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HOW SAFE IS GADOBUTROL? EXAMINING THE EFFECT OF GADOLINIUM DEPOSITION ON THE NERVOUS SYSTEM

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
August 2021

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ACKNOWLEDGEMENTS

"Anything you dream is fiction, and anything you accomplish is science, the whole history of mankind is nothing but science fiction." ― Ray Bradbury

I would like to thank Dr. Nada Lawand for her continued guidance and support throughout my project. You have taught me so much and given me the opportunity to grow on personal, professional, and academic levels. Thank you for believing in me when I doubted myself and for being my mentor. No matter where I go, I will always remember the impact you had on me dearly.

I would also like to extend my gratitude to Mr. Bassem Najem and Ms. Batoul Darwish for their guidance and help in the lab. You have taught me that anything can be perfected with practice and patience.

Thank you to Mr. Jimmy Asroui from the Environment Core Laboratory at the American University of Beirut for the inductively coupled plasma mass spectrometry analysis.

Thank you to the Diana Tamari Sabbagh Scholars Program (DTSSP) for their generous funding of the present work.

Finally, I would like to thank my family who deserve more than words can ever express and more gratitude than I can ever repay so enough said.

ABSTRACT
OF THE THESIS OF

Maya Wafik Jammoul for Master of Science
Major: Neuroscience

Title: How Safe Is Gadobutrol? Examining the Effect of Gadolinium Deposition on the Nervous System

Background and objective: Repeated administration of Gadolinium based contrast agents (GBCAs) has been shown to cause Gadolinium (Gd) deposition in the nervous system. It is unclear whether neurotoxic effects can arise from this deposition. The aim of this study was to evaluate the safety of the GBCA, Gadobutrol, by assessing its effect on the central and peripheral nervous system under normal conditions and to investigate whether neuroinflammation can exacerbate the effect of Gadobutrol.

Methods: As such, 24 male Sprague Dawley rats were divided into 4 groups that consisted of a saline group, Gadobutrol group, lipopolysaccharide (LPS) + saline group and an LPS + Gadobutrol group. Gadobutrol and saline were administered intraperitoneally for 20 days at a dosage of 2.5mmol/kg while LPS was given intraperitoneally at a dosage of 5mg/kg followed by Gadobutrol or saline after 1 hour and for 20 days. Behavioral tests that include the heat hyperalgesia test, beam walking test, and spontaneous alternation T-maze test were conducted weekly over a period of 4 weeks to evaluate pain sensitivity, motor function, and cognitive ability. Gd concentration was measured in the brain and peripheral nerves, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), one week following the last set injection. Additionally, electromyography (EMG) and an assay of lactate dehydrogenase (LDH) activity in the sciatic nerve and hippocampus were carried out. One-way ANOVA followed by Tukey's test in addition to multiple t-tests and unpaired t-tests were utilized for statistical analysis.

Results and conclusion: Results showed that Gadobutrol did not cause behavioral deficits under normal conditions; however, the cumulative effect of LPS and Gadobutrol significantly impaired pain sensitivity over time. ICP-MS showed Gd deposition in the cerebrum and peripheral nerves in addition to greater deposition in the cerebrum of the LPS + Gadobutrol group. LDH activity was significantly higher in the hippocampus of the Gadobutrol group but not in the sciatic nerve and EMG revealed that Gadobutrol led to a decreased activation threshold in the sciatic nerve. In conclusion, repeated administration of Gadobutrol is safe under normal conditions; however, inflammation can exacerbate the effect of Gadobutrol over time leading to central and peripheral manifestations.

