodium, potassium and calcium ions in artificial cerebrospinal fluid (aCSF) was also performed using bio-impedance spectroscopy at frequencies 100, 500, 1K, 5K and 10K Hz. The *in-vivo* bio-impedance analysis suggests that the impedance is consistently increased during occlusion as compared to normal condition. The *in-vitro* study revealed that

the impedance escalates with an increase in the concentration of potassium and calcium ions and reduces with an increase in the concentration of sodium ion in aCSF. Further electrolytes analysis suggested that the level of sodium and chloride ion is significantly decreased as well as the level of calcium and potassium is significantly increased during occlusion as compared to the normal condition. These findings suggest that increase in impedance during occlusion may be due to changes in ionic concentration of rat brain. Above *in-vivo* and *in-vitro* studies successfully demonstrated and interrelated the change in impedance with corresponding changes in ionic concentration.

6.1 INTRODUCTION

Cerebral ischemic insult is a major cause of mortality and morbidity worldwide [1]. Ischemia occurs due to occlusion in blood vessels that causes the deprivation of oxygen and glucose to the nerve cells and ATP production failure [53]. The interruption in cerebral blood flow (CBF) results in termination of evoked and spontaneous electrical activity within 20 seconds that disrupts normal tissue ion homeostasis [54]. Within several minutes of electrical activity disturbance, marked an increase in the concentration of potassium ion and a sharper decrease in calcium and sodium ions in the extracellular space is reported [55,253]. Also, excessive efflux of glutamate after ATP and ion homeostasis failure causes NMDA receptor-mediated excitotoxicity resulting in mitochondrial dysfunction, oxidative stress, signal transduction alteration due to higher influx of calcium and sodium ions into the cell [254,255]. The rapid movement of calcium and sodium ions from extracellular to intracellular space as well as the release of calcium from the endoplasmic reticulum and mitochondria significantly increase the intracellular free calcium and sodium [256]. High level of intracellular calcium ions is responsible for the breakdown of cell-membrane and organelles by activating the harmful

endonucleases, calpains, proteinases, and phospholipases [257]. Ischemic pathophysiology mechanisms and its therapeutic interventions can be understood by using experimental rodent models. The rodents have been widely used as models for focal and global ischemia to study the disease pathophysiology and recovery after drug treatment [75,246,258]. Now-a-days, electrical impedance spectroscopy is an emerging tool to differentiate between normal and stroke conditions [40,41,259]. The bio-impedance analysis is a non-invasive method, which is used to measure the linearly varying electrical response across a specific dielectric medium (such as the cell, tissue, etc.), to yield useful information of that particular medium [129]. Previous studies have demonstrated that the impedance of rat brain is increased during occlusion and reversed during reperfusion [41] [44] but there is lack of studies that demonstrated and compared the changes in bio-impedance with change in electrolyte concentration of rat brain. It is reported that the impedance of rat brain increases during occlusion and is restored after reperfusion in global ischemic condition [41] [44] but the reasons behind this is not well elaborated. It is also known that brain electrolyte concentrations vary during induction of cerebral ischemia in both occluded and reperfused state. So it might be possible that the brain impedance change is closely related to changes in brain electrolyte concentrations. Hence, the present study is designed to investigate that the possibilities related to changes in impedance during ischemia-reperfusion condition is dependent on changes in concentrations of various electrolytes present in rat brain.

6.2 EXPERIMENTAL SETUP

6.2.1 Instruments Used:

INL191 Blood Flow Meter (AD Instruments Pvt. Ltd, Australia) with OxyFlow needle probe and NI-Elvis II Platform (National Instruments, USA) with two stainless steel screws and connecting wires were used to perform the experiments.

6.2.2 Animals

Inbred Charles–Foster (CF) albino rats (240 ± 20 g) were used for the present study. All rats were kept under conditions of controlled 12-h light/dark cycle, constant humidity, temperature (25±2 °C), and with free access of food and water. The surgical procedures were performed as per the protocol for animal use and approved by the Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA).

6.2.3 Cerebral blood flow measurement

The laser Doppler blood flow meter (INL191; AD Instruments Pty Ltd, Australia) is used to measure the regional cerebral blood flow (rCBF) of normal, occluded and re-perfused conditions. Before BCCAO, the anesthetized rat was fixed on a stereotaxic instrument (INCO, Ambala India) in the prone position and head was prepared for bio-impedance and cerebral blood flow measurement (Fig 6.1a). The skull bone was exposed through a midline incision (about 2 cm in length) in the scalp's skin. The tissues on the skull bone were gently removed using a sterile cotton swaps soaked in povidone-iodine and dental scraper. A burr hole of 1.5 mm diameter located at 4 mm lateral and 0.5 mm caudal to the bregma was formed (by keeping the dura intact) using a dental drill for the placement of laser doppler flow (LDF) probe (Fig 6.1c). To measure the rCBF, LDF probe was placed 0.5 mm above the dura surface.

6.2.4 Global ischemia induction

The surgical procedure to induce global cerebral ischemia using BCCAO is discussed in section 5.2.3 of chapter 5. In this study, the artery is occluded for forty minutes followed by reperfusion of forty minutes.

