Adverse Aftereffects of Methotrexate as Chemotherapy on Cognitive Deficits in Rat Models

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Acceptance of Senior Honors Thesis

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Abstract

Patients with acute lymphoblastic leukemia have a high five-year survival rate thanks to methotrexate (MTX). However, cognitive side effects are reported, characterized as chemo brain. The study investigated if impulsiveness is part of the aberrant cognitive functions after being exposed to MTX in the early stage of physical development. Adolescent rats were injected with either phosphate-buffered saline (PBS) or MTX. The novel object recognition (NOR) task was conducted a month after the injections to measure the memory deficits. The discounting task was performed after the rats completed training on a fixed-ratio one schedule for both levers. The NOR test showed both the PBS and MTX rats recognized the novel object; however, the PBS group spent more time inspecting the novel object than the MTX group. Initial results from two rats (one from each group) for the discounting task showed that the PBS-treated rat preferred the immediate reward at the beginning and had increased omissions during the later trials. The MTX rat showed a consistent preference for the immediate lever across the entire procedure. These composite findings suggested cognitive deficits are not apparent within one month of MTX treatment, yet the impulsiveness is more apparent after MTX exposure.

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Childhood Leukemia and Chemotherapy

It has been known that childhood acute lymphoblastic leukemia (ALL) has a relatively good prognosis with over 80% of the patients having a five-year-after-cancer survival rate (Nathan et al., 2004; van der Plas et al., 2015). While the cure of the malignant cancer has been targeted as a priority in the past, psychiatrists have questioned the association of the cognitive dysfunction as an aftereffect of the chemotherapy in the pediatric brains (Ikonomidou, 2018). The main chemotherapy utilized for ALL patients is methotrexate (MTX). The effect of the chemotherapy can vary from one patient to another based on gender, age, and other demographics, yet the common adverse effects include disturbances in recalling memories, loss of ability to concentrate, and poor academic performance (van der Plas et al., 2015). These cognitive dysfunctions are commonly referred to as chemo brain or chemo fog since “fogginess” impairs the normal operation of the brain (Ikonomidou, 2018). Although there are many cases associated with the cognitive dysfunctions followed by traumatic brain injuries, including strokes, neurodegenerative diseases, and cerebral infarctions, the chemotherapy is a human intervention to treat an existing disease. Because the treatment is meant to minimize possible aberrant effects, it is important to consider ways to minimize any side effects resulting from the treatment. For instance, antiemetic drugs are given to treat the nausea associated with many chemotherapeutics (Dupuis, et al., 2016).

The common age group affected by ALL ranges from two to five years old, which underlines the importance of protecting the quality of future life after overcoming this deadly disease. The young age of these patients demands further studies concerning the vulnerability of the developing brain to the toxicity of MTX. It also suggests that developing novel treatments to

prevent these impairments are of great importance for the medical community (Krull et al., 2011).

Methotrexate Mechanism of Action in Cancer

MTX is the main chemotherapy for patients with ALL. MTX is often given orally, which is then directly digested by the stomach and undergoes metabolic processes (such as first-pass metabolism), reducing the degree of the toxicity of MTX to the body (Angelov et al., 2009). Intravenous (IV) administration of MTX is efficient in distributing the drug throughout the systemic circulation with the half-life of approximately 8-12 hours. Due to its lack in lipophilic property, the penetration of the drug across the vascular endothelium will occur slowly and require a high dose, which is around 1000mg/m^2 (Bleyer, 1977). An alternative way to administer MTX is an injection through the subcutaneous tissue or by deep intramuscular injection, which will stimulate faster and higher intensity of the drug effects. To target brain tumors, scientists have developed a specific pathway to administer MTX,

which is to inject it intracerebroventricularly. This study suggests that the likelihood of MTX, naturally crossing the blood-brain barrier (BBB) is low as the tight endothelial junctions will prevent the larger molecules from being passed without specific transporters (Angelov et al., 2009). However, IV administration of MTX with the high dose (>1000mg/m^2) can reach the required concentration for the therapeutic effect on the brain tumor. This is because the plasma composed of three to ten percent cerebral spinal fluid (CSF), which allows the high dose of MTX to leak into the CSF (Comandone et al., 2005).

MTX is a folic acid analogue, inhibiting the dihydrofolate reductase (DHFR) enzyme. This enzyme is involved in several essential pathways: to produce purines, pyrimidines, and interconversion of amino acids. DHFR enzyme catalyzes the reduction of dihydrofolic acid

(DHF) to tetrahydrofolic acid (THFA) that acts as the precursor of the formation of thymidine, purines, and methionine, ultimately leading to RNA and DNA synthesis (Breedveld et al., 2004). When MTX is introduced to those pathways, MTX plays a role as a competitive inhibitor of DHF because MTX and DHF are similar in their chemical structures. In addition, MTX has the affinity for DHFR about 1000-fold that of DHF, which then efficiently prevents the reaction progress. The lack of THFA due to the pathway inhibition by MTX blocks the DNA, RNA, and protein syntheses, consequently leading the affected cells to undergo apoptosis. This blockage of the pathway is especially fatal to the malignant tumor cells as they require significantly higher amount of DNA replications than the normal cells (Dheen, Kaur, & Ling, 2007).

The further mechanism that is affected by a decreased amount of THFA is the lack of methionine protein availability in the cells, which is regulated by the conversion of THFA. Methionine protein is an essential component for RNA synthesis (specifically for transfer RNA), but more importantly playing its role in polyamines. Polyamines are found in high concentrations in tumor cells with the function of the proliferation of the cell divisions while decreasing the prospect of cellular apoptosis. To counteract the increased number of polyamines, the reduced amount of methionine will decrease the function of tRNA as methionine dictates the initiation of the polyamine synthesis. Disruption of producing a protein chain will ultimately destroy the fast-growing cancer cells due to no working proteins in the cell (Hagner & Joerger, 2010).

Epigenetics on MTX

The use of MTX is also prominent in inflammatory autoimmune diseases such as rheumatoid arthritis (RA). DNA methylation is part of an epigenetic mechanism, which regulates and manages the expression of the gene. Clinical studies have discovered the significant increase in differential DNA methylation following the MTX treatment. An alteration of gene expression

allows the tendons around the affected joints to be repositioned, which then consequently strengthens the tendons around the patients' joints. Although it is not completely mapped out, MTX's wide range of influence in a biological system underlines the importance of investigating its complex mechanism in the body (Nair et al., 2019).