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ABBREVIATIONS

GBCAs: Gadolinium-based contrast agents

MRI: Magnetic resonance imaging

Gd: Gadolinium

FDA: Federal and drug administration

NSF: Nephrogenic systemic fibrosis

Gd^{3+}: Gd ions

Ca^{2+}: Calcium ion

VGCC: Voltage-gated calcium channels

Zn^{2+}: Zinc ion

Cu^{2+}: Cupric ion

B_{ex}: External magnetic field

RF: Radio-frequency

T1: Longitudinal relaxation

T2: Transverse relaxation

CT: Computed tomography

PNS: Peripheral nervous system

CNS: Central nervous system

ROS: Release reactive oxygen species

LDH: Lactate dehydrogenase

CSF: Cerebrospinal fluid

BBB: Blood-brain-barrier

CVOs: Circumventricular organs

MS: Multiple sclerosis

ATPases: Adenosine triphosphatases

IL: Interleukin

LPS: Lipopolysaccharide

IACUC: Institutional Animal Care and Use Committee

LEAF: Laboratories for the Environment, Agriculture, and Food

ICP-MS: Inductively coupled plasma spectrometry

PFA: Paraformaldehyde

CMAP: Compound muscle action potential

EMG: Electromyography

PBS: Phosphate buffered saline

SEM: Standard error of the mean

ANOVA: Analysis of variance

CHAPTER I

INTRODUCTION

Gadolinium-based contrast agents (GBCAs) are drugs widely used for Magnetic Resonance Imaging (MRI) to enhance the quality of scanned images and to improve the accuracy of the diagnosis. The use of Gadolinium (Gd) as a possible contrast agent was introduced by Weinmann and colleagues in 1984 (1). Soon, the U.S. Federal and Drug Administration (FDA) approved the first GBCA, Gadopentetate Dimeglumine (Magnevist), in 1988 (2). Other types of GBCAs followed such as Gadodiamide (Omniscan), Gadoterate Meglumine (Dotarem), and Gadobutrol (Gadavist) (3). All these agents were considered safe until 2006, when reports of adverse reactions such as nephrogenic systemic fibrosis (NSF) in patients with a history of renal dysfunction were published (4). As a result, the FDA recommended that the administration of specific GBCAs should be avoided in patients with a history of kidney problems (5). In 2017, the FDA issued another warning on GBCAs as evidence of Gd retention in various body tissues emerged, which prompted limiting multiple dosages of GBCAs (6).

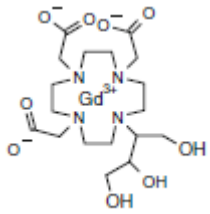
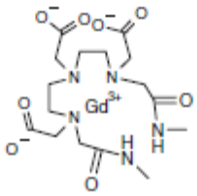
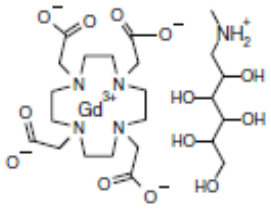
A. Chemical Structure of GBCAs

GBCAs consist of Gd ion (Gd^{3+}) and specific chelating ligand complexes (7). Gd is a lanthanide metal that possesses seven unpaired electrons responsible for its excellent paramagnetic properties (7). On the other hand, chelating ligands are cyclic or linear polyaminocarboxylic acid complexes bound to Gd^{3+} with 8 coordinate bonds, which allows a water molecule to occupy the ninth coordination site (7). These ligands are carriers that encapsulate the Gd^{3+} ion to prevent its dissociation and release (7). Free

Gd^{3+} can disrupt many biological processes when deposited in tissues and causes toxicity (7). The similarity between the ionic radius of Gd^{3+} (108ppm) and that of the calcium ion (Ca^{2+}) (114ppm) , enables the Gd^{3+} ion to compete with Ca^{2+} ions and inhibit specific enzymes or block voltage-gated calcium channels (VGCC) (3). It is hypothesized that transmetallation of Gd^{3+} with Zinc (Zn^{2+}) , cupric (Cu^{2+}) , or Ca^{2+} ions is a possible mechanism by which Gd^{3+} dissociates from chelating ligands (3). Pre-clinical studies have highlighted the effectiveness of chelation in decreasing Gd toxicity by showing a ten times increase in the median lethal dose (LD50) of Gd following chelation (8). Nonetheless, the beneficial effect of chelating compounds on eliminating retained Gd from human tissues remains to be studied (8).

GBCAs are classified into macrocyclic or linear and ionic or non-ionic depending on the chemical structure of the bound ligands (9). In macrocyclic GBCAs, ligands completely surround the Gd^{3+} ion forming a cage-like structure, while in linear or open chain GBCAs, the ligands partially encapsulate it (10). Macrocyclic GBCAs like Gadoterate Meglumine and Gadobutrol are considered more stable than linear GBCAs due to their lower dissociation rate and higher thermodynamic and kinetic stability (3). Moreover, GBCAs can be classified according to their net charge. In ionic GBCAs, the chelating ligands possess more than three negatively charged elements that form electromagnetic interactions with the central Gd^{3+}(10). Therefore, the net negative charge of ionic GBCAs implies that the complex is less likely to dissociate (10). On the other hand, nonionic GBCAs are neutrally charged with three or less negatively charged moieties interacting with Gd^{3+}, which results in a weaker bond than that of ionic GBCAs (10). Examples of ionic GBCAs include Gadopentetate Dimeglumine and Gadoterate Meglumine while Gadodiamide and Gadobutrol are non-ionic GBCAs (3).

The kinetic stability of different types of GBCAs in human serum was assessed by measuring the amount of free Gd^{3+} before and after adding 1mmol/L solution of GBCAs over 15 days (11). At physiological pH and temperature, the dissociation of Gd^{3+} in non-ionic linear GBCAs was shown to be 10 times greater than in linear ionic GBCAs. On the other hand, the release rate of Gd^{3+} in macrocyclic GBCAs was almost null indicating stability even when 10mM phosphate was added to the serum (11). Thus, the results confirmed the predicted stability of the different types of GBCAs, based on their chemical properties, and showed that macrocyclic and ionic GBCAs tend to be more stable than their linear and non-ionic counterparts respectively.