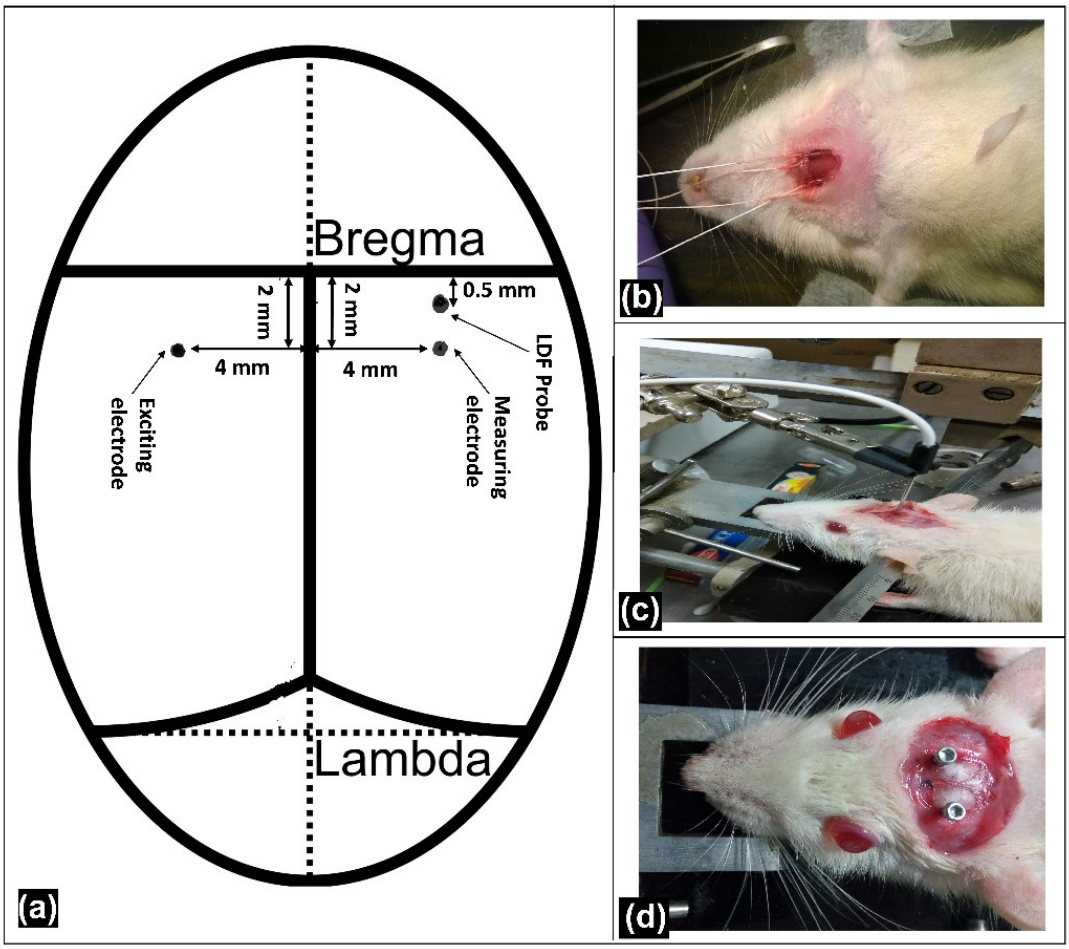

Fig. 6.1: Animal preparation for bio-impedance and blood-flow measurement

6.2.5 *In-vitro* impedance Measurement

The electrical impedance of artificial cerebrospinal fluid (aCSF) was measured *in-vitro* at different frequencies 100, 500, 1K, 5K, and 10K Hz using two electrode impedance system. The aCSF was prepared according to Cold Spring Harbor protocols (http://cshprotocols.cshlp.org/content/2011/9/pdb.rec065730.full). The prepared aCSF

contains 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 119 mM NaCl, 2.5 mM KCl, 2.5-mM $CaCl_2$, 10 mM glucose and 1.3 mM $MgCl_2$. Also aCSF solutions containing 2X and 4X concentration of each calcium, sodium and potassium ion were prepared to analyze the changes in impedance due to change in ionic concentration of aCSF. For measurement of impedance, a petri-dish of 2 ml volume was used with a fixed distance of stainless steel electrode (8 mm) that mimic the coordinates used for *in-vivo* impedance measurement in rat brain. The aCSF was used for the study since it resembles with the concentrations of electrolyte and osmolality found in real CSF and can be used as a physiological perfusate during endoneurosurgery [260].

6.2.6 *In-vivo* Bio-impedance Measurement

The bio-impedance of the rat brain was measured using the NI-ELVIS II platform. The NI-ELVIS II platform has a built-in impedance analyzer module. A two-point impedance measuring system configuration has been employed as an electrical bio-impedance Spectroscopic Data Interpreter and a two-terminal electrode system configuration is used in this work. The electrode system consists of two electrodes, one as a measuring electrode and other as a stimulating electrode. To measure the bio-impedance, both the stainless-steel electrodes (with insulated thread) were fixed at 4 mm lateral and 2 mm posterior to the bregma after forming burr hole of 1 mm diameter (Fig. 6.1a and 6.1d). The electrodes tips were placed in the cortex region prone to ischemic insult during BCCAO [261]. The measurement was done by manually varying frequency i.e. 100, 500, 1K, 5K, and 10K Hz in normal, occluded, and reperfused state with an simulating current of about 10 μA. The *in-vivo* bio-impedance analysis was performed to study the anisotropic effect of the brain in normal, occluded and re-perfused conditions. After completion of the study, all animal was euthanized using a high dose of chloroform.

