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| 13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) | In the first year, this program has supported two graduate students (one from EE Department and one from Biochemistry Department) and a postdoctoral fellow (Radiology Department). They have been introduced to the Biomedical NMR Laboratory and the Howard University Cancer Center. The trainees learned the theory and instrumentation of Nuclear Magnetic Resonance imaging and spectroscopy. The students have also rotated through the clinical services in the hospital to learn the mammography procedures. They have participated in the seminar series in the Cancer Center and throughout the campus. The trainees have been introduced the ongoing research projects in the lab. They all have started their research projects with the PI. Based on the preliminary findings, two papers and two posters have been presented in the University Research Forums and in the National Scientific Meetings. From these initial research results and contacts with scientists at Georgetown University, a collaborative partnership grant has been developed, submitted and funded by USAMRMC. |
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IV. Reports

There are two Ph.D. graduate students (Mr. Emmanuel Agwu and Ms. Lisa Kinnard) and one postdoctoral research associate (Dr. Jianwei Zhou) supported by this grant. Mr. Agwu is a 5th yr MD/PhD student pursuing his Ph.D. degree in Biochemistry. Ms. Kinnard is a graduate student from the Department of Electrical Engineering. Dr. Zhou is a chemist in the Department of Radiology. The reports and accomplishments for each individual are listed separately according to the Statement-of-Work in the proposal. The individual reports are followed by a summary of the reportable outcomes including presentations/publications, awards and grant.

1. By Lisa Kinnard, Howard University, Department of Electrical Engineering, Ph.D. student

- Introduction to the Biomedical NMR Laboratory and Cancer Center
  During the first several months, Dr. Paul Wang introduced me to the Biomedical NMR Laboratory. At this time, Dr. Wang explained the technical capabilities of the equipment as well as the group's current research projects. At this time I also met with mentors to learn the current status of breast cancer research and to determine what possible contributions could be made to the field.

- Learn NMR instruments
  From the fourth month through the end of the year, I learned the physics and instrumentation of Mammography and MRI. Additionally I learned the about picture archiving communication system (PACS) with regard to its significance in the radiology field and its general impact on hospitals.

- Seminar Presentation
  In March and April of 2001, I worked on an image processing project and coauthored a poster presentation in the annual meeting in The Annual Pediatric Academic Societies Meetings, May 1-5, 2001, Baltimore, MD.

- Clinical Preceptorship
  In December of 2000 I began a radiology internship with Dr. Eva Duckett of the Howard University Hospital Radiology department. During this internship, Dr. Duckett trained me in the following areas: 1) Patient management, 2) Screening/Diagnostic procedure, 3) Breast cancer image patterns, 4) Understanding of typical cases versus clinically indeterminate cases, 5) Understanding of geometric distribution (physical locations of tumors), 6) Image patterns of cysts, fibroadenomas, 7) Image pattern analysis of masses vs. microcalcifications and 8) Biopsy procedures. Dr. Matthew Freedman (and various other radiologists) of the Georgetown University Medical Center (GUMC) currently answers any of my questions concerning the mammography images; however, in the future, Dr. Rebecca Zuurbier (director of breast imaging, director of Betty Lou Ourisman Breast Health Center) of the GUMC will be interviewed concerning the screening/diagnostic procedure, patient management, and biopsy procedures of the GUMC.

- Summary of Accomplishments
  The objective of the research is to develop a system that will act as a consultation system to assist radiologists in determining the likelihood of malignancy. This work can be divided into three parts: determination of image segmentation (separation of tumor from surrounding tissue), calculation of image statistics, and determination of a classification method. During the past year I have completed the following tasks toward the completion of the research:
  1. Written report on Mammography which covering:
a. Physics/Instrumentation of traditional X-ray
b. Summary of Mammography
   i. Physics/Instrumentation
   ii. Film-Screen Combinations
c. Breast Anatomy/Mammographic Analysis
d. Clinical Procedures
e. Summary of image patterns in benign and malignant calcifications in mammography
f. Summary of image patterns in benign and malignant masses in mammography

2. Performed literature search on image features used for breast cancer diagnosis
3. Programmed statistical equations
4. Digitized mammography films
5. Ran statistics on phantom images
6. Correlated patient records with images in order to locate tumors
7. Surveyed image segmentation techniques
8. Completed first prototype of image segmentation phase

By Emmanuel Agwu, an MD/PhD student, PhD candidate in the Biochemistry Department

- **Introduction to the Biomedical NMR Laboratory and Cancer Center**
  I was given a tour of the Biomedical NMR Laboratory so that I was able to locate laboratory materials as well as major equipments (e.g. 200 and 400 MHz NMR machines). I was also given a tour of the Cancer Center in order for me to know where other necessary biomedical equipments were located and to meet other scientists conducting research in breast cancer. I was briefed on the on-going projects in our laboratory and give the grant proposal as well as related journals for thorough review.

