

USE OF AN OSMOTIC PUMP IN A RAT MODEL OF CHEMOTHERAPY INDUCED
CNS DAMAGE IS ASSOCIATED WITH NEURONAL INJURY

By

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Use of an Osmotic Pump in a Rat Model of Chemotherapy-Induced
CNS Damage is Associated with Neuronal Injury

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Abstract

The purpose of this study was to determine if the utilization of an osmotic pump in a rat model designed to study CNS damage secondary to intrathecal methotrexate causes cortical neuronal injury. This study was designed to mimic methotrexate treatment in children diagnosed with acute lymphoblastic leukemia. Previous research in our laboratory examined rats treated with methotrexate or artificial cerebral spinal fluid as a control. This study served to compare this previously compiled data with an additional control group, rats not receiving osmotic pump insertion. Brains from three Fischer 344 rats were perfusion-fixed and processed for histology. Microscopic images were digitally captured of the superior, medial, and inferior areas of the cortex using a 40x objective. Neurons were counted and categorized as either healthy or damaged. Statistically significant differences were found. In the superior cortex, there were more healthy neurons for the perfusion-fixed cortex (mean \pm SD = 0.771 ± 0.149) compared to the artificial CSF controls using the osmotic pump (mean \pm SD = 0.508 ± 0.309). In the inferior cortex, there were also more healthy neurons for the perfusion-fixed cortex (mean \pm SD = 0.815 ± 0.192) compared to controls (mean \pm SD = 0.517 ± 0.341).

**Use of an Osmotic Pump in a Rat Model of Chemotherapy-Induced
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Chapter 1: Introduction

Introduction and Background

Acute lymphoblastic leukemia (ALL) is a type of cancer characterized by the production of malignant, immature white blood cells. It is currently the most common leukemia and cancer in children with a peak incidence between two and five years of age (National Cancer Institute, 2013). The American Cancer Society (2014) estimates approximately 6,020 new cases of ALL in adults and children in the United States for the year 2014. ALL is known as a cancer of the blood and bone marrow as the bone marrow is responsible for producing blood stem cells. These stem cells further develop into either a lymphoid or myeloid stem cell. A lymphoid stem cell then becomes a lymphoblast, which develops into one of three types of lymphocytes. Lymphocytes play an integral role in the body's defense mechanisms. In ALL, these cells continuously multiply thereby crowding out normal cells, leaving the body unable to effectively protest against infections (National Cancer Institute, 2013).

Between 1975 and 2010, mortality rates for children with cancer have decreased by more than 50% (American Cancer Society, 2014). For ALL, the five-year survival rate has increased over the same time frame from approximately 60% to an estimated 90% for children younger than 15 years of age (National Cancer Institute, 2013). While survival rates have significantly increased over the years due to improved treatment methods, long-term cognitive deficits still remain. One treatment method for ALL includes intrathecal chemotherapy directly administered into the central nervous system (CNS). Although this treatment has greatly improved survival,

significant and persistent neurocognitive deficits occur including changes in attention, working memory, and executive functioning (Robinson, 2010).

In a previous study conducted by Humphrey, Merkle, Moore, and Ross (2012), the goal was to determine if intrathecal methotrexate therapy causes neuronal injury in children with ALL. Efforts to understand the severity and mechanism behind these changes included the use of rat models. The animal experiment allows researchers to manipulate variables in order to analyze the affects of methotrexate on the brain. In Humphrey et al. (2012), only the rats treated with methotrexate and artificial CSF as a control were analyzed. In this study, a second control group without the insertion of an osmotic pump was analyzed and compared to previously attained data.

Purpose and Significance

Previous research in our laboratory shows differences between neuronal injuries in the cortex of rats after intrathecally-administered methotrexate compared to controls with intrathecally artificial CSF alone. However, in order to deliver the methotrexate and control artificial CSF, an osmotic pump is inserted into the ventricle of the brain and is used to deliver the methotrexate or artificial CSF. Thus, it is possible that the surgical procedure, osmotic pump insertion, and/or delivery through the osmotic pump causes neuronal injury. The purpose of this study was to determine if the number of injured neurons in the cortex is different between that of rats exposed to both the osmotic pump insertion and artificial CSF delivery process and control rats without the procedure. This is clinically significant in order to refine current research methods in order to better understand neurocognitive deficits in children treated for ALL.