6.2.7 Electrolytes analysis of rat brain

Brain was carefully isolated after cervical dislocation of anesthetized rats (normal, occluded and reperfused state) and placed in labelled tubes after washing with normal saline for the measurement of calcium, sodium, potassium and chloride electrolytes. The whole brain was homogenized in phosphate buffered saline using tissue homogenizer. The homogenized samples were centrifuged at 2100 RCF for 5 min. at 4°C and supernatants were collected and transferred to a clean tube for electrolyte analysis [247]. The concentration of calcium, sodium, potassium and chloride ions were measured using HDC-Lyte electrolyte analyzer (HD Consortium India Ltd., New Delhi).

6.2.8 Equivalent circuit model of rat brain

Generally, the equivalent circuit models are used to measure the factors, which are involved in inducing a measurable behavioral changes in any medium/system. Hence, choosing an appropriate electrical model to quantify these behavioral changes happened in a medium (such as biological tissue, Organ, etc.) is an important aspect. So, to fit the impedance data to an appropriate model, researchers used various models [262–264] but in this case, we have used Hayden model (Fig. 6.2) to represent the complex biological medium (such as Organ, Tissue, Cells, etc.) present in-between an electrode system with its equivalent electrical components. Usually, the resistance and capacitance offered by the cell-membrane (C), is termed as R_m & C_m and the resistance offered by both extracellular region/fluid and intercellular region/fluid is termed as R_e & R_i respectively. In the Hayden model, the extracellular resistance (R_e) will be in the upper branch, in parallel with intracellular resistance (R_i) and cell-membrane (C) in the lower branch. While the cell-membrane (C) can be represented by its membrane resistance (R_m) in parallel with corresponding membrane capacitance (C_m). But, to represent the non-

homogeneous constant present in the biological tissue, the cell-membrane (C), that is membrane capacitance (C_m), and membrane resistance (R_m) were generally replaced with a Constant Phase Element (CPE_m). Thus, the Hayden model gets modified as shown in fig. 6.2b and these CPE_m is used to regulate the non-uniformity, or non-homogeneity changes present between the cells in a complex biological tissue/medium. Hence, the modified Hayden model will have the electrical components in such a way that the extracellular resistance (R_e) will be in the upper branch, whereas the intracellular resistance (R_i) and Constant Phase Element (CPE_m) will be in the lower branch in parallel to that.

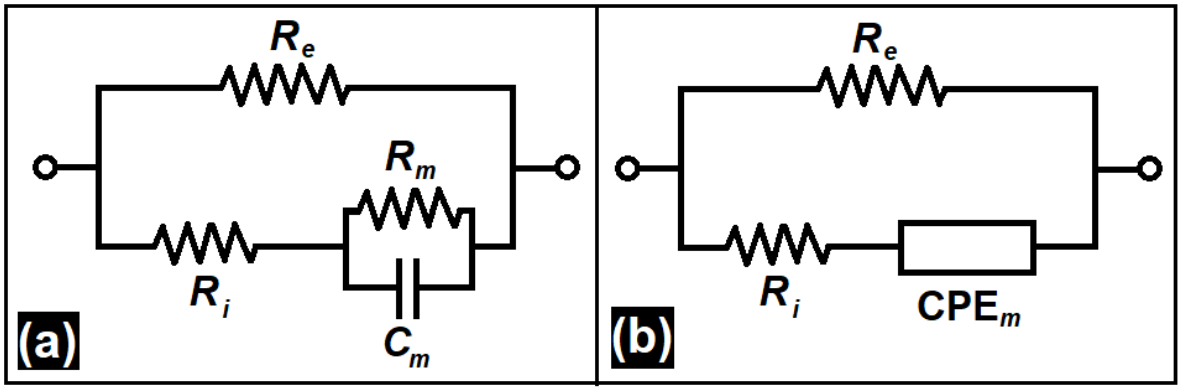

Fig. 6.2: Equivalent circuit model of rat brain (a) Hayden Model; (b) modified Hayden Model.

Here in this work, the measured impedance data were fitted using Non-Linear Curve Fitting Method, to analyse the non-uniformity change in various state of rat brain. The Hayden model's equivalent circuit can be defined as in equation 6.1:

$$Z(j\omega) = R_e || R_i + C_m \qquad (6.1)$$

Expanding equation 6.1, we get equation 6.2

$$Z(j\omega) = \frac{1}{\frac{1}{R_e} + \frac{1}{R_i + \frac{1}{C_m}}} \qquad (6.2)$$