Common name	**Chemical structure**	**Chemical name**	**Type**
Gadavist		Gadobutrol	Macrocyclic, non-ionic
Omniscan		Gadodiamide	Linear, non-ionic
Dotarem		Gadoterate Meglumine	Macrocyclic, ionic

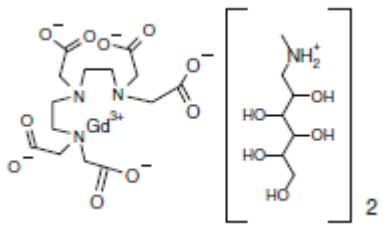
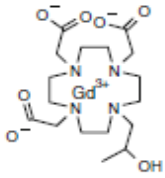
Magnevist		Gadopentetate Dimeglumine	Linear, ionic
ProHance		Gadoteridol	Macrocyclic, non-ionic

Table 1: Names, structures, and types of different GBCAs.

B. Role of GBCAs in Imaging

In 1977, the first scan of the human body was generated using a whole body MRI, which led to the introduction of a revolutionary imaging technique (12). MRIs function by utilizing the effect of an external magnetic field (B_{ex}) and radio-frequency (RF) field on the magnetic moment of hydrogen atoms in water molecules (13). Hydrogen atoms have a random nuclear spin that creates a nuclear magnetic moment analogous to a magnet bar (13). When a B_{ex} is applied, hydrogen atoms realign with its direction then undergo an energy transition upon the addition of a RF field (13). More specifically, when the RF emitter is off, hydrogen atoms release a RF that is detected by a coil receiver and consequently analyzed by a computer to create an image (13). The emission of a RF signal indicates a transition from a high energy state to a low one or what is termed relaxation of the hydrogen atoms upon realigning with the original B_{ex} (13). Moreover, the shorter the relaxation time, which is composed of the longitudinal (T1) relaxation and transverse (T2) relaxation, the better the signal (14).

The significance of GBCAs arises from their ability to decrease T1 and T2 or their high relaxivity (14). This characteristic depends on the number of water molecules bound to Gd^{3+} in the inner sphere and their mean residency time, in addition to the number water molecules and their residency time in the surrounding coordination spheres (15). To induce relaxation, GBCAs create a fluctuating magnetic field due to the relaxation or excitation of Gd^{3+} electrons and the "tumbling" of the molecule that is described by its rotational correlation time (15). The water molecule bound to Gd^{3+} in the inner sphere is the first one to undergo relaxation by the unpaired electrons of Gd^{3+} (14). The faster it is exchanged with other water molecules, the better the GBCA (14). Relaxivity can be enhanced by increasing the number of water molecules in the inner sphere or by decreasing the tumbling rate of the molecule by using a larger one (16). However, the stability of the GBCA can be compromised by decreasing the bonds between Gd^{3+} and its chelating ligand along with the limitation imposed by other factors when tumbling rate is slowed (15, 16). All FDA approved GBCAs bind one water molecule in their inner sphere and are administered at a dose of 0.5mmol/ml except for Gadobutrol, which is administered at a dose of 1mmol/ml (16). This is attributed to the slightly larger size of Gadobutrol compared to the other GBCAs and the consequent lower tumbling rate (16). Knowing that the efficiency of a GBCA depends on its concentration and relaxivity, Gadobutrol is thus considered the best GBCA owing to its high concentration and relaxivity (16).

C. Bio-distribution and clinical manifestations of GBCAs

While Gd deposition following GBCA administration became expected in people with renal dysfunction, proof of such deposition in people with normal renal

function soon emerged. For example, Gd was shown to be deposited in different tissues like the skin, bone, and liver (17-19). In 2014, Kanda and colleagues found a correlation between the total dose of GBCAs and an increased signal intensity in the dentate nucleus and globus pallidus of patients injected with GBCAs (20). Moreover, postmortem studies detected Gd deposition in the brains of subjects without renal dysfunction (21). These studies prompted further investigation to understand the effect of GBCAs and identify the mechanisms by which Gd is deposited in the aforementioned organs, especially the brain. First, it is vital to understand the potential bio-distribution of GBCAs. GBCAs are extracellular fluid contrast agents (22). Thus, following an intravenous injection, GBCAs are transported in the blood and the extracellular space to be finally eliminated by the liver and kidneys (22). Gadobutrol is excreted via glomerular filtration with a half-life of 1.33-2.13 hours, and, within 12 hours, 90% of the intravenous injection is eliminated in the urine (23). However, GBCAs avoid first pass clearance and circulate in the body before excretion, which allows them to reach other compartments (24).

In addition to organ deposition, GBCAs can cause other side effects like physiological reactions and allergic-like reactions depending on the type of GBCA used (25). Physiological reactions include nausea, dizziness, headaches, chills, chest pain, and burning pain whereas allergic-like reactions are comprised of anaphylaxis, wheezing, and other throat symptoms (25). Studies have shown that the rate of adverse reactions following GBCA administration is low with most of the reactions being mild (26-28). For example, the rate of allergic reactions following Gadobutrol administration was found to be 0.32% (29). Pain development has also been observed in the extremities including the hips, elbows, knees, and wrists directly following GBCA

injection (30). One case report describes a man who suffered from severe arm pain after Gadopentetate Dimeglumine administration (31). Four cases of women with normal renal function demonstrated Gd deposition along with acute or chronic symptoms after GBCA injections (32). These symptoms included torso pain, a headache, arm pain, leg pain, and skin thickening in the extremities (32). Moreover, such occurrences of chronic symptoms have been supported by other reports whereby patients complained of headaches, bone pain, and joint pain (33). The aforementioned results further highlight the need to investigate the link between peripheral acute or chronic pain onset and Gd exposure. This is especially true as an association between fibromyalgia and Gd toxicity has been postulated (34).