Chapter 2: Review of Literature

Epidemiology and Treatment of Acute Lymphoblastic Leukemia

ALL is classified as the most common cancer in children with a prevalence of approximately 25% of cancer diagnoses among children younger than 15 years of age (National Cancer Institute, 2013). With an annual rate of 35 to 40 cases per 1 million people in the United States, that is approximately 2,900 new diagnoses of ALL each year (National Cancer Institute, 2013). A sharp peak can be seen among children aged 2 to 3 years of age with more than 90 cases per 1 million per year (National Cancer Institute, 2013). Additionally, the incidence of ALL is highest among Hispanic children with approximately 43 cases per 1 million, and about threefold higher incidence in white children than in black children (National Cancer Institute, 2013).

Current treatment regimens for childhood ALL can vary depending on age, health status at the time of diagnosis, and the results of the cytogenetic testing. Treatment can be divided into four phases with the goal being a cure: induction therapy, consolidation therapy, maintenance therapy, and central nervous system prophylaxis. Induction chemotherapy is used to rapidly kill leukemia cells residing in the blood and bone marrow, with a goal of putting the leukemia into remission (National Cancer Institute, 2013). A complete remission is attained when the blood and bone marrow do not show evidence of persistent leukemia and blood counts return to normal. Once remission is achieved, the consolidation phase is started with the intention of destroying any remaining leukemia cells, and prevents any new cells from growing. This step typically includes delivery of multiple cycles of intensive agents over a period of six to nine months (National Cancer Institute, 2013). Following this intensive chemotherapy regime, the maintenance phase begins which involves taking oral chemotherapy pills for approximately 18 to

24 months and having monthly blood tests performed. Maintenance therapy serves to destroy any new or remaining leukemia cells that could lead the patient into relapse (National Cancer Institute, 2013). Lastly, central nervous system prophylaxis is also used to prevent metastasis of ALL in the spinal fluid through the use of intrathecal chemotherapy. This is performed by infusing chemotherapy directly into the clear spinal fluid by inserting a needle between the vertebrae of the lower back.

Cognitive Deficits in Children Treated for ALL

There are a number of studies attempting to uncover the extent and resulting consequences of cognitive decline due to central nervous system treatment. Although current treatment regimens for ALL are associated with a decrease in adverse side effects compared to previous methods, survivors are still at risk for increased cognitive impairments secondary to disease and treatment (Ashford et al., 2010). Cognitive processes such as memory, attention, intelligence quotient (IQ), and white matter volume are being evaluated to determine the long-term affects of chemotherapy treatment.

One of the changes observed within the brain of children treated for ALL include decreases in white matter volumes (Ashford et al., 2010). These decreases have been associated with deficits in attention, memory, lower IQs, and declines in academic achievements (Ashford et al., 2010). Treatment-induced white matter changes play an integral role in a child's cognitive functioning. This can affect their ability to excel in school. For example, one of the most frequently observed deficits for childhood survivors of ALL is difficulty with mathematics (Harshman et al., 2012).