- **Learn NMR**
  Dr. Wang provided me a week of intensive training on NMR. Aside from many hours of on-hands training, I also enrolled in a NIH graduate course on NMR. Furthermore, I was provided with several textbooks on NMR and MRI theory and applications. During the spring of 2001, I attended courses on MRI theory and application taught by Dr. Wang to the Howard University Radiology residents.

- **Start Biochemical Departmental Course Work**
  I am a fifth year MD/PhD student. I have completed the first two years of medical school. I was also enrolled in the Biochemistry Department as a PhD student. I have completed 21 institutional credit courses and passed the qualified examination. I gave a seminar each semester both in the Biochemistry Department and the MD/PhD program.

- **Clinical Preceptorship**
  As required by the MD/PhD program, I attended a clinical rotation with a clinical preceptor once a week for half a day. This is necessary to keep me abreast on clinical issues while understanding my Ph.D. training.

- **Report to MD/PhD Committee and Biochemistry Department on Research Progress**
  The MD/PhD program committee hosts a student research forum every year. In the research forum, the students in the program report on the progress of their research to the rest of the university. I presented a talk on May 17, 2001, entitled “MRS Study of $^{31}$P Metabolism in MCF7 Breast Cancer Cells”. I also presented a paper in the 12th Annual Research Day in the
Biochemistry Department, entitled “An Improved NMR Perfusion System For Breast Cancer Cell Study”. I have submitted and am being accepted to present a paper on October 12, 2001 at the Academic Minority Physicians 15th Annual Scientific Meeting in Washington, DC.

3. By Jianwei Zhou, Ph.D., Research Associate, Department of Radiology, Howard University

- **Introduction of Biomedical NMR Laboratory and Howard University Cancer Center**
  In the beginning of February 2000, I joined the Howard University Hospital Cancer Center, as a research associate in the Biomedical NMR Laboratory. At first, I spent one month to learn about the ongoing research projects in this lab. Breast cancer is one of the major research areas of the Cancer Center. The Biomedical NMR Laboratory has been involved in breast cancer research using NMR imaging and spectroscopy techniques since 1989. Our major research interests include a study of metabolism and the responses of perfused breast cancer cells under the drug treatment. I have studied related literature and frequently discussed with Dr. Wang, Dr. Shridhar, and other group members in the lab to appreciate the complexity of the research. As a chemist by training, I have learned the cell culture techniques required for the research such as subculture cells, harvesting cells, how to freeze the cells for storage and to prepare agarose thread containing breast cancer cells for NMR studies. I have learned how to use the shared facilities, such as cold room, incubator, and handling the cells under the hood in the Cancer Center.

- **Participate in weekly Cancer Center seminars**
  I participated in many Cancer Center weekly seminars. I also participated in many NMR and cancer related seminars on campus. I attended a workshop entitled “Animal Models in Breast Cancer Imaging” sponsored by the Howard University Cancer Center and Walter Reed Hospital.

- **Learn to use three NMR instruments in the laboratory**
  As a NMR spectroscopy chemist, it took me a relatively short time to be familiar with the Varian NMR instruments in the lab. I learned how to obtain good spectra with high signal to noise ratio, how to transfer data between NMR machines and satellite workstations, and data analysis.

- **Take NMR and other courses**
  Besides the instrumentation training in the lab, I have learned NMR imaging and spectroscopy theories taught by Dr. Wang. I also took the biochemistry course at NIH. I also learned the cell culture procedures from the Dr. Asafa’s lab at the Cancer Center.