Other than peripheral pain, GBCAs can heavily impact the brain by inducing neurotoxicity (35). This was demonstrated in patients with an iodine allergy who require an intrathecal injection of GBCAs instead for neuroaxial procedures (35). One report describes the case of a man, with a history of chronic pain, who suffered from aphasia and confusion (36). Initially, an acute subarachnoid hemorrhage was suspected, yet a computed tomography (CT) angiogram disproved this, and the patient was diagnosed with Gd-induced encephalopathy due to the use of intrathecal analgesia pump (36). Similarly, another report describes a patient who developed seizures, confusion, and respiratory difficulties after receiving 2 (4ml) intrathecal injections of Gadodiamide for an epidugram (37). The patient was diagnosed with Gd-induced encephalopathy after a non-contrast MRI revealed intracerebral and intraventricular Gd deposition (37). In the two cases, the patients improved after a few days of hospitalization; however, a fatal case of Gd-induced encephalopathy has been reported after the development of acute Gd neurotoxicity (38). The patient died 18 days after

receiving a second intrathecal injection of (5ml) Gadoteridol (38). Symptoms of the deceased included confusion, seizures, tachycardia, and respiratory distress while an autopsy revealed infarctions in the cerebral cortex, changes in the cerebellum, and gliosis in the hippocampus and pons (38). Thus, guidelines have been updated to allow a maximum of 0.5 mL of 0.5 mmoL/mL GBCAs to be injected intrathecally, following CSF aspiration, and even to use iodine contrast agents instead along with allergy management (39). Also, as exemplified above, both linear and macrocyclic GBCAs can result in neurotoxicity, which indicates that the resultant neurotoxicity is independent of the nature of the chelating agent.

The clinical safety of Gadobutrol has been widely demonstrated. This is supported by a prospective study on 3710 adults and children, whereby only 0.59% reported a minimum of one adverse reaction that mostly appeared 1hour post injection (40). However, this study lacked a comparator group. When Gadobutrol was compared to other types of GBCAs in 42 (phase II to IV) studies, it was found that the rate of drug related adverse reactions in both was 3.5% (41). Nonetheless, this study was limited by the small sample size of the comparator group. Also, both studies did not investigate the effect of multiple injections of Gadobutrol and the long term effects associated with Gd deposition. On the other hand, a retrospective study that looked at the effect of 10-34 injections of Gadobutrol in 25 children compared to a control group of children with no GBCA exposure showed no T1 hyperintensities in the dentate nucleus or globus pallidus (42). The results were independent of the number of Gadobutrol injections, dosage, or time between MRI scans (42); however, whether other brain areas or other parts of the nervous system exhibited T hyperintensities following Gadobutrol injection has not been indicated. A follow-up study is warranted to determine the long term effect

of deposited Gd and trace their presence in other areas of the nervous system. For example, given the high rate of Gd deposition in bone, which is 23 times that in the brain, and the fact that for > 8 years, Gd can remain in bone, Gd in the bones of children can be mobilized with bone growth to reach the peripheral nervous system (PNS) or the central nervous system (CNS) (43, 44). Interestingly, intrathecally injected Gadobutrol displayed similar safety to an iodine contrast agent with a recommended dose of 0.5mmol/ml (45, 46). Lower extremity pain and spasms have been observed immediately following an intrathecal injection of Gadobutrol (1mmol/ml) (47). In addition, intrathecal Gadobutrol induced seizures, confusion, and aphasia with signal hyperatenuation in the subarachnoid space and other brain cisterns (35). Thus, given that the recommended concentration of Gadobutrol is twice that of other GBCAs, more research is required to determine the intrathecal dosage with minimal side effects.

D. Pre-clinical studies on the effects of GBCAs

Following 20 daily injections of GBCAs in rats at a dosage of 2.5mmol/ml, it was demonstrated that linear GBCAs result in greater Gd deposition in the brain than macrocyclic GBCAs (19). This is supported by other studies that detected Gd accumulation in the spinal cord and peripheral nerves of rats in addition to the back skin of mice (48, 49).