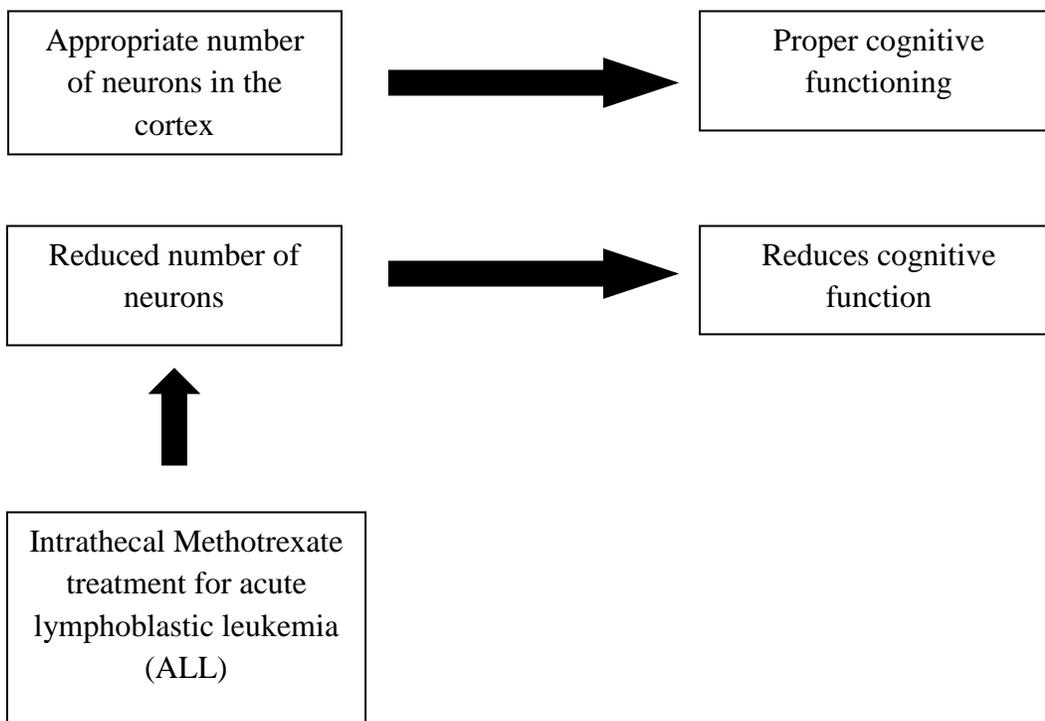
Another area for consideration is the emotional development of the child. In a study conducted by Campbell et al. (2009), the objective was to examine the role of executive function

in coping and behavioral outcomes in children survivors of ALL. Changes occurring within the brain may have negative consequences on emotional development and regulation. This can affect the capacity for coping, which may impact the child and family's quality of life. Some reported attributes for children cancer survivors who were treated for leukemia included elevated levels of emotion distress, depression, anxiety, and social issues (Campbell et al., 2009).

Conceptual Framework

Proper cognitive functioning involves the adequate number of neurons functioning correctly within the cortex of the brain. The delivery of intrathecal methotrexate in order to treat ALL, results in neuronal injury thus reducing the number of healthy neurons in the cortex. This neuronal injury resulting in a decline in the number of functioning neurons contributes to a decline in cognitive function. Below is a diagram representing this conceptual framework.

Figure 1: Diagram of conceptual framework (Humphrey et al., 2012).



Chapter 3: Methodology

Original Animal Experiment

Protocol developed by this laboratory was used in the study conducted by Humphrey et al. In the current study, the same protocol was utilized. A total of nine Fischer 344 rats (Harlan Laboratories, Inc., Indianapolis, IN) were randomly assigned to one of three groups. A total of three rats were assigned to each of the following groups: rats receiving methotrexate, rats receiving artificial CSF, and rats receiving perfusion only, which served as a control group. The treatment animals analyzed in this study received a methotrexate dosage of 4mg/kg for five days. This dosage is comparable to current practices for children being treated for ALL (Humphrey et al., 2012).

Rats receiving either methotrexate or artificial CSF were anesthetized prior to receiving treatment. Treatment was delivered by use of an osmotic pump (Alzet Micro-Osmotic, Model 1003D) placed directly into the ventricle of the brain. Following that, all animals including treatment and control, were sacrificed by being placed into a gas anesthetic (isoflurane) vaporizer. The rat brains were then perfused with 50mL PBS, followed by 100mL of paraformaldehyde. Once the brains were removed and fixed, they were placed in a vial of room temperature formaldehyde to allow for preservation.

To prepare the slides for study, the rat brains were removed from the beaker with a large spatula and carefully placed into the brain slicer. Microtome blades were placed in the grooves every 2mm, beginning at the front of the brain. Once sliced, the blades were individually removed and small forceps were used to slide the brain slice into a six-well plate. These plates were labeled with the date, storage solution, orientation of brain slices, and identification of the assigned group.

Section Processing

The six-well plates were then sent to Histology or Tissue Acquisition Cellular/Molecular Analysis Shared Service (TACMASS) for staining. Sections were 5 μ m in size with a 50 μ m section skipped in between each subsequent section. The 5 μ m sections were then formalin fixed and paraffin embedded. Sections selected for staining were deparaffinized using xylene and ethanol and then stained with hematoxylin and eosin. The sections were mounted onto slides with cover slips placed on top and allowed to dry for seven days.