- **Start research program**
  I participated in the $^{31}$P NMR study of breast cancer cells including MCF7 wild type (wt), MCF7 drug resistant, MDA231, KB-V-1, and KB-3-1 cells. We presented a poster at the 42nd ENC Conference. Some of the major findings and achievements are listed as follows:

1. The high signal to noise ratio spectra are obtained for all kinds of breast cancer cells.
2. The NMR T1 measurements of phosphorus metabolites for MCF7/wt and MDA231 cells.
3. Constructed a new perfusion system for NMR studies to resolve the bubble problem.
4. Build a gas bubbling system for the cell oxygenation experiments.
5. Start the oxygenation experiment, which is designed to understand the cell oxygenation conditions in our cell perfusion system.
6. Start the drug treatments studies using Doxorubicin.
7. A special spin lock system/technology has been introduced in the cell perfusion NMR experiment.
V. REPORTABLE OUTCOMES

PUBLICATION


AWARDS

1. Mr. Emmanuel Agwu received the Association for Academic Minority Physician, 2001 Minority Medical Student Research Summer Fellowship, a Merck/AAMP scholarship.
2. Mr. Emmanuel Agwu received a 2001 Scandrett Scholarship Award, a scholarship for disable students.

GRANT

Dr. Paul Wang with Dr. Mohamed Chouikha (P.I.) in the Department of Electrical Engineering has submitted a partnership training grant to USAMRMC and it was funded for 2001-2004. This program is a training partnership between Howard University and Georgetown University (Dr. Ben Lo and Dr. Matthew Freedman) to train faculty and students in breast cancer imaging, digital image database library techniques and network communication strategy.

VI. APPENDIX

Appendix 1. The poster from the 42nd Experimental NMR Conference, March 11-16, 2001, Orlando, FL. Entitled “An Improved NMR Perfusion System For Breast Cancer Cell Study”.

Appendix 2. The poster from The Annual Pediatric Academic Societies Meetings, May 1-5, 2001, Baltimore, MD. Entitled “Early EEG and Diffusion MRI (dMRI) Changes in an Experimental Model of Severe Periventricular Leukomalacia (PVL)”. 

7
A Prolonged Perfusion System For Breast Cancer Cells

Abstract

In this paper, an improved NMR cell-perfusion system is presented. The perfusion system is driven by diaphragm pump. The portion of the system before the pump is under negative pressure, while the portion after the pump is under positive pressure. This design helps with the removal of air bubbles from the perfusion system. The long-perfusion system, a cell viability study of the MCF-7 breast cancer cell line was extended successfully for more than a week. The 1H NMR spectrum of the MCF-7 showed three distinct phases: phase 1 and 2. Further characterization of the long-perfusion cell population system suggests the cells utilized aerobic respiration while under perfusion. This was done by perfusing the cells with either 0.1 mM lactate or 10 mM Pentobarbital. The spectra showed the disappearance of phosphate metabolites until the eventual cell death. The results clearly demonstrate that the long time bubble-free NMR cell perfusion system could be a useful tool for breast cancer research.

Introduction

NMR has been used to monitor the metabolism of cells as well as the responses of cells under drug treatment (1,2,3,4). Usually, the NMR study requires data acquisition over a long period of time, hours or even days. To assure even distribution of nutrients, the cells under study are encased in agarose while being perfused with serum-containing medium (5). Over a long acquisition period, the air in the perfusion medium gets released as it comes in contact with the agarose, giving rise to bubble formation. Air bubbles destroy the magnetic homogeneity of the system thus decreasing the signal-to-noise ratio of the spectrum. This discourages preclinical cell studies in agarose-encased systems. So, it is crucial to have an air-tight perfusion system to avoid bubble formation inside the NMR tube if a prolonged acquisition period is desired. Hence, an improved long time NMR perfusion system is presented. This system releases and traps air bubbles from the perfusion medium prior to the medium reaching the agarose-encased cells in the NMR tube.

Experimental Procedure

Sample Preparation: The breast cancer cells, MCF-7, were grown to 90-95% confluence in 10 tissue culture flasks containing MEM growth medium complemented with 10% FBS. The cells (~2x10^6 cells) were harvested with trypsin-EDTA, and resuspended in a 15 cm tissue culture plate giving ~0.7 mL per cell. The pellet was placed in a 37°C water bath and mixed with 0.7 mL cold temperature-gelling agarose dissolved in PBS (1% v/v). The agarose-cell mixture was exposed to an agarose bath, through a 0.5 mm inner diameter plastic tube into an NMR tube containing MEM complete medium. While transferring through the plastic tube, the agarose-cell mixture solidifies producing a thread resembling lemon. The NMR tube containing the agarose-encased cells was then placed inside the magnet probe and perfused at the rate of 0.5 ml/min throughout the acquisition period. Depending on the study, the cells were perfused with MEM complete medium, MEM complete medium containing 0.1 mM lactate, or MEM complete medium containing 10 mM pentobarbital.