Toxicity of MTX

Damage in Microglial Cells

Microglial cells are known to initiate and manage the inflammatory responses in the brain, playing essential roles in recognizing neuronal damage and phagocytizing dead particles. However, many studies have found the correlation between the neuronal loss in the consequence of the overactivated microglia in the brain. Physicians have found long-term neurological deficits in young patients who underwent chemotherapy (Dheen et al., 2007). The exact mechanism of microglial activation due to anti-metabolites, such as MTX is not well understood. A study suggests that MTX is accompanied by oxidative stressors in the brain, which restricts essential pathways taken in the cellular reactions (Rajamani, Muthuvel, Senthilvelan, & Sheeladevi, 2006). Microglia are triggered in the brain, which gather cytokines and other immune responses to the affected area (Wen et al., 2018). This immunological signal accompanies neuronal damages introducing the synergetic effect of the apoptosis of the neurons (Seigers et al., 2010). Due to activated microglia's detrimental effects, the future direction of the study can be targeting the inhibition of the microglial activation by administering a "rescue drug" that can compensate the toxicity of the MTX to the brain.

Hindrance in Astrocytes and Oligodendrocytes

Reactive oxygen species that cause oxidative stress in the cellular level further instigate the malfunction of astrocytes, whose roles are to connect the BBB and neural cells for the

purpose of delivering nutrients and communicating between the cells (Shao, Tan, Shi, & Zhou, 2019). Oxidative stress induced by the chemotherapy in the brain not only provokes an apoptotic pathway in neurons but also disrupts the differentiation of oligodendrocytes (Mueller et al., 2013). In the mouse models, the thickness of myelin sheaths showed significant reduction upon the MTX treatment compared to healthy controls. This phenomenon was persistent or became worse after six months of stopping the MTX treatment. Reported damage in white matter in the brain showed consistent results with the previous observation as incompletely differentiated oligodendrocytes will be hindered from synthesizing myelin sheaths (Kovalchuk & Kolb, 2017).

Impulsivity and Brain Damage

Failure to tolerate delays for better rewards indicates impulsiveness. This behavior can often be found in children, ranging approximately from three to five years old (Cardinal, Robbins, & Everitt, 2000). The most represented experiment to measure the delay-gratification for children in such ages was conducted at Stanford in 1972, called the Stanford marshmallow experiment by Walter Mischel. From the experiment, young children struggle not to eat a small marshmallow placed in front of them after they are told that they will be given two marshmallow pieces if they can wait for a few more minutes (Mischel, Ebbesen, & Zeiss, 1972). An ability to control one’s desire for better outcomes in the later time becomes more perfected as the brain develops throughout the growing phase from the youth to the period of adolescence and even to young adults. However, MTX disrupts the production of the normal level of the brain amines, including dopamine and serotonin (Madhyastha, Somayaji, Rao, Nalini, & Bairy, 2002, 2005). Insufficiency in such amine molecules in the developing brain can be detrimental, causing dysfunction of prefrontal cortex and basal ganglia, which are known to manage memory and behaviors (Langen et al., 2012; Whitaker-Azmitia, 2001; Zhang et al., 2010).

A similar trend happens with smokers. They tend to choose an immediate monetary reward rather than waiting a little longer to receive a greater value of rewards. This test is called the Kirby delay-discounting task (Jaroni, Wright, Lerman, & Epstein, 2004). The study shows that discounting the profit of delayed reward is prominent among smokers and drug abusers. They focus on the physical desire rather than to evaluate the aftermath of such decision, characterizing impulsive behavior. In addition, further analysis following the discounting task suggests that the impulsive conduct is correlated with worse performance in their academics and low attention abilities. This relationship indicates that testing impulsivity can predict the damage in releasing certain types of neurotransmitters that regulate rational thinking and intrinsic motivation. Such inputs are dopaminergic, serotonergic, noradrenergic, and cholinergic to execute proper prefrontal cognitive function. The low-quality performance can be treated and improved by targeting the neurotransmitters that are altered in relation to impulsive behavior as well as applying a behavior modification approach. This finding is essential for young survivors of ALL who exhibit similar phenotypic result in their cognitive abilities (Logue & Gould, 2014).

Operant Conditioning and Delay Discounting Task

Operant conditioning was first defined by B.F. Skinner in 1937 in a context of learning behavior by relating the environment and behavior. This method is indeed an efficient tool to investigate the behavioral changes based on either reinforcement or punishment with the intent of either inducing the targeted behavior or removing the targeted behavior. Two types of interventions (whether to add or eliminate) and two types of behavioral responses (whether to induce or prevent) can be combined and result in four possible experimental designs, including positive punishment, positive reinforcement, negative punishment, and negative reinforcement. *Positive* means adding an influential factor, while *negative* means taking away an influential

factor. Punishment is the concept to decrease the behavior, while the reinforcer is to increase the behavior. Then, positive punishment means adding something to decrease the behavior; positive reinforcement means adding something to increase the behavior; while negative punishment is to remove something to discourage the behavior; and negative reinforcement means to remove something to encourage the behavior. One important feature regarding operant conditioning is that the environmental conditions responsible for the behavior are known and that behavior can be very stable for long periods of time when conditions are not altered. Extinction of the behavior can occur under certain procedures despite no changes in the reinforcements to maintain behaviors such as with high-ratio schedules, yet some behaviors may be persistent in spite of reinforcement or punishment (Staddon & Cerutti, 2003).

The delay-discounting method to assess impulsivity utilizes the positive reinforcement, where animal models can be reinforced by the number of sucrose pellets granted during the experimental session. Rats are encouraged to wait longer to receive more pellets and discounting occurs when a rat chooses the immediate lever instead of waiting longer for the larger reward. The behavior necessary for receiving either an immediate reward or a later reward can be the same, such as pressing the lever once. In this situation, only the delay serves as a factor. Additional factors can be used, such as differential interval time rather than fixed interval time to determine the cutoff where the amount of delaying time compared to the reward (the number of sucrose pellets) is no longer effective (Thompson et al., 2017).

Novel Object Recognition and Executive Function

Novel object recognition (NOR) is to measure the recognition memory of experimental animal models who may have suffered from brain injury. Studies have shown that the rodents tend to spend more time with the novel object as they find it more interesting than the object with

which they are familiar. This study was first done by Ennaceur and Delacour (1988) and was continued to evaluate the behavioral and cognitive changes of the subjects (Grayson et al., 2015). This test will function as a positive control in the MTX-induced-rats study because the study aims to investigate the impulsive behavior, while the cognitive dysfunction is expected based on previous studies.

Literature Gap and the Purpose

The pathophysiology of the chemotherapy MTX on brain dysfunction is incompletely understood especially among the younger age group of patients. Cognitive deficits due to MTX are problematic especially for pediatric patients since the common age group affected by ALL is two to five. Recognizing phenotypic disorder (abnormal behavioral changes such as impulsiveness) induced by MTX will provide additional information on the MTX effect on the cognitive dysfunctions. Furthermore, observing MTX's toxicity on an anatomical abnormality in the developing brain may inform the key region of the brain that can be protected. This study, therefore, asks whether the MTX-induced subjects show behavioral changes, including cognitive deficits and impulsivity by utilizing adolescent rat models to mimic the developing brain.