Microscopy

Microscopic images were digitally captured using a Leica Microsystems (type 090-135.002) microscope. By placing slides in the same orientation, images could be properly identified and labeled in the same manner for each section. Each slide contained three sections of the particular rat being studied. After identifying the section as right or left, each side of the brain was further divided in three sections of the cortex labeled as superior, medial, or inferior. Three consecutive images were captured at each position using a 40x objective. Images were then saved and labeled with the rat number, slide number, slice number, HE1 designation, magnification, section number on the slide, left or right cortex, superior, medial, or inferior area, and picture number.

Cell Counts

Similar to how Humphrey et al. (2012) performed cell counts, neurons were counted and categorized as either healthy or damaged using the ImageJ program downloaded from the National Institutes of Health. The Analysis and Cell Counter plug-ins were additional tools downloaded for use. A region of interest (ROI) set at 200.08 μ m by 200.08 μ m, which corresponded to 820 pixels x 820 pixels, was used for each picture. The location of the ROI was

selected by entering x and y coordinates, 380 by 100 respectively, in order to locate the center of the picture. Neurons were then counted according to their classification and recorded in a Microsoft Excel spreadsheet. A healthy neuron was defined as having a nucleus or nuclei as clearly defined and the nucleus being within a clearly defined cell body. Injured neurons shrink, become eosinophilic due to the condensation of mitochondria and the nuclei become pyknotic, therefore, a damaged neuron was defined as having no distinguishable nucleus and the cell being heavily stained with a withered structure indicating cell rupture. Inter-rater reliability was performed by a second person randomly selecting and counting 15 percent of the total images.

Statistical Analysis

The number of pictures was used as 'n' for the statistical analysis. First, the mean percentages of healthy neurons for the three areas of the cortex were calculated. The student's t-test was used to evaluate possible differences between the means using a significance level of $p < 0.05$. Additionally, a correlation coefficient was calculated for the inter-rater reliability.

Chapter 4: Results

The purpose of this study was to determine if the utilization of an osmotic pump in a rat model designed to study CNS injury secondary to intrathecal chemotherapy causes neuronal injury in the rat cortex. Thus, images at the superior, medial, and inferior areas of the cortex of the perfusion-fixed rat brains were captured. A total of 159 images were captured, and each image was counted to determine the percentage of healthy neurons. Inter-rater reliability was performed by counting 15 percent of the total images. This is done in order to compare neuron counts with another researcher who is more experienced. As shown in Table 1, a correlation coefficient of 0.979 was calculated for the inter-rater reliability. This represents a strong positive correlation of the counted neurons, indicating that both researchers attained similar results for the number of neurons counted. This value is also demonstrated by the R squared values for each category, displayed in Table 1. Figures 2-4 are a graph representation of neuron counts in each category, which compare the percentage of neurons counted by each researcher.

Table 1: Inter-rater reliability correlation coefficients.

	Healthy Neurons	Damaged Neurons	Percent of Healthy Neurons
Correlation	0.963	0.971	0.979
R-Squared	0.927	0.944	0.959

Figure 2: Inter-rater reliability for healthy neuron cell counts.