Gd can block VGCC, which could affect the transport of synaptic vesicles and release of neurotransmitters across synapses (50). In sensory neurons, disruption of Ca^{2+} flow across voltage gated or ligand gated channels can affect pain processing (51). Also, alterations in Ca^{2+} concentrations and neuronal death are suggested to play a role in the pathophysiology of neuropathic pain (52). This is supported by an in vivo study

that showed a decrease in intraepidermal nerve fiber density and an increase in terminal axon swellings 4 weeks after a single GBCA injection (48). It also indicated that small fiber neuropathy, which is accompanied by pain in the lower extremities, could be caused by GBCAs (48). Nonetheless, the study did not investigate whether these changes were permanent and affected by multiple GBCA injections. Furthermore, it has been shown that multiple Gadodiamide injections in rats induce pain hypersensitivity to thermal and mechanical stimuli (49). However, the study found no effect of GBCAs, whether linear or macrocyclic, on spatial working memory or hippocampal neurogenesis (49). In addition, motor disturbances such as ataxia have been observed in rats injected with Gadopentetate Dimeglumine in the lateral ventricles at a dose of 5 to 15 µmol/g brain (53). Gadopentetate Dimeglumine showed a neurotoxic effect that spread in the nervous system to cause lesions in the thalamus, brain stem, and spinal cord which is consistent with the presentation of ataxia (53). On the other hand, injecting Gadodiamide intraventricularly produced a similar neurotoxic effect but with lesions mainly located in the cerebellum and none in the spinal cord or brain stem (54). Thus, given the variation in GBCAs characteristics and structures, it would be of great consequence to detect and analyze the subtle morphological changes in the brain induced by the intraventricular injection of both macrocyclic and linear GBCAs. In addition, the need for comprehensive studies that investigate the differential effect of GBCAs on Gd retention in neural tissue and the resultant sensorimotor and cognitive impairment cannot be over emphasized

The use of Gd has displayed an environmental harm as seawater that is contaminated with Gd caused accumulation of Gd in mussels (55). The mussels exhibited neurotoxicity, an increase in oxidative stress, and a decrease in metabolic

ability (55). Evidence of Gd-induced neurotoxicity is supported by in vitro studies as well. For example, Gd was shown to induce neurotoxicity in rat cortical neurons by impairing mitochondrial function and increasing oxidative stress (56). More specifically, Gd^{3+} triggered the mitochondria to release reactive oxygen species (ROS) that directly led to cell death, or indirectly by activating a cascade of cytochrome c and caspase-3 release (56). DNA fragmentation and an increase in lactate dehydrogenase (LDH) release were also observed (56). In a similar study Gd^{3+} was shown to increase oxidative stress in rat cortical neurons leading to LDH release and cell death (57). However, they demonstrated that the ROS induced activation of the endoplasmic reticulum further enhanced oxidative stress and neuronal apoptosis (57). Both studies have provided evidence that Gd^{3+} leads to neurotoxicity in neuronal cells. Erdogan and colleagues (2020) investigated whether GBCAs could lead to a similar outcome (58). Indeed, macrocyclic and linear GBCAs resulted in neuronal cell death with the effect being more pronounced in the linear group compared to the macrocyclic one that included Gadoterate Meglumine and Gadobutrol (58). Neurotoxicity increased with GBCA dosage and was evident even at micro levels of GBCAs (58).

E. Routes of GBCAs entry into the nervous system

After metals enter the body, they accumulate in tissues that require metals such as the brain or heart, store metals like bone, or eliminate metals such as the liver and kidneys (59). In the nervous system, it is plausible that GBCAs enter the brain through the blood-cerebrospinal fluid (CSF)-barrier, blood-brain-barrier (BBB), and the circumventricular organs (CVOs). The blood-CSF-barrier allows access to the CSF through the fenestrations characterizing the choroid plexus cells and the lack of tight

junctions (60). Indeed, different types of GBCAs were found to enter the brain through the CSF regardless of their properties (61). The glymphatic system plays an important role in the distribution of toxins possibly carried by the CSF in the nervous system (60). Once in the CSF, GBCAs move to the subarachnoid space and enter the brain through the spaces surrounding the cerebral arteries (60). Bulk flow movement distributes the CSF across the brain parenchyma through Aquaporin 4 channels to reach different parts of the brain and possibly the spinal cord (60).

However, another portal for GBCAs to reach the brain is through the BBB, which has been evidenced by transmission electron microscopy (62). The BBB is made up of endothelial cells bound by tight junctions and lined by different cells including pericytes and astrocytic end feet (63). However, inflammation compromises the integrity of the BBB by acting on its cellular components and the basement membrane (64). The various mechanisms by which this may occur include the downregulation of tight junction proteins or membrane transporters in addition to the upregulation of leukocyte adhesion molecules and the disruption of astrocyte function (64). Multiple pre-clinical studies examined the effect of intravenous GBCA administration after the osmotic disruption of the BBB. Results indicated a dose-dependent effect whereby macrocyclic GBCAs injected at dosages of 0.2, 0.3, and 0.6 mmol/kg did not cause neurotoxic effects whereas macrocyclic and linear GBCAs given at dosages of 1 and 3 mmol/kg induced neurotoxicity (65-67). Also, the type of the GBCA used affected the results. For example, the macrocyclic GBCA, Gadoteridol, showed the greatest neurotoxic effect among other GBCAs at the same dose following BBB disruption (67). It was lethal at a dose of 1mmol/kg and had a greater neurotoxic effect than Gadobutrol and Gadopentetate Dimeglumine while Gadodiamide did not show any effect (67).