The modified Hayden model's equivalent circuit can be rewritten using equation 6.1 as shown in equation 6.3 to replace cell-membrane with a Constant Phase Element

$$Z(j\omega) = R_e \| R_i + CPE_m \qquad (6.3)$$

And, the CPE_m can be simply written as in equation 6.4

$$CPE_m = \frac{1}{(j\omega)^{\alpha} \cdot Q_m} \qquad (6.4)$$

Expanding equation 6.3, we get equation 6.5

$$Z(j\omega) = \frac{1}{\frac{1}{R_e} + \frac{1}{R_i + \frac{1}{(j\omega)^{\alpha} \cdot Q_m}}} \qquad (6.5)$$

where the angular frequency (ω) is defined by $\omega = 2\pi f$, (f = Frequency) with the imaginary value j. Solving equation (6.5), then we get the total impedance of the rat brain (equation 6.6):

$$Z(j\omega) = \frac{R_e \cdot R_i}{R_e + R_i} + \frac{R_e - \frac{R_e \cdot R_i}{R_e + R_i}}{1 + \left\{ j\omega \left[(R_e + R_i) \cdot Q_m \right]^{1/\alpha} \right\}^{\alpha}} \qquad (6.6)$$

Equation 6.7 is the corresponding phasor value $(j\omega)^{\alpha}$ through which the complex impedance offered across the two-electrode system was estimated (equation 6.8)

$$(j\omega)^{\alpha} = \omega^{\alpha} \left[\cos\left(\frac{\alpha\pi}{2}\right) + j \sin\left(\frac{\alpha\pi}{2}\right) \right] \qquad (6.7)$$

$$Z(j\omega) = \frac{\mathrm{Re}\left\{ 1 + \omega^{\alpha} \cdot Q_m \left[(2R_i + R_e) \cdot \cos\left(\frac{\alpha\pi}{2}\right) + \omega^{\alpha} Q_m R_i (R_e + R_i) \right] \right\} - j \left\{ \omega^{\alpha} Q_m R_e^2 \cdot \sin\left(\frac{\alpha\pi}{2}\right) \right\}}{\left(\omega^{\alpha} Q_m (R_e + R_i)\right)^2 + 2\omega^{\alpha} Q_m (R_e + R_i) \cdot \cos\left(\frac{\alpha\pi}{2}\right) + 1}$$

(6.8)

The time distribution constant (τ) using the phasor value is defined by equation 6.9

$$\tau = \left[\left(R_e + R_i \right) \mathrm{g} Q_m \right]^{1/\alpha} \qquad (6.9)$$

When. α = 1, the CPE_m becomes ideal capacitor that is C_m and α < 1 indicates the level of non-uniformity or inhomogeneity present in dielectric medium.

At low frequencies, the magnitude of the total impedance offered is dominated by lower branch i.e., $R_e \ll |Ri + CPE_m|$, so the medium will experience a maximum level of current penetration across the resistor $R_e$at extracellular region and yields R_e. Whereas at higher frequencies, the magnitude of the total impedance offered is dominated by $R_i \gg |CPE_m|$, so the medium will experience maximum level of current penetration across R_e & R_i, involving both intracellular and extracellular region to approximately yields the values $[(R_e . R_i)/(R_e + R_i)]$.

6.2.9 Statistical Analysis

The electrolytes concentration data were analysed using Graph Pad Prism 7.0 software with one-way analysis of variance (ANOVA) using Bonferroni's Multiple Comparison Test. The results are expressed as the means ± SD. P-values <0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Cerebral Blood Flow measurement

The rCBF is measured as per mentioned coordinate shown in fig. 6.1a and 6.1c for normal, ischemic and reperfusion conditions of rats. Figure 6.3a demonstrates the values obtained for regional CBF. During occlusion, the rCBF is decreased significantly up to 80% as compared to the normal state. Whereas, during reperfusion the rCBF is restored near to the normal rCBF values in 40 minutes.

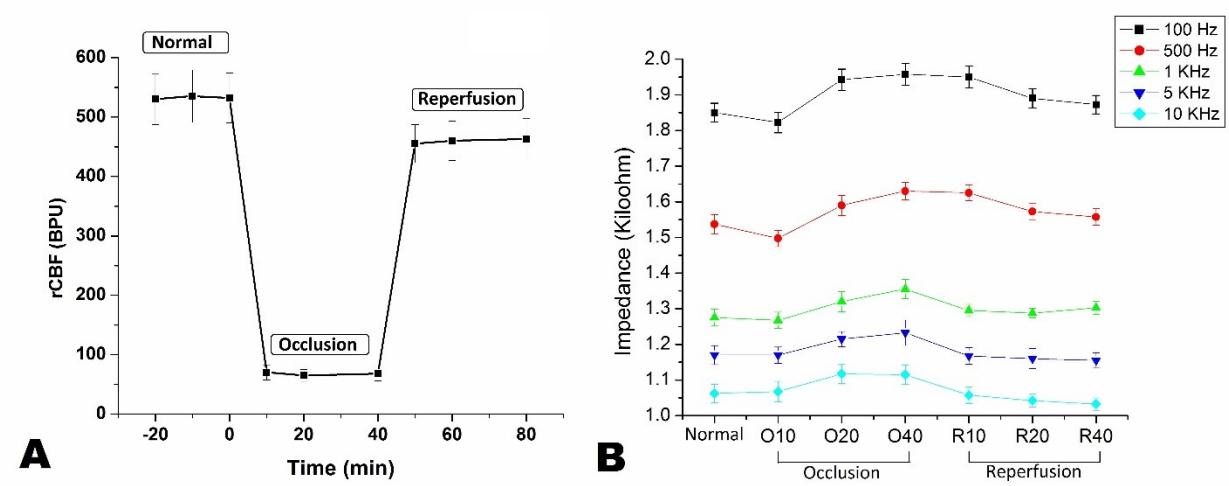

Fig. 6.3: Regional cerebral blood flow and bio-impedance of rat brain (A) rCBF is measured by LDF. The blood flow is expressed in blood perfusion unit (BPU). (B) Bio-impedance of rat brain in normal, occlusion (10, 20 and 40 minutes) and reperfusion (10, 20 and 40 minutes) state at 100, 500, 1k, 5k and 10k Hz frequencies.