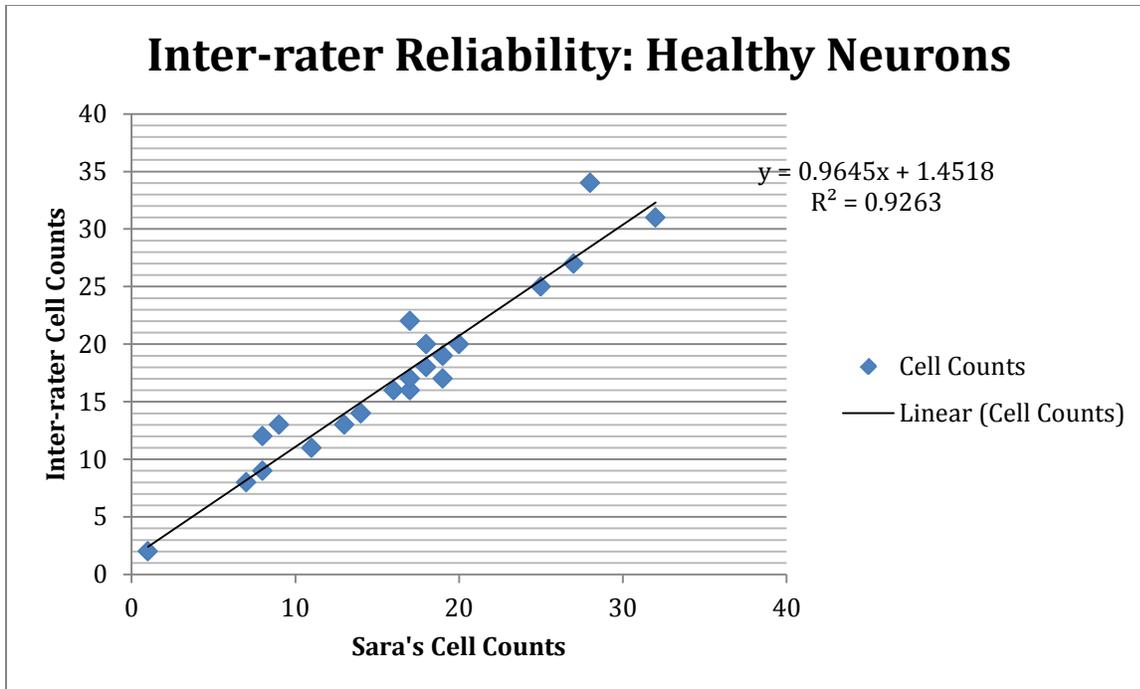


Figure 3: Inter-rater reliability for damaged neuron cell counts.