More recent studies that examined the relation between neuroinflammation and linear GBCAs showed that inducing inflammation in rats increases the amount of Gd deposition in the brain following 8 injections of 2.5 mmol/kg of Gadodiamide (68). However, whether the use of a macrocyclic GBCA could yield such a result is yet to be investigated.

On the other hand, GBCAs can reach the brain through structures that lack a BBB such as the CVOs. Once termed “the windows of the brain”, CVOs are characterized by their high capillary permeability and the absence of a BBB (69, 70). CVOs are so called since they surround the third ventricle (neurohypophysis, vascular organ of the lamina terminalis, subfornical organ, pineal gland and subcommissural organ) and the fourth ventricle (area postrema) (70). They function to regulate molecular transport, carry out an immunological response against invaders, and interact with autonomic centers like the hypothalamus (71). Thus, CVOs can act as a direct portal for GBCAs to enter the brain without an anatomic barrier.

There are not enough studies that describe the possible mechanisms of spread of Gd deposition between the CNS and PNS. However, given the evidence presented previously, it can be speculated that Gd can utilize the glymphatic system to travel from the brain to the spinal cord and possibly reach the peripheral nerves. Another route of peripheral invasion is transport across nerves. Conversely, Gd can travel from non-neuronal tissues, as mentioned previously, into the peripheral nerves and then get transported retrogradely into the spinal cord and brain. Thus, research on the route of GBCAs spread between the CNS and PNS is needed to help understand and mitigate the observed neurological effects.

F. Possible mechanisms of Gd toxicity

Understanding the mechanism by which Gd can induce neurotoxicity and its consequent adverse effects is key to recovery and prevention. Apoptosis and oxidative stress are one of the mechanisms utilized by Gd (56, 57). Gd can impair the normal function of mitochondria and/or the endoplasmic reticulum to exert its neurotoxic effect. Gd was shown to reduce the mitochondrial membrane potential by increasing the levels of intracellular ROS (72). A study that examined whether GBCAs act similarly found that both macrocyclic and linear GBCAs decrease mitochondrial membrane potential and increase neurotoxicity in dopaminergic neurons of the basal ganglia (73). Whether or not GBCAs induce the same outcome in hippocampal neurons and affect cognitive ability and memory function remain to studied.

Moreover, given the similarity in ionic radius between Gd^{3+} and Ca^{2+} ions, another mechanism by which Gd could be acting is through the interference of Gd with the calcium homeostasis of the endoplasmic reticulum. In support of this claim, it was found that Gd chloride increases intracellular levels of Ca^{2+} and the activity of caspase-4 leading to apoptosis via the ER-stress pathway (72). Gd can also block Ca^{2+} activated enzymes like adenosine triphosphatases (ATPases), kinases, and dehydrogenases (74). Additionally, whole patch clamp studies on rat and human thyroid cells showed that Gd and other lanthanide ions can decrease current flow across T-type VGCC (75). Increasing extracellular Ca^{2+} levels reduced this blockage, which means that Gd^{3+} acts as a competitive antagonist to Ca^{2+} resulting in a closed channel pore (75). Based on the above data, one could speculate that Gd deposits can modulate the function of VGCC found on sensory and motor neurons, leading to the disruption of synaptic communication and normal neuronal function.

Another possible mechanism by which Gd acts is through transmetallation. This is supported by the case report of a man with numbness in the extremities, ataxia, and zinc poisoning from a dental cream, who revealed Gd retention following an injection of Gadopentetate Dimeglumine for an MRI (76). The patient's Gd urine levels remained elevated even after 2 and a half years post Gd exposure (76). Also, an in vitro study on transmetallation found that Zinc-dependent Angiotensin-converting enzyme (ACE) was shown to be significantly inhibited by linear but not macrocyclic GBCAs (77). Normal brains contain different metals such as aluminum, iron, copper and zinc various regions such as the globus pallidus, caudate nucleus, substania nigra, and hippocampus among others (78). The highest concentration of Zinc is found in the hippocampus, amygdala, and other regions (78). Therefore, it is reasonable to assume that transmetallation with Gd at these sites can have deleterious effects on memory

Lastly, Gd can affect the function of white blood cells. For example, a study showed that linear and macrocyclic GBCAs act on monocytes to increase the release of different inflammatory interleukins (IL) such as IL-4, IL-6, and IL-13 (79). Exposing these activated monocytes to human fibroblasts resulted in an increase in types I and III collagen in addition to fibronectin, which are needed to initiate fibrosis and eventually NSF (79). Another study found that micro-concentrations of GBCAs (2.5μmol/L) cause Gd deposition in murine macrophages and lead to an increase in ROS and a reduction in mitochondrial membrane potential (80). Only Gadopentetate Dimeglumine significantly increased the levels of IL-6 while it suppressed the level of IL-10 along with Gadobutrol and Gadodiamide (80). Interestingly, when macrophages were exposed to Lipopolysaccharide (LPS), all GBCAs tested, except Gadobutrol, upregulated the resultant inflammation by reducing the levels of IL-10, IL-6 and increasing the levels of

IL-1β (80). Thus, Gadobutrol caused the least toxicity in macrophages. The clinical implications of such finding are vital for people with underlying inflammatory conditions who may require GBCA administration.