Materials and Methods

Animal Subjects

Preliminary Study for Appropriate Sex Choice of the Rat Models. Adult male (n=12) and female (n=12) rats from Charles River were used for the preliminary study. Upon arrival, rats were group-housed by sex (two rats/cage) and were acclimated for one week with food and water ad libitum. The rats were grouped by their sex. Rats were singly housed during operant conditioning studies.

MTX Impact Study. Adolescent male (n=6) and female (n= 6) Sprague Dawley rats from Charles River were used for the MTX study. Upon arrival at postnatal day 20, the rats were singly housed and acclimated for one week. The temperature was kept consistent (22°C minimum & 25°C maximum), and a controlled light cycle was applied to regulate the sleep cycle of the subjects. Then, the 6 rats were randomly selected (3 males and 3 females) as a control group (PBS, vehicle) and the rest as an MTX-induced group. The demographic data, including sex, weight and the physical appearance or abnormality were recorded.

NOR Test Setting

Novel object recognition (NOR) test was performed in a cardboard box (24-in x 18-in x 18-in) with a Plexiglas bottom. The day before the actual NOR trial, the rats were allowed to explore the box for 5 minutes in order for them to be habituated to the experimental environment without objects in the box. The next day, all rats underwent a paired experiment—a sample trial took precedent over a test trial. The objects were 35 cm apart (Figure 1). Each trial was conducted for 3 minutes and the box was cleaned at the end of the experiment. For the sample trial, two identical objects were placed. There was a one-hour breaktime between the sample and the test trial. During the test trial, one object was the same as the previous test and the other object was a new object (novel object). All objects were fixed to the floor so that they could not be moved by the rats (Fardell, Vardy, Logge, & Johnston, 2010).

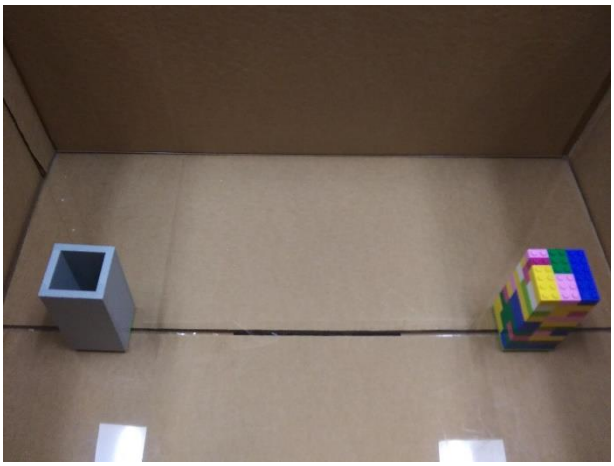

Figure 1. The NOR test trial box. The object in the left was the used as the familiar object, while the object in the right was used as the novel object (the box dimension: 24-in x 18-in x 18-in).

Operant Conditioning Chambers

Six identical chambers were used (Med Associates). Each apparatus was equipped with an overhead houselight (2.8-W). Two retractable levers with two stimulus lights above the levers were positioned opposite from the houselight, at a height easily reachable by the rats. Between the levers, a tray was located. Behind the tray, a tube was connected through which sucrose pellets were delivered from the pellet dispenser (Cardinal et al., 2002).

Saline (PBS) and MTX Injection

For MTX impact study, all rats were injected on postnatal day 26 into the intraperitoneal cavity. The amount of MTX was measured based on the rat weight (250mg of MTX in PBS (pH 7.4) / kg of body weight). The solution was made one hour prior to the injection.

Training

Preliminary Study for Appropriate Sex Choice of the Rat Models. Rats were trained under a fixed-ratio 1 (FR1) schedule of reinforcement. In order to be included in subsequent experiments, rats had to achieve three days of responding for at least 50 pellets in a one-hour

time period. All 12 male rats and 11 female rats successfully completed this training within one month (3-4 weeks).

MTX Impact Study. The animal subjects started training on postnatal day 30 because an adolescent period for the rats marks around 28-42 days (Hammerslag & Gulley, 2014). The rat models were put under a FR1 schedule of reinforcement. In order to be included in multiple subsequent experiments, the subjects had to first respond for 40 sucrose pellets with the left lever and then for 40 sucrose pellets on the right lever. In both cases, only one lever was available. The rats who completed the left lever training were moved to the right lever training. All chambers had the houselight on throughout the session and the session lasted for one hour, and rats were tested five times a week until the criterion was met. Four male rats (three control rats and one MTX-induced rat) and four female rats (three control rats and one MTX-induced rat) successfully completed this training within 3-4 weeks by reaching 40 pellets from the left lever and 40 pellets from the right lever.

Experimental Sessions

Preliminary Study for Appropriate Sex Choice of the Rat Models. Rats were run in the chamber for 30 minutes three times a week. Rats were initially tested under an FR1 schedule that was increased to an FR2, 4, 8 and 16 each subsequent week. Rats were then tested in a variable-ratio 5 (VR5) schedule, fixed-interval 30 second (FI30) schedule, and variable-interval 30 second (VI30) schedule over the next three weeks.

MTX Impact Study. The experimental design for the choice procedure is based on the one developed by Evenden and Ryan (1966), which allows multiple modifications in days, time periods, and signaling methods. Rats were run in the chamber until completion of the procedure which can last from sixty to one-hundred minutes. The equal session took two times a week.

The overall experimental design is illustrated below (Figure 2). This schematic map is modified based on the previous study by Cardinal, Robbins, and Everitt. The session was initiated with the houselight switched off and the levers retracted. Each trial began with the houselight on and both levers extended. The rat was required to press either of the levers. If the rat missed the trial by not responding within 10 seconds at the start, it was recorded as a Type 1 omission and the chamber was reset to the initial state in preparation for the next trial. The rats were offered two choices. The left lever was designed for the delayed, but greater rewards, five sucrose pellets (the time of delay increased as the trial continued for an hour), whereas the right lever was denoted for the immediate fulfillment of the request, but with less value, one sucrose pellet. The total number of sucrose pellets desired by the rats was counted. Also, rats' choices between two levers as the delays became longer throughout the session were recorded to observe the threshold of when the value of instant one sucrose pellet was greater for the rats than the value of the long-waiting for the five sucrose pellets (Cardinal et al., 2002).