NMR Measurement: All NMR data were collected in a Varian VNMRS-400 NMR instrument at 16.07 MHz for 1H channel and 400.13 MHz for 13C channel. The temperature of the magnet was kept constant at 37°C throughout the duration of acquisition. The magnetic field lock signal was provided by the 5.0 ppm, which surrounded the outside of the RF coil.

An Improved NMR cell-perfusion system

According to Henry's law, the mass of a slightly soluble gas that dissolves in a definite mass of a liquid at a given temperature is directly proportional to the partial pressure of that gas, namely:

\[ P = K \cdot X \]

Where, \( K \) is the Henry's law constant, which is a function of temperature. At the equilibrium state, the mole fraction of dissolved gas (X) in solution is dependent on the partial pressure of the gas (P) on the solution surface and the temperature. For example, CO, O, and N, at 37°C, the equilibrium partial pressure is 1.38 kPa, 4.07 k Pa, and 38°C, the equilibrium partial pressure is 2.0 ml. The solubility of all gases is a function of the pressure, and the amount of gas dissolved increases as the pressure increases. MCF-7 cells were gently washed with PBS and resuspended in MEM medium containing lactate or pentobarbital. The NMR data were collected using a 1H, 13C, and 31P NMR spectrometer.

Application II: After establishing the baseline characteristics of MCF-7 cells in the improved agarose-encased cell perfusion system, we set out to determine the respiratory conditions of the cells using this improved system. The MCF-7 cells were perfused with medium containing either 0.1 mM lactate or 10 mM Pentobarbital. The behavior of the 13C metabolites were quiet interesting (see Fig. 3, 4, and 5). For example, when the cells were perfused with lactate, there was an initial increase in the signals of t-P, t-C, GPC, and T-ATP. The reason for this is not understood. A week later, the signal in GPC was no longer detectable, suggesting that lactate metabolized the proteins involved in ATP production pathways. The signal of inorganic phosphate (Pi) was clearly seen when Pi concentration decreased. This was the case until the cells died. Cell death was supported from the fact that after treatment with lactate, the inorganic Pi signal gradually became extracellular Pi (see Fig. 3C; pay attention to the change in the chemical shift of Pi suggesting either cell lysis or cell membrane leakage).

Even though Pentobarbital did not kill the cells, it had a similar effect on 13C metabolites as did lactate (see Fig. 6). All 13C metabolites (except Pi) decreased in signal intensity after perfusion with Pentobarbital. Both of the drugs used in this experiment inhibited the enzymes critical to aerobic respiration of the cell suggesting that the MCF-7 cells in the improved perfusion system utilize aerobic respiration for energy production. More experiments have to be conducted to further support this argument.

Figure 1: The typical 1H-P spectrum of MCF-7 breast cancer cells during the perfusion. The metabolic components in Fig. 3 are phosphoethanolamine (PE), phosphocholine (PC), phosphatidylcholine (PC-OH), and glycerophosphocholine (GPC). Figure 4 shows the spectra of MCF-7 cells perfused with lactate or pentobarbital. The spectra show a decrease in the signals of GPC and ATP, while a slight increase in the signals of T-ATP and PC is observed. This suggests that the MCF-7 cells use the time to adjust to the environment of the agarose gel. Within phase 3, the cells are in metabolic equilibrium, and all the 1H metabolites maintain steady signal intensity. Phase 3 is the best stage to conduct drug treatment experiments. Continued until the cells die and all the metabolites decay. This process may last a few days.

Reference:
EARLY EEG & DIFFUSION MRI (dMRI) CHANGES IN AN EXPERIMENTAL MODEL OF SEVERE PERIVENTRICULAR LEUKOMALACIA (PVL)

P. Ting, M.D.\(^1\), P. Wang, Ph.D.\(^1\), L. Kinnard, M.S.\(^1\), M.M. Herman, M.D.\(^2\), R. Cohn, M.D.\(^1\)

\(^1\) College Of Medicine, Howard University, Washington D.C., \(^2\) IRP NIMH, NIH, Bethesda, MD

OBJECTIVES

The incidence of severe PVL in preterm neonates is 8 to 15%. The poor neuro-developmental outcome, especially, spastic diplegia occurs in majority of these infants. In addition, impaired visual and cognitive functions are also significant morbidity. While the etiology of PVL is multifactorial, cerebral ischemia (clinically silent) plays a dominant role. Therefore, early identification of ischemia is important for timely therapeutic intervention. The objective of the current study is to monitor EEG and dMRI profiles in the early hours of cerebral ischemia in an experimental PVL model.