G. Aim of the Study

The objective of this study is two-fold: 1) to investigate the impact of repeated Gadobutrol administration on the peripheral and central nervous system under normal conditions, and 2) to investigate whether neuroinflammation exacerbates the effect of Gadobutrol.

Our hypothesis is that repeated exposure to gadobutrol leads to Gd deposition in the peripheral and central nervous system and results in symptoms of neurotoxicity. Inflammation of the nervous system is presumed to exacerbate the effect of Gadobutrol.

To assess this hypothesis, the concentration of Gd in the brain and sciatic nerves was measured following the administration of Gadobutrol in normal and inflamed rats. Biochemical, behavioral and electrophysiological tests were also conducted to assess the effect of the resultant Gd deposition on the integrity of the nervous system under normal and inflammatory conditions

CHAPTER II

METHODS

A. Animals

In total, twenty-four male Sprague-Dawley rats weighing around 150-250g were used in this study. The rats were housed in a room maintained at a constant temperature (20-22°C) and with a 12-hour light/dark cycle. Also, standard chow and water were provided ad libitum. All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut.

B. Experimental design

Twenty-four rats were divided into 4 groups that consist of a saline group(n=8), Gadobutrol group (n=8), LPS + saline group (n=4), and an LPS + Gadobutrol group (n=4). Gadobutrol and saline were administered intraperitoneally for 20 days at a dosage of 2.5mmol/kg. For the LPS groups, LPS was given intraperitoneally at a dosage of 5mg/kg followed by Gadobutrol or saline after 1 hour and for 20 days. This dosage of Gadobutrol is based on the dosage needed to achieve brain MRI enhancement in rodents (81). One day after the final injection, the saline group (n=4) and Gadobutrol group (n=4) underwent electrophysiological recording then they were cardially perfused with 0.9% saline followed with 10% formalin to fix the tissues. Brain, and peripheral nerves (trigeminal and sciatic nerves) were extracted and sent to the Laboratories for the Environment, Agriculture, and Food (LEAF) at the American University of Beirut for Gadolinium measurement using Inductively Coupled Plasma Mass Spectrometry (ICP-

MS). All groups were assessed using behavioral tests once per week over a period of 4 weeks. One day after the last test, the rats belonging to the saline group(n=4) and Gadobutrol group (n=4) were anesthetized and decapitated to collect fresh samples of the sciatic nerve and hippocampus for LDH activity measurement.

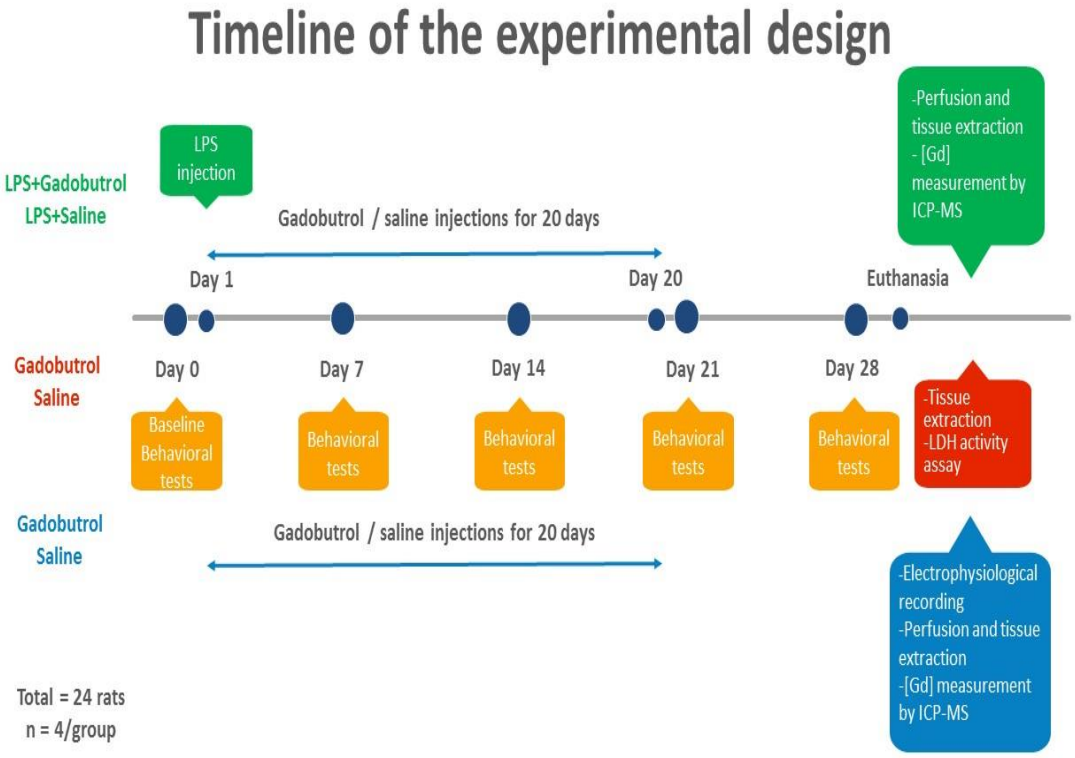


Figure 1: Timeline of the experiment.