6.3.2 *In-vivo* Bio-impedance of rat brain

The bio-impedance analysis of rat brain was carried out using two-point impedance measuring system at frequencies 100, 500, 1K, 5K, and 10K Hz. Generally, at the lower frequency range (10Hz to a few kHz), the maximum amount of current will flow in the extracellular region, and this can be termed as α-dispersion. At mid-frequency range (>1 kHz to few MHz), the maximum amount of current will flow through the extracellular region while some portion of current will enter into the cell membrane and intracellular region, and this can be termed as β-dispersion. At higher frequency range (>10 MHz), all the current enters into the intracellular region and lesser amount of current will pass through the extracellular region, this can be termed as γ-dispersion [133]. The α- and β- dispersion have been widely used as the region of interest to study the parametric changes in the biological tissue/medium [134]. Therefore, in the present study, we used frequency ranges of 100-10,000 Hz that encompass α- and some portion of β-dispersion. The impedance at different time point was plotted to divulge how the

impedance value changes in rat brain in normal, occlusion and reperfusion conditions. The impedance changes in different states at different time points at frequencies of 100 and 500 Hz (α dispersion) as well as 1K, 5K, and 10K Hz (β dispersion) are illustrated in fig. 6.3b. Results suggest that the impedance of rat brain decreases with increase in frequency. The impedance value decreases during the first ten minutes of occlusion after that it gradually increases up to forty minutes of occlusion thereafter a sharp decrease was observed during the 40 minutes of reperfusion, reestablishing the values near to the normal state.

6.3.3 Determination of electrolyte concentration in whole rat brain

The concentrations of sodium, chloride, calcium and potassium ions of rat brain in normal as well as at forty minutes of both occlusion and reperfusion were determined using electrolyte analyzer (fig. 6.4). A significant reduction in concentration of sodium and chloride ions was observed in occlusion as compared to normal state ($p<0.01$). Also a significant difference in concentrations of sodium ($p<0.01$) and chloride ($p<0.05$) ions were observed in the occluded and reperfused states. A significant increment in concentrations of calcium ($p<0.001$) and potassium ($p<0.05$) ions was observed during occlusion as compared to the normal state. In reperfusion, the level of calcium ($p<0.01$) and potassium ($p<0.05$) ions was significantly reduced as compared to that during occlusion.