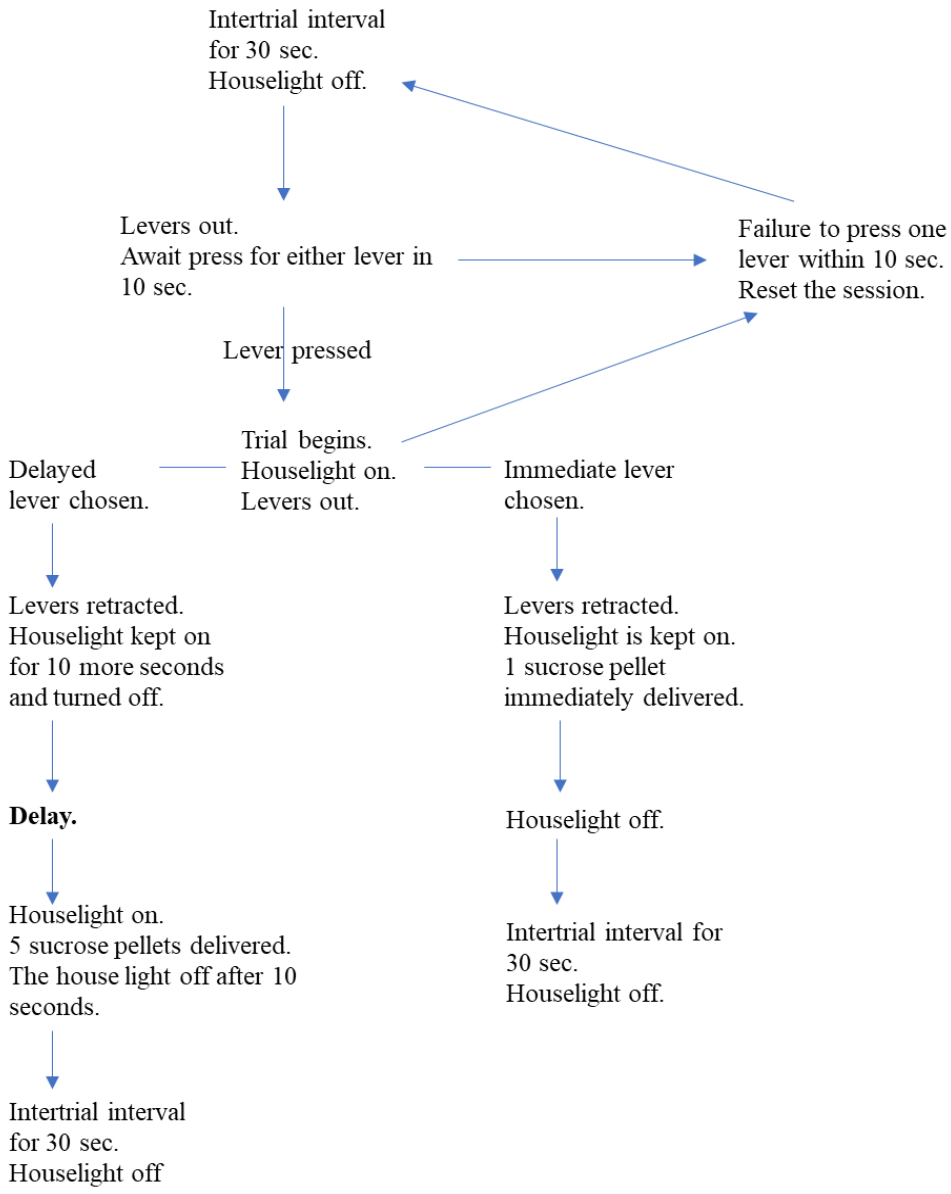

Figure 2. The schematic map each trial. The comparison between immediate and delayed responses are presented side by side in order to deliver two different pathways more clearly. The intertrial interval time is indicated as well as whether the houselight is switched on or off. The exact time of the delays is not represented since the delays will vary (increasing) throughout the session.

Data Collection and Statistical Analysis

Data were collected using the MED-PC® V Software Suite. Cumulative records were plotted usingSoftCR™ Pro. Graphs were produced using Microsoft® Office Excel 2016. Data were analyzed using IBM SPSS Statistics 24.

A general linear model was used to analyze behavioral data by testing an analysis of variance (ANOVA). Missing values due to omissions or absence of responses were excluded from analyses. Homogeneity of variance was tested and confirmed by Levene's test. Mauchly's test of sphericity was used to analyze repeated measures throughout the experiment. If the sphericity assumption was violated, the Huynh-Feldt epsilon was used to be corrected with more conservative values. The significant values were set to be $\alpha=0.05$ throughout. The statistical difference of preference between immediate and delayed levers was compared between the control (PBS saline) and MTX-induced group, using a T-test. To observe the significant effect of MTX, ANOVA was used to measure the impact of saline and MTX on impulsivity across three session pairs. In the case of a significant result, a further test was done using Bonferroni. The correlation between the time from the MTX injection date and the incidence of omission was tested using linear regression.

Results

The study results were novel in that choice procedures were performed as a measure of impulsiveness targeting the impact of MTX in the animal models. Impulsiveness is the aberrant cognitive deficit additional to the executive function, measured by the NOR trials. In addition, studying adolescent rats, regarding the fact that they are in a critical stage of life in developing their brains, contributes to the future direction in determining the possible factors triggered by MTX on the impulsiveness of the rats.

The Preliminary Study for the Choice of Sex of the Rat Models and Baseline

In the preliminary experiment, the animal subjects were tested to demonstrate the intake of the number of sucrose pellets per rat to create the baseline. In addition, the sex difference of the animal models was evaluated to determine the best-fit animal models for the operant

conditioning. In the past, female rats were excluded from experimental studies primarily due to misconceptions about their ability to perform operant procedures, whether due to questions of stamina or cognition (Becker, Prendergast, & Liang, 2016). Sucrose pellets are often used as positive reinforcers; therefore, females might respond for half as many pellets because the body mass per height is twice as much as males. Exclusion of female rodents in preclinical studies may explain failures to generalize preclinical studies to clinical studies since half the US population is female. To analyze the natural tendency of the sucrose consumption based on sex, the rats were divided into male and female groups. The data suggest that the female rats, despite overall small body sizes compared to the male rats, showed an overall higher number of responses to sucrose than male rats (Figure 3).