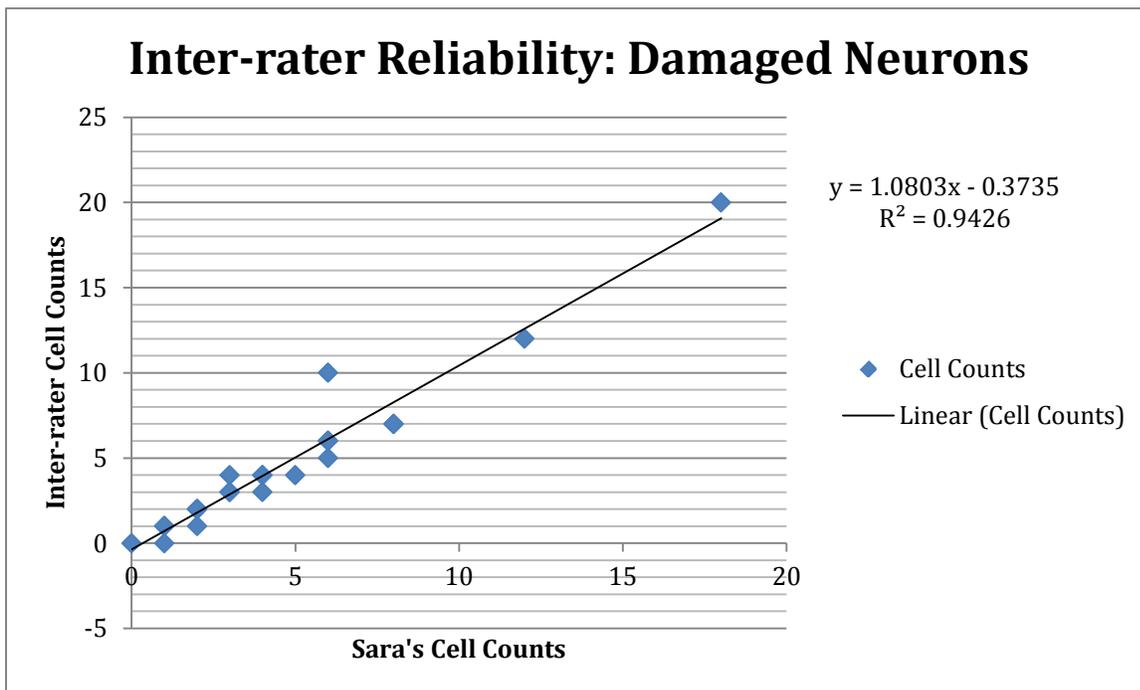
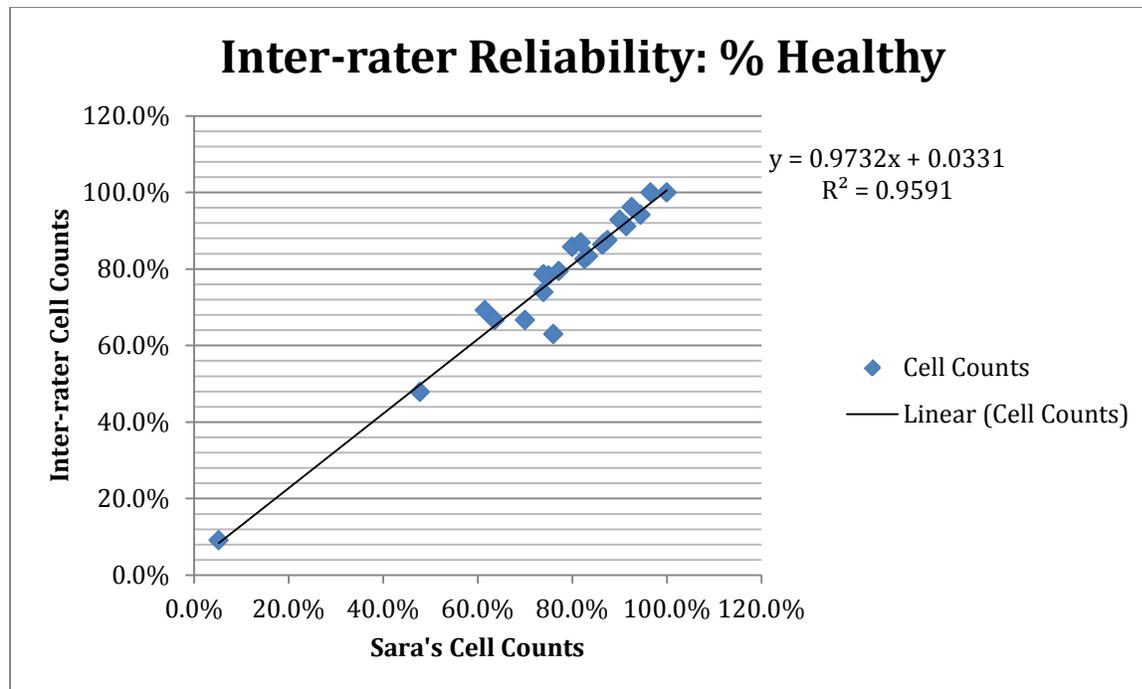


Figure 4: Inter-rater reliability for the percentage of healthy neurons.



The results from Tables 2 and 4 show that statistical significance ($p < 0.05$) was found for the superior and inferior areas of the cortex. In the superior cortex, there were more healthy neurons for the perfusion-fixed cortex (mean \pm SD = 0.771 ± 0.149) compared to the artificial CSF controls using the osmotic pump (mean \pm SD = 0.508 ± 0.309). In the inferior cortex, there were also more healthy neurons for the perfusion-fixed cortex (mean \pm SD = 0.815 ± 0.192) compared to controls (mean \pm SD = 0.517 ± 0.341). These results suggest that the insertion of an osmotic pump to deliver artificial CSF does cause neuronal injury demonstrated by the fewer number of healthy neurons present in the cortex when compared to the perfusion-fixed cortex.

While there were still a greater percentage of healthy neurons in the medial area of the cortex for the perfusion-fixed rats (mean \pm SD = 0.762 ± 0.211) compared to CSF controls (mean \pm SD = 0.717 ± 0.218), no statistical significance was found. The results from Table 3 represent this.