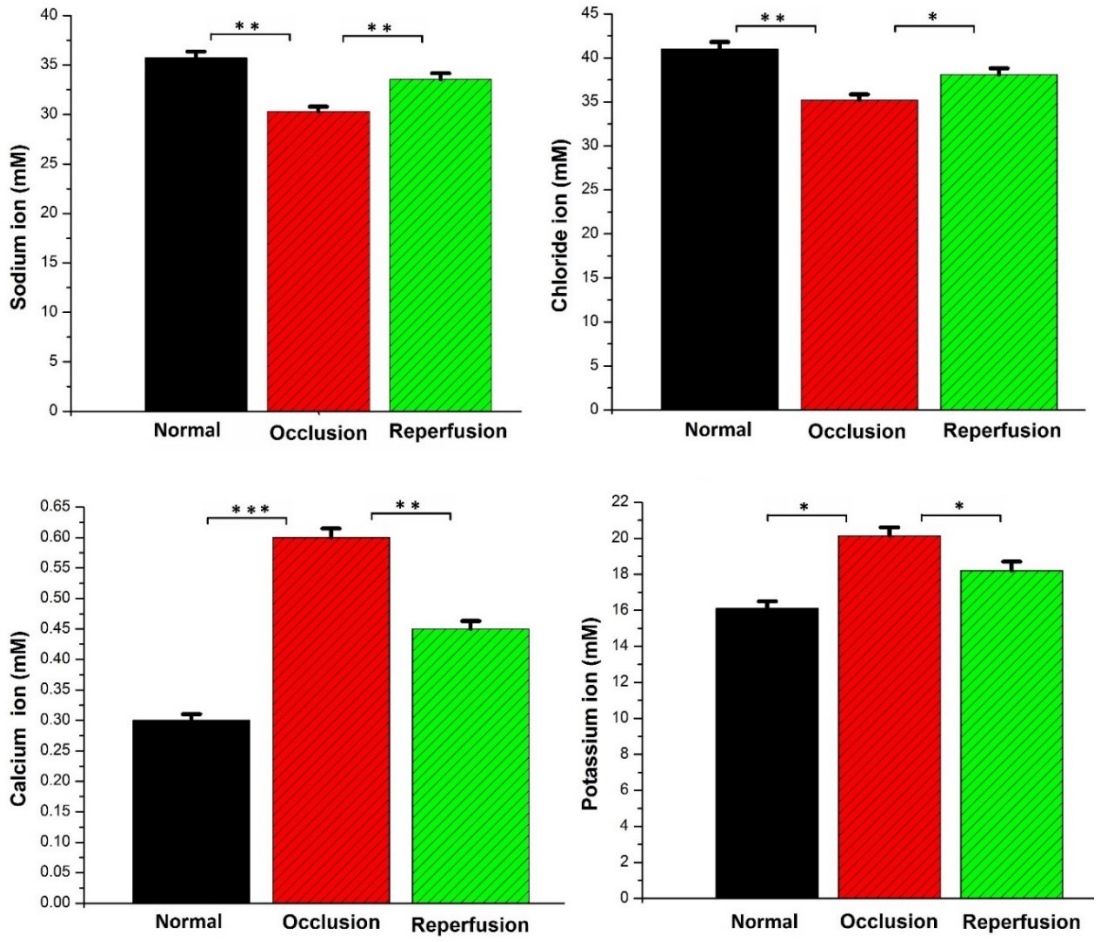

Fig 6.4: Concentrations of Sodium, Chloride, Calcium and Potassium of whole rat brain in normal, occlusion and reperfusion conditions. (***p<0.001; **p<0.01; *p<0.05; ns: non-significant)

6.3.4 *In-vitro* impedance of aCSF

The impedance of the 2ml solution of aCSF and aCSF with 2X and 4X concentration of calcium, sodium, and potassium ions was successfully measured which is illustrated in fig. 6.5. Results suggest that an increase in ionic concentrations of calcium and potassium ions in aCSF increased the impedance. However, an increase of sodium ions decreases the impedance.

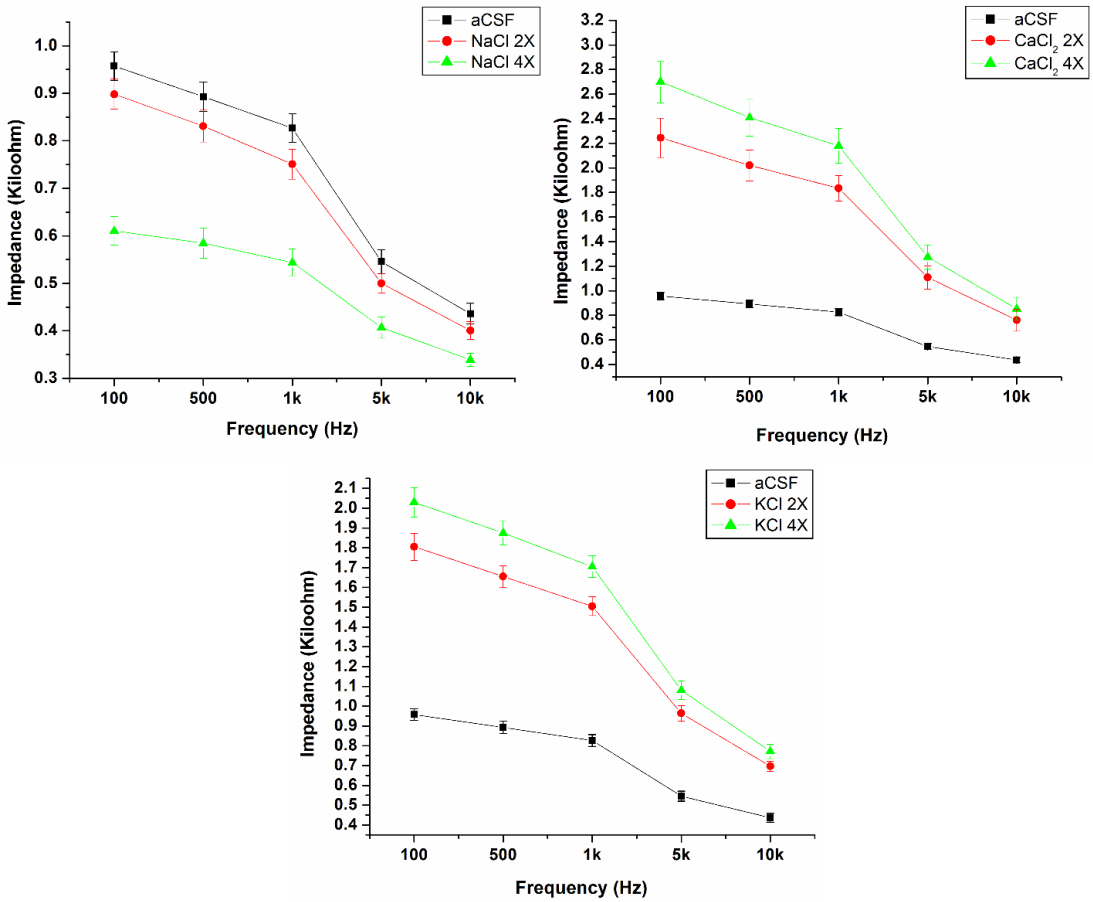

Fig 6.5: Impedance of aCSF and aCSF solutions containing 2X and 4X concentration of sodium, calcium and potassium ions at 100, 500, 1k, 5k and 10k Hz frequencies.