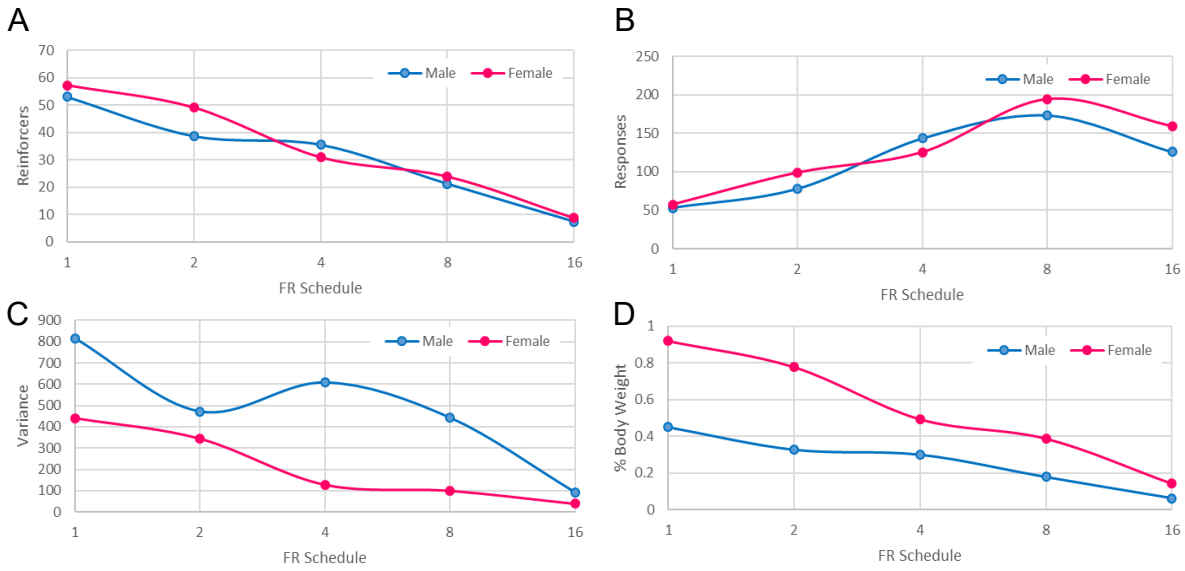

Figure 3. As a percent body weight, female rats respond more for sucrose pellets under a fixed-ratio (FR) schedule of reinforcement in operant conditioning. (A) shows the number of reinforcers received, (B) shows responses, (C) shows variance between sexes for reinforcers received, and (D) shows amount of sucrose received as a percent body weight. A mixed two-way ANOVA was used for (A), (B) and (D) and Levene's test for (C). (D) showed a significant interaction of sex and schedule ($F_{4,84} = 5.64$, $p < 0.01$). Variance among females was lower than males; however, this was only significant with an FR4 ($F_{1,21} = 4.39$, $p = 0.049$).

Additional data indicate that female groups generally showed higher self-control compared to male groups with less frequent reinforcements (pressing the levers) due to delays. Enduring delays allowed the female rats to reach a higher number of sucrose pellets outcomes compared to the male rats (Figure 4).

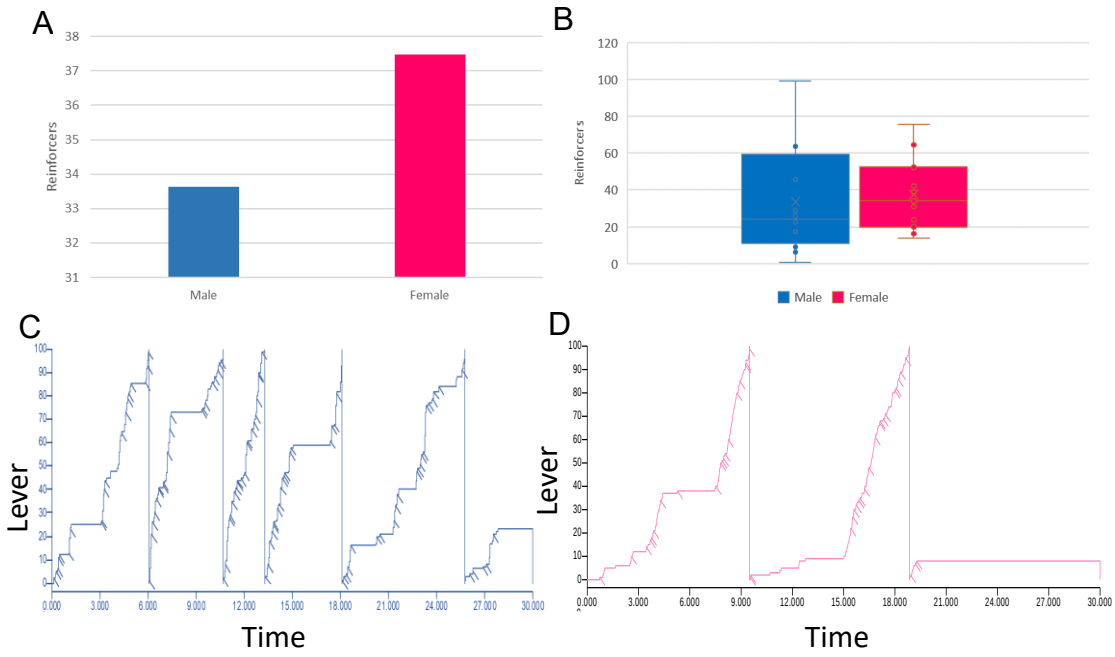

Figure 4. Responding by male and female rats under a variable-ratio 5 schedule. (A) shows average reinforcers received, (B) shows a box-and-whisker plot demonstrating the distribution of responses, (C) shows a representative cumulative record for a male rat, and (D) shows a representative cumulative record for a female rat.

Based on the results illustrated from the graphs, the use of female rats provides as equally accurate operant conditioning response as the use of male rats. The baselines of sucrose consumption by males and females were established. The following study of the MTX-induced rat models, therefore, used both male and female rats.

Sex Data and Abnormality of Stomach

A bodyweight of the control group (saline-injected) increased throughout the experiment. The MTX-induced group had a period of weight decrease. However, the rats who tolerated the chemotherapy soon began to increase the bodyweight. The individual bodyweight measured

daily was averaged across each group (Figure 5). After the day 48, all rats showed consistent bodyweight increase regardless of saline or MTX injections.