DESIGN/METHODS

- Under ketamine-xenobenzamine (KAX) isoflurane anesthesia, and strict aseptic technique, 15 mongrel dogs (1-3 wk old) underwent either sham operations without ischemia (SOC, N=4) or permanent bilateral common, external and internal carotid arteries occlusion (ISCH, N=11).
- Rectal temperature and transcutaneous \(O_2\) saturation (\(O_2\) sat) were monitored during surgery and MRI measurements. The temperature was kept at 37-38\(^\circ\)C, and the \(O_2\) sat was maintained > 95%.
- Flecainide 100,000 units/kg IM qd was given for 5 days, started 1 day prior to surgery.
- Fluid and nutrition:
  - RL 150cc/dfl iv & sc
  - Gavage fed until it was returned to its dam
  - Weaned to puppy's chow after a month of age
- Body weight was recorded periodically.
- EEGs were obtained via four scalp electrodes (Grass) placed in the extreme frontal and occipital regions of the head. The EEGs were recorded on paper and simultaneously into a digital computer programmed to read the frequency and duration of all defined waves. These waves were compiled in a usable form by the computer in a time span of 10 seconds. This essentially produces on-line frequency distributions as well as statistical and other displays. EEGs were recorded prior to, and within 6h, 2, and 7 days after surgery. The animal was pre-sedated with KA and cricoid hydrate for intubation for the dMRI study. A 4.7T, 33 cm, horizontal bore Varian MRI machine was used. Serial diffusion-weighted images were obtained prior, and within 7 h of surgery, and then repeated within 12 h after surgery. The diffusion-weighted MRI technique was a modified spin-echo EPI imaging technique with two identical diffusion gradients were added before and after the 190\(^\circ\) RF pulse. Four diffusion gradients ranging from 0 to 6 Gau&uuml;sin/cm, the x, y, and z directions were used to generate a series of diffusion weighted images (Fig 2). The apparent diffusion coefficients (ADC) were derived from these images shown in Figure 3. The Regions of Interest (ROI's) were selected from the white matter in centrum semiovale, internal capsule, and optic radiation, in predetermined coronal MRI slices.
- Dogs were sacrificed at 3 months of age.
- Statistical analysis - One-way ANOVA analysis of variance per JMP program.

RESULTS

All 4 SOC survived, but, 7 out of 11 ISCH dogs died (5 at < 1 wk, and 2 at 3-4 wk post-ischemia).

The EEG data were integrated over 1-2 cycles per second frequency band (CPF). Compared to normal dogs prior to surgery (NSOC, N=17), there was a significant increase in \(\leq 3\) CPSF in EEG within 6h postischemia in both SOC (N=4) and ISCH (N=9). However, it was more marked in ISCH (Fig.4 A-C, p \leq 0.05). In addition, ISCH and SOC had respectively a significant decrease in 8-14, and 11-14 CPSF. However, the EEG of SOC and ISCH dogs normalized toward NSOC values 1 wk after surgery.

The ADC from the selected white matter areas were measured in SOC and ISCH dogs. The values of SOC were 0.065 to 0.121 mm/s/sec. For the normal, non-involved areas of ISCH, the ADC values were between 0.066 to 0.085 mm/s/sec. On Day 7 and Day 9 the ADC of the ischemic regions were 0.111 and 0.131 mm/s/sec. The relative ADC value, which was the ratio of ADC from the ischemic region to the non-involved opposite side of the brain were plotted as a function of time (Figure 6).

CONCLUSION

- Significant abnormal EEG patterns were observed within 6 hours of ischemia or sham-operation, but, the ISCH experienced marked increase in 1 to 3 CPSF than the SOC dogs. Normalization of the EEG occurred 1 week after surgery in both groups. Anesthesia and sedation had profound effects on EEG.
- The relative ADC values decreased at 24 h of ischemia in the periventricular white matter (centrum semiovale), but, increased to above SOC values on 27 days of ischemia.
- All ISCH dogs revealed abnormal triphenyltetrazolium chloride stain in the PV white matter and thalamus 6 h after ischemia, and were subsequently associated with cystic PVL and motor deficits. SOC were normal.

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