6.3.5 Impedance modelling

The impedance data of rat brain in normal, ischemic and occlusion states was fitted using modified Hayden model to quantify the R_e, R_i, CPE coefficient and amplitude of CPE exponent. The results of this model is illustrated in Fig. 6.6 & Fig. 6.7. The values of R_e and CPE coefficient is increased during occlusion and decreased gradually in reperfusion. Whereas, the values of R_i and amplitude of CPE exponent is decreased during occlusion and increased in reperfusion. The bio-impedance magnitude and phase plot of rat brain is shown in Fig. 6.7. Modelling of impedance data yielded similar results as found in vivo. The magnitude of impedance increases gradually in occlusion and reaching near to the normal value in reperfusion condition.

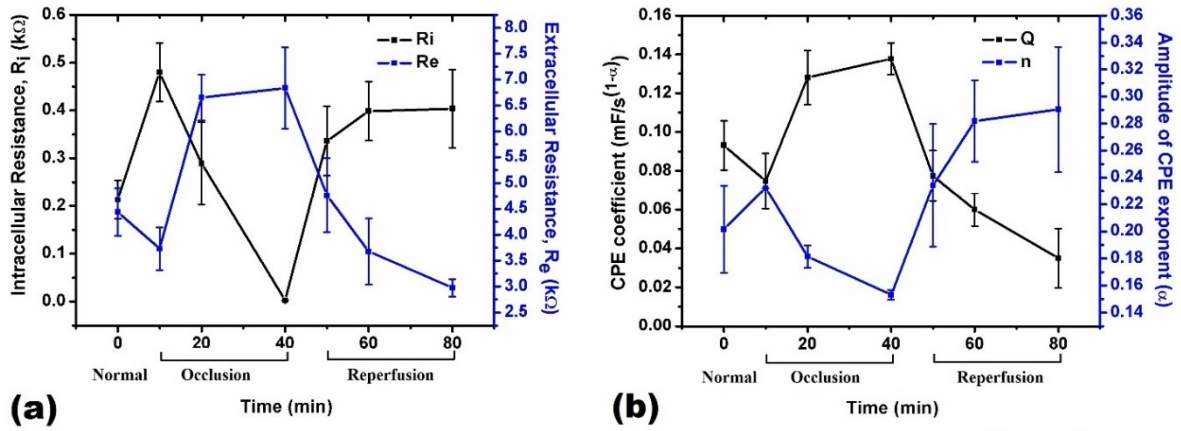

Fig.6.6: Bio-impedance model parameters for rat brain in different conditions. (a) Intracellular Resistance, R_i and Extracellular Resistance, R_e. (b) CPE coefficient (Q) and Amplitude of CPE exponent (n).