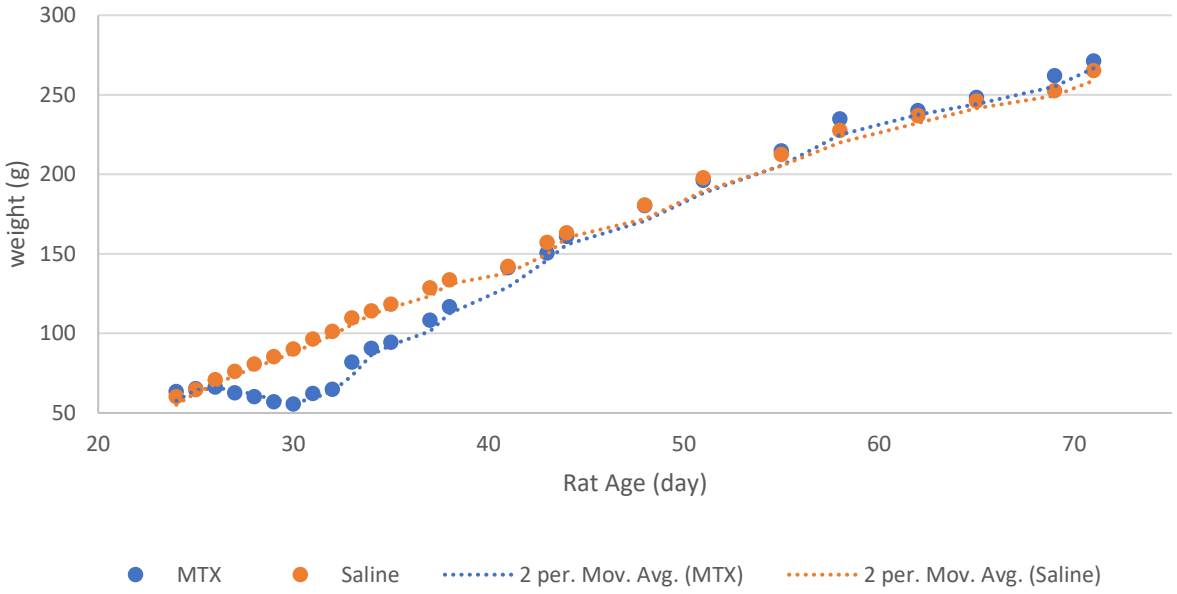

Figure 5. The weight change from age 20 day to 71 day. Injection of either PBS or MTX was performed on the rats on the day of 25. The decrease in body weight is evident on the day 26 and continues until the day 31. The MTX-induced rats began to show similar weight average as the saline-injected rats approximately on the day 48 after the birth.

In the MTX-induced group, two male and two female rats did not tolerate the drug well and as a result, deceased throughout the span of 28 to 33 days. During this time, any abnormal behavior or physiological phenotype was noted. However, the rats who endured the MTX injection soon caught up the bodyweight of the control group.

When the deceased rat models had undergone the dissection of the brain, their internal organs were also evaluated. Three out of four rats had an evidently enlarged stomach and did not have diarrhea, while the other rat suffered from diarrhea. These rats refused to consume sucrose pellets and did not show any bowel movements. The rat who had diarrhea and died showed normal size stomach. Such findings are consistent with studies that suggest constipation, abdominal pain, and esophageal disturbances are prominent among patients who ingest low-dose

MTX to treat rheumatoid arthritis (Tsukada, Nakano, Miyata, & Sasaki, 2013). The evidence of the enlarged stomachs from the MTX-injected rats underscores the toxicity of MTX on the intestinal lining. The rats who tolerated the MTX injection (n=2) started gaining weight again and were able to complete the training.

Novel Object Recognition

The time spent between the same objects during the sample trial was not significantly different as expected ($p > 0.05$). NOR test results showed that both saline-received and MTX-induced rats showed similar cognitive function as they spent significantly longer on the novel object compared to the old object ($p < 0.0001$ and $p = 0.0193$, respectively). The amount of time spent for each object during the 3-min trial distinguished the preference of the novel object, indicating the intact working memory and proper cognitive functions (Figure 6).

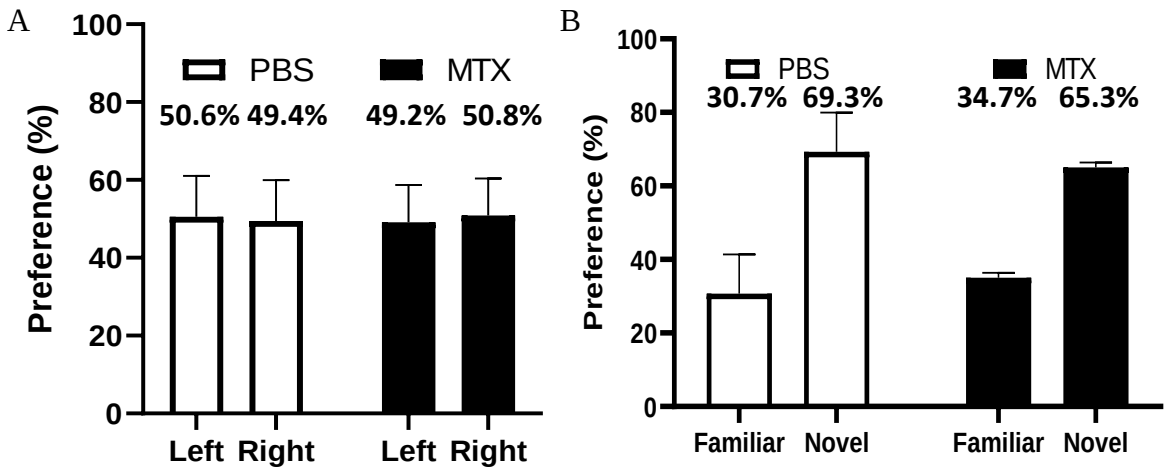

Figure 6. The preference between the familiar and the novel object based on the time the rats spend on each. A) The sample trial showed not much difference in their fondness on either left or right object in both groups, as expected ($p > 0.05$). B) The test trial, after replacing one familiar object with the novel object resulted in a significant difference in the preference of the object in both PBS and MTX ($p < 0.0001$ and $p = 0.0193$, respectively). Although the test trial showed the significance in both groups, the MTX-induced rats showed a little less significant result compared to the PBS-injected rats.

Both control and experimental groups seemed to recognize the novel object which displaced the old object they were familiar with. These results suggested that all rats demonstrated the minimal cognitive ability that is required to recall the familiar condition and the brand-new condition.

Training and Sensitivity to Delay for Rewards

All subjects learned to press the lever for food. A cumulative record for one rat is shown in Figure 7. However, only female rats completed the training course for both groups as the male rats did not reach the set-goal of the number of the sucrose pellets.

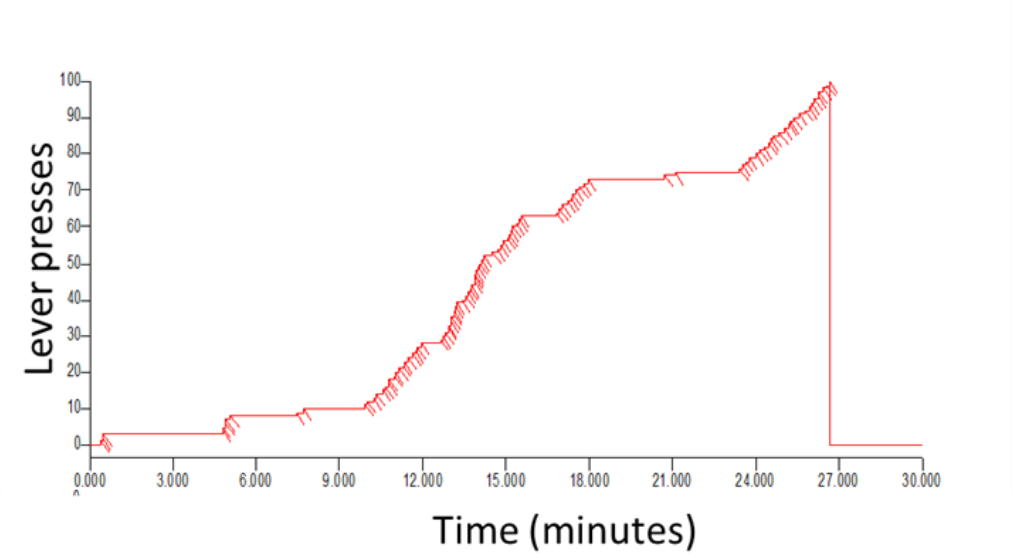

Figure 7. The trend of rats pressing the lever throughout the course of training. After 3 days of training, the majority of the rats began to press levers. The number of lever presses is also consistent with the sucrose pellets provided.