Table 2: Comparison (Based on Percentage of Healthy Neurons) in the Superior Cortex

	Group 1: MTX (4+1 day) Superior Cortex	Group 2: Artificial CSF Superior Cortex	Group 3: No Osmotic Pump Superior Cortex
n	45	52	54
Mean	0.609	0.508	0.771
Standard Deviation	0.315	0.309	0.149

*P Value: Group 1 vs. Group 2 = 0.056; Group 1 vs. Group 3 = 0.001; Group 2 vs. Group 3 = 0.0001

Table 3: Comparison (Based on Percentage of Healthy Neurons) in the Medial Cortex

	Group 1: MTX (4+1 day) Medial Cortex	Group 2: Artificial CSF Medial Cortex	Group 3: No Osmotic Pump Medial Cortex
n	52	46	54
Mean	0.556	0.717	0.762
Standard Deviation	0.329	0.218	0.211

*P Value: Group 1 vs. Group 2 = 0.003; Group 1 vs. Group 3 = 0.0002; Group 2 vs. Group 3 = 0.298

Table 4: Comparison (Based on Percentage of Healthy Neurons) in the Inferior Cortex

	Group 1: MTX (4+1 day) Inferior Cortex	Group 2: Artificial CSF Inferior Cortex	Group 3: No Osmotic Pump Inferior Cortex
n	54	54	51
Mean	0.661	0.517	0.815
Standard Deviation	0.250	0.341	0.192

*P Value: Group 1 vs. Group 2 = 0.007; Group 1 vs. Group 3 = 0.0006; Group 2 vs. Group 3 = 0.0001

Chapter 5: Discussion

Summary of Findings

The purpose of this study was to determine if the utilization of an osmotic pump in a rat model designed to study CNS injury secondary to intrathecal chemotherapy causes neuronal injury in the rat cortex. The superior and inferior areas of the cortex reached a level of significance, indicating that insertion of the osmotic pump to deliver artificial CSF causes greater neuronal injury compared to perfusion-fixed controls. However, while the medial areas of the cortex also had a higher percentage of healthy neurons in the perfusion-fixed controls, no statistical significance was reached.

Importance of Findings to Oncology Nursing

The results suggest that the insertion of an osmotic pump with delivery of artificial CSF alone causes cortical neuronal injury. This has important implications for studies on the effects of intrathecally-introduced methotrexate through an osmotic pump in a rat model. Hence, previously collected data attained using this model should be reevaluated to better understand neurocognitive deficits in children treated for ALL. This includes determining where methotrexate causes neuronal injury in the brain in order to understand its effects on cognitive functioning. Discerning the mechanism behind how intrathecally administered methotrexate impacts the brain will be important in preventing neuronal damage. This will influence the oncologic nursing care received by these patients, as well as, preventative and interventional treatments.

Strengths and Limitations

Strengths of this study included the ability to compare to previous work done by Humphrey et al., 2012. Looking at all three groups provided a greater picture of neuronal injury caused by either the use of the osmotic pump to deliver methotrexate or artificial CSF.

Additionally, the dose of methotrexate used in the treatment group was comparable to doses used in children to treat ALL. An additional strength of this study was the fact that various areas of the cortex (superior, medial, inferior) were examined, providing a more comprehensive look at how the use of an osmotic pump affects a rat brain.

Limitations of the study include the small sample size. Only three rats were used in each group examined. Furthermore, throughout the study several images captured had to be excluded due to excessive tissue damage, which affects the ability to accurately count neurons. This could have affected the mean percentage of healthy neurons, and thus the significance of the results may have been altered. Additionally, the study only examined the cortex of the rat brain. Future research should include other areas of the brain such as the hippocampus, which serves as an important area for learning and memory. Acquiring knowledge on what areas of the brain are negatively affected by this treatment will serve as an important component in the development of interventional measures and preserving the quality of life in children treated for ALL.

Conclusions

In conclusion, this study suggested that the insertion of an osmotic pump to deliver fluids into the ventricle of a rat brain does cause neuronal injury within the cortex. Both the superior and inferior areas of the cortex reached a statistical significance, with a higher percentage of healthy neurons existing in the perfusion-fixed control compared to both the artificial CSF and methotrexate group. These studies will provide important insight on the effects of intrathecally-introduced methotrexate through delivery of an osmotic pump in a rat model. Determining the mechanism of how intrathecally methotrexate affects the brain will be important in preventing neuronal damage and understanding why children have cognitive deficits after receiving

chemotherapy. This will allow for preventative and/or interventional measures to be implemented into oncologic nursing care.

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