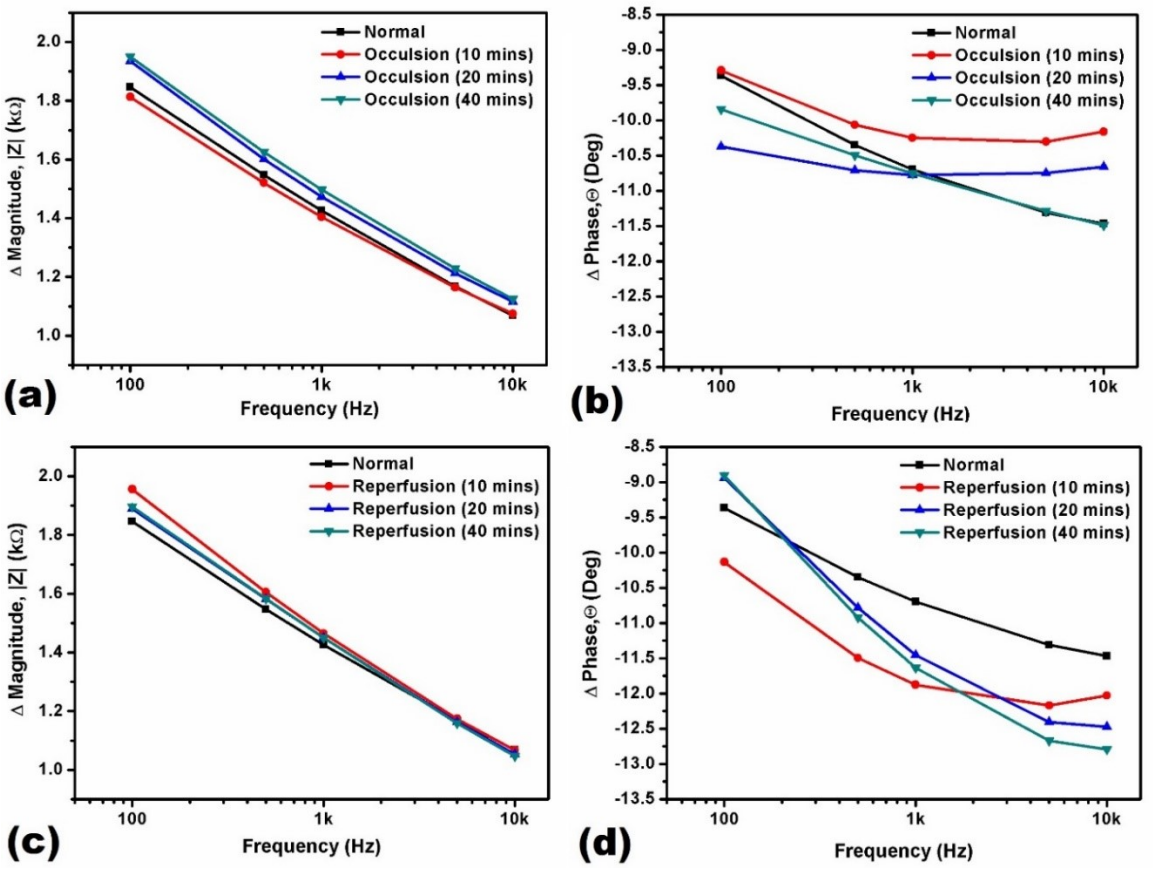

Fig. 6.7: Bio-impedance magnitude and phase plot of rat brain**.** (a) Magnitude vs. frequency plot and (b) Phase vs. frequency plot of normal and occlusion state. (c) Magnitude vs. frequency plot and (d) Phase vs. frequency plot of normal and reperfusion state.

6.4 DISCUSSION

The present work possibly constitutes the first reported analysis of impedance recording that tries to establish a relation between changes in impedance with change in electrolyte concentration in normal, occlusion and reperfusion conditions of rat brain. Several literatures also suggest that the impedance is increased during occlusion and reversed after reperfusion [41,265]. We also found similar results in the present study. The *in-vivo* study demonstrated that the concentration of sodium ion is decreased and calcium ion is increased during occlusion in whole rat brain homogenate. Whereas, an increase in sodium ion concentration increases the conductivity hence lowers the resistance of solution due to a raise in the number of charge carriers. Meanwhile, we observed that an increment in calcium concentration increases the impedance because calcium is a poor electrical conductor [266]. Measurement of impedance in aCSF solution revealed that an increase in concentration of calcium and potassium ions increased the impedance of solution whereas, an increment of sodium ion concentration lowers the same. The electrolyte analysis of sodium, chloride, potassium and calcium ions in whole rat brain in normal, occluded and reperfused state demonstrated that the concentrations of sodium and chloride ions are decreased and the concentrations of potassium and calcium ions increase during occlusion. The above mentioned phenomena might be responsible for increase in impedance during occlusion or ischemia.

Modelling of impedance revealed that extracellular resistance (R_e) of rat brain during ischemic condition is increased and reversed after reperfusion, while intracellular resistance (R_i) of rat brain is decreased during ischemic condition and reversed after reperfusion. This phenomena can be understood by relating it with the influx/efflux of ions across cell membrane during ischemia. We know that the concentration of sodium and calcium ion in the extracellular

space is relatively high as compared to the intracellular space. Usually, the calcium ion [Ca^{2+}] concentration in extracellular space is approximately 40,000-fold higher than that of intracellular space, which ranges from 1 to 2 mM [267]. In the abnormal condition like ischemia, the membrane's depolarization state gets disturbed due to ion homeostasis. Further, due to the efflux of glutamate neurotransmitter, energy failure occurs resulting in excitotoxicity which leads to neuronal cell death [54]. The extracellular calcium is translocated into the cell through various ion channels like the NMDARs, voltage and agonist operated ion channels as well as other unspecified ROS-activated ion channels [268]. The extracellular space/region is also reduced by 50% of the normal condition which also signifies an influx of almost total extracellular calcium ions into the cell. These conditions lead to an increase in electrical conductivity resulting in a decrease in resistance and electrical conductivity (increase in resistance) in the extracellular and intracellular space respectively. These phenomena are clearly observed in the first ten minutes of occlusion which is illustrated in Fig.6.8 through mathematical modeling of brain impedance. In the normal state, the sodium ion (Na^+) is predominantly present in the extracellular space [269]. It can enter into the cells through a variety of routes including NMDA receptors, voltage-gated cation channels, gradient-driven co-transporters and membrane exchangers. Whereas, in abnormal conditions like ischemia, sodium ions influx overcome the Ca^{2+}-induced inhibition of NMDA receptors. It potentiates the inward Ca^{2+} flow and activates NMDAR activity through AMPARs, non-selective cation channels, and voltage-gated Na^+ channels [270]. A high influx of Na^+ ions and efflux of potassium ions reduces the conductivity (increase in resistance) in the extracellular space and enhances the conductivity (decrease in resistance) in the intracellular space. The present study is supportive of this phenomenon resulting in the increase of extracellular resistance [R_e] and

a decrease of intracellular resistance [R_i] with an increase in CPE_m coefficient during occlusion as compared to the normal physiological condition.

6.5 CONCLUSION

The study demonstrated the changes in brain impedance with corresponding changes in electrolyte concentrations in rat brain. The extracellular and intracellular ionic concentrations determine the characteristic behavior such as resistivity, conductivity, and permeability of the brain tissue. The impedance changes in *in-vivo* condition has a relation with the characteristic behavior of impedance change observed in the *in-vitro* study. The present study establishes that the changes in bio-impedance across rat brain can be inter-related with the changes in both intracellular and extracellular ionic concentrations using impedance modelling. Further studies regarding precise quantitation of electrolyte concentrations in extracellular and intracellular space during occlusion and reperfusion of rat brain are required for better understanding of the current findings.