Based on the time it took for the rats to complete their training sessions, it was observed that the female rats overall achieved the goal of the number of sucrose pellets better than the male rats, which can be affected by their active hormonal changes or other differences in biological differences.

During the initial training in the choice procedure, all female rats showed a greater preference for the immediate lever (Figure 8).

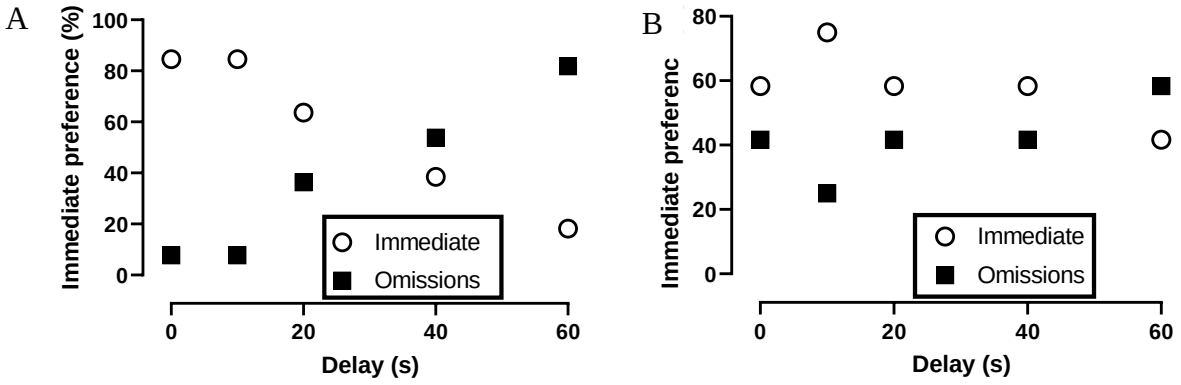

Figure 8. The relationship between a choice for the delay lever vs the immediate lever among the PBS and MTX groups. A) Choice procedure performed by the PBS-injected rat. B) Choice procedure performed by the MTX-induced rat. An empty circle represents the choice for the immediate lever, while the filled square represents the omission during the trial. Preference for an immediate lever was negatively correlated with an increased time of delays, while the number of trial omissions was positively correlated with the increased time of the delays in the PBS group, whereas the MTX group showed a less clear relationship.

Interestingly, the number of immediate lever presses decreased as the delay time increased. This relationship was unexpected as the reward for the delay lever was fixed at five sucrose pellets regardless of the length of the delay period. However, this correlation can be explained by the number of omissions. As the trial went on, the choice for the sucrose pellet itself decreased as the omission increased with the increase in the delay time. This behavior needs further investigation. Although the correlation was clear in the PBS individual, the MTX-induced rat did not show as much difference in this relationship.

After they learned the delay-lever functions, the further choice procedure showed different responses between the control group and the MTX-induced group (Figure 9).

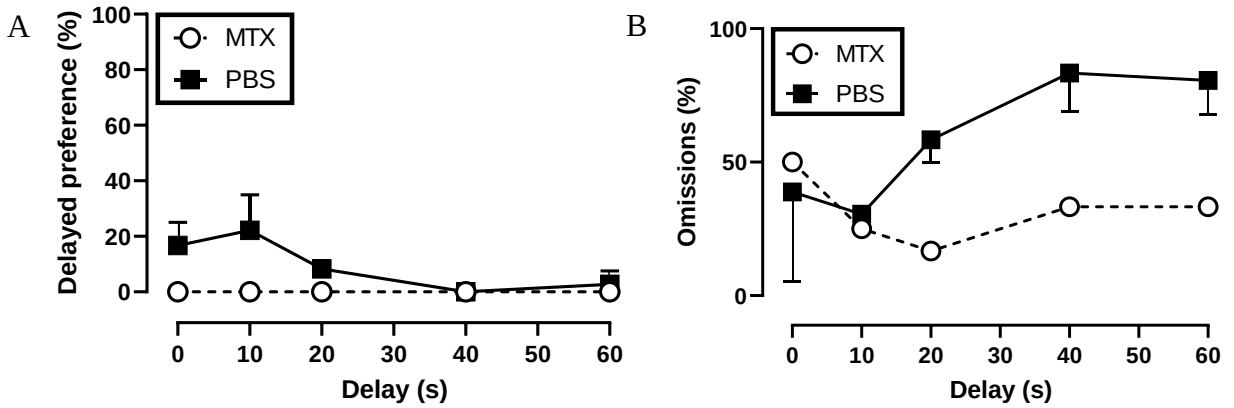

Figure 9. The preference for the delay lever and the number of omissions in relation to the time of delay. A) The control group (PBS-treated) showed the preference of the delay lever at the beginning of the session, while the MTX-treated group did not choose the delay lever throughout the session. B) Both groups still indicated a high percentage of omission especially towards the end of the experiment.

The MTX group exclusively preferred the immediate reinforcer, while the control group chose the delay lever for the first quarter of the session. This decrease in delay-lever preference overtime was expected, as the delay period gets longer, while the delay-reward is fixed to five sucrose pellets. The overall omissions of the trials were still high, which may be related to their age.

Discussion

Administering MTX to the rats who are undergoing an active development of the brain structure had a significant impact on various areas of the cognitive functions. The extent of which MTX chemotherapy damages the neuronal development, as well as other hormonal regulations, is not well examined. This study, then, was able to demonstrate the phenotypic characteristics of the MTX-treated rats by observing behavioral changes. The task was validated as the prolonged trials still allowed the subjects to remain sensitive to the signals of the number of sucrose pellets.

The preliminary studies showed that both male and female rats will produce the results that will be more generalized among the population. The female rats consistently pressed for more sucrose pellets compared to the male rats, which resulted in a higher number of ingestions of sucrose pellets for the female rats than the male rats. An exclusion of the female rats as the animal models from the study, which has been the case for a long time in the past due to the fact that the females experience a menstrual cycle every four to five days can skew the generalizability of the results.

NOR test was performed once a month (total three months) to observe any development or deterioration of working memory as well as cognitive functions. This experiment served as a positive control as the defects in brain development were expected from the rats who are disturbed by the MTX toxicity at an early age. Although the MTX-induced rats seemed to recognize the novel object, it was clear their response to the new subject was less noticeable compared to the non-affected group. This result is consistent with the clinical findings as the cancer-survivors in their young age with early exposure to MTX experience partially abnormal performance in their academic fields compared to healthy peers (Ikonomidou, 2018; van der Plas et al., 2015). This result supported the difference in cognitive functions between the PBS and the MTX groups prior to learning their difference in impulsiveness.

In the discounting task, all rats recognized the consequences of pressing a certain lever (the left for 5 sucrose pellets and the right lever for 1 sucrose pellet) within the first two weeks of training. The completion of training confirmed that the adolescent rats were able to comprehend the signals and respond appropriately regarding their desirable choices. At the beginning of the choice test, all rats tend to prefer the immediate lever more than the delay lever. The correlation test was performed based on the percent preference of the immediate lever and the length of the

delay time of the delay lever. The female rat who was not exposed to MTX in her early stage (23 days old) showed over 80% of preference for the immediate lever at the beginning of the session, expressing her desire to consume the sucrose pellets. This preference decreased as the session proceeded, while the omission of the trial increased simultaneously. This association can suggest that instead of choosing the delay lever, the rat may have recognized her satiety and decided to stop eating more sucrose pellets. In contrast to the PBS rat, the MTX rat showed little relation between the preference of the immediate lever and the time of delay throughout the discounting task session. No significant difference in choosing the lever in the experimental rat can demonstrate the overall withdrawn ability to perform tasks with the appropriate understanding of the levers. The comparable responses between the control group and the MTX-exposed group were observed, indicating that the altered executive functions to consume less number of sucrose pellets by choosing the immediate lever in the MTX rats show impulsive behavior, which can be associated with the MTX impact on daily tasks.

The MTX-induced impulsive behavior became more evident in the experimental models throughout the choice procedures. The MTX-administered rats completely ignored the choice of having a higher reward (five sucrose pellets), but rather chose to receive one sucrose pellet. MTX's impact on the rats suggested intolerance against the temptation of having food instantaneously. Although the MTX injection took place on day 24 after birth, the cognitive deficits were still evident on day 71 after birth. This finding suggested that the time it took for MTX to alter a behavioral aspect—in this case, impulsiveness—far exceeded the half-life of MTX. The novel discovery that MTX contributes to the development of the impulsiveness at a later time for the rat models was consistent with other studies where the aberrant cognitive functioning persistently decreased in the clinical patients who survived ALL. The experimental

design was to mimic the developing brain with an independent variable of injecting MTX without the presence of cancer. This condition exclusively targets the influence of MTX disregarding the possible negative effect by cancer itself, highlighting that prevalence of neurocognitive dysfunction among young patients who had undergone the chemotherapy is most likely induced by the drug.

Considering the half-life of methotrexate, the rats' metabolic activity will be able to eliminate MTX from the biological systems in approximately 90 minutes (Fahrig, Brasch, & Iven, 1989). There was only a one-time injection of MTX at the beginning of the experiment before the rats underwent the training and the experimental sessions. Then, the long-lasting effect of MTX may be via other intermediate components. Such interpretation is also implied by other studies, demonstrating that MTX not only halts the active cell divisions but also has an indirect effect on cellular apoptosis observed in patients with rheumatoid arthritis (Spurlock et al., 2011). In fact, reactive oxygen species are commonly found in the body exposed to MTX treatment. Those molecules cause oxidative stress in the cell and if the condition is not reversed, the affected cell shows an increase in sensitivity to apoptosis. Rheumatoid arthritis patients are usually administered a low dose of MTX, while the majority of MTX chemotherapy suggested treating for ALL and other cancers require a high dose MTX, which is known to affect the young female patients the most (Holmboe, Andersen, Morkrid, Slordal, & Hall, 2012). In terms of the MTX impact of brain cell deaths, the reliance on temporal precedence between MTX injection and the development of behavioral disorders explains that there are missing keys to instigate the abnormal responses in the brain. The intermediate molecules that mitigate the brain function in response to MTX impact should be investigated further (Angelov et al., 2009).

The behavioral analyses were performed by investigating the frequency of omission during the trials. The unexpected omissions after successful training of recognizing the lever functions suggest a few interpretations. Ignoring the signals from the PBS rats can be due to the loss in appetite, as they have acquired more sucrose pellets during the first few trials of the session, which provided them with over five times what they will normally ingest during their training session. On the other hand, the MTX-administered rats showed a consistent percentage of omissions, indicating that their behavior depends on random failure to press the lever due to lack of attention ability. Additionally, the decrease in physical activity could be assessed as if the rats were choosing not to press the lever or were challenged, moving from place to place, and pressing the levers. Since the omissions were prominent throughout the session in the MTX group compared to the control group, a possible interpretation of withdrawal in physical activity can point to the presence of the pain, although this factor was not specifically targeted in our study. The reduction in physical strength or alteration in an ability to pay attention due to the unbalanced biological homeostasis may be able to point at the sensation of pain.

In short, the mechanism of action for MTX in the adolescent rat models demands longer than a month to undermine cognitive deficits, but the impulsive behavior was present within two months after the MTX injection. This timeline of one to two months in the rats is equivalent to two and a half years to five years in human years, supporting that five years after the MTX exposure will show phenotypic symptoms in cognitive behaviors in young children (Sengupta, 2013).

This study was limited due to the small sample size of the animal models, questioning the generalizability. However, using both male and female rats helped minimize biased results.

As the study on the adolescent rat models were never done in the past, the appropriate doses of MTX per body mass was not accurately determined and the doses that were used were proven to be fatal for some rat models.

The adolescent rats not only have developing brains, but also growing bodies, indicating that accounting for the possible hormonal influences playing the role in these rats can be confounding factors.

Conclusion

The chemotherapy, MTX, despite its effect on curing various cancers including ALL, implies some detrimental disorders in cancer survivors. In this study, the degree of the negative effect of MTX on the actively developing brain was closely investigated. The experiment was conducted, according to the baseline obtained for the male and female rats' tendency to consume sucrose pellets. Administration of MTX to the animal models accompanied a decrease in working memory evidenced by the NOR test, which served as a positive control. MTX's impact on impulsive behavior was evident from the discounting task. From the choice procedure, the MTX rats exclusively chose the immediate lever to receive one sucrose pellet and discounted the values of the rewards after delays. Additionally, the degree of discomfort could have been a factor based on the frequency of omission. MTX group's random omissions could point to a decrease in attention skills or physical discomfort. The collaborative results indicate that MTX requires longer than a month to show the distinctive cognitive deficits in the adolescent rats, but the drug accompanies impulsive behavior within two months of being exposed to chemotherapy, comparable to the drug's effect on young children.

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