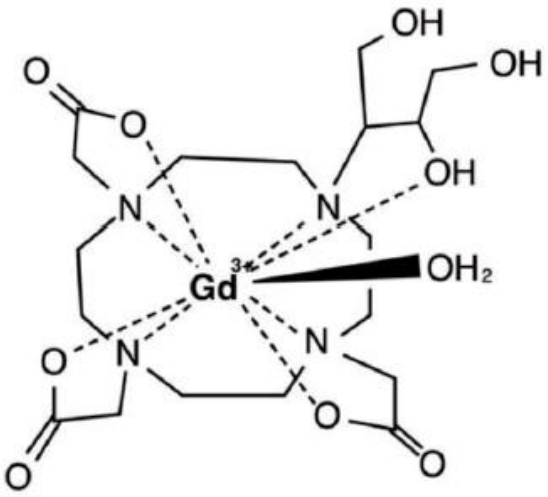


Figure 2: Chemical Structure of Gadobutrol.

C. LPS administration

Rats in the LPS + saline group (n=4), and LPS + Gadobutrol group (n=4) were given an intraperitoneal injection of LPS at a dosage of 5mg/kg. The LPS injection was prepared as recommended by Ramírez and colleagues (2019) by dissolving LPS in 0.9% saline solution (82). The weights of the rats were recorded before and after the LPS injection and their behavior was monitored. One hour after the LPS injection, the rats received an intraperitoneal injection of 0.9% saline or Gadobutrol.

D. Animal Perfusion

Prior to perfusion or surgical procedures, the rats were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg, Ketalar; Panpharma, Luitré, France) and xylazine (10 mg/kg, Xyla; Interchemie, Harju County, Estonia). Following anesthesia, the rats were perfused through the left ventricle with a saline (0.9%) solution with added heparin followed by 10% formalin for tissue fixation. The brain and

peripheral nerves were extracted and stored in 4% paraformaldehyde (PFA) overnight then in 30% sucrose solution for storage at 4°C.

E. Behavioral tests

1. Heat Hyperalgesia test

Heat hyperalgesia was assessed in all groups once a week over 4 weeks. To carry out the test, the rats were accommodated for one hour in a clear plastic cage. A heat stimulus, at an intensity of 35 infrared units, was then applied to the plantar surface of the right and left hindpaws. Foot withdrawal latency was measured over 5 trials for both paws with a 10 min interval between each trial to avoid paw injury. A cut-off time of 20 seconds was set to avoid tissue damage.

2. Beam walking test

The Beam walking test was performed to assess motor coordination and balance. One day prior to testing, the rats were trained to walk across the beam and familiarize with the apparatus and new height. During training, the rats were first placed halfway across the beam till they reach the finish point and then they were placed at the starting point. In order to encourage the rats to cross the beam, they were always placed at the same starting point and only moved back to their cage once the finish point was reached. Also, the cage was placed next to the final point so that it is more strongly associated with the safety of the cage. The time required to cross the beam from one side to another was recorded during 3 trials for each rat at the set time points.

3. Spontaneous Alternation T-Maze Test

To assess spatial working memory, the rat groups underwent the spontaneous alternation T-maze test. This is a test driven by spontaneous alternation so no habituation was needed (83). To carry out this test, rats were placed at the start area and were allowed to choose a right or left goal arm. After a choice was made, the rat was confined for 30 seconds by quietly sliding down a guillotine door. Then, the rat was carefully removed, the door was raised, and the rat was replaced at the start area facing away from the goal arms. The chosen goal arm was noted and the percentage of successful alternation was calculated for 3 trials.

F. Electrophysiological Assessment

For electromyographic recordings, each rat in both Gadobutrol and control groups was placed under general anaesthesia (ketamine, 100 mg/kg and xylazine, 10 mg/kg). A small incision was made in the skin at mid-thigh level and the muscle was blunt-dissected to expose the sciatic nerve. Evoked compound muscle action potentials (CMAPs) were evoked in the gastrocnemius muscle in response to sciatic nerve stimulation at 1V, 5V and 7V. Electrical stimulus was delivered using bipolar stainless steel wire electrodes placed directly under the sciatic nerve trunk. Electromyography (EMG) responses were recorded using a microneedle electrode placed in the belly of the gastrocnemius muscle, while the reference electrode was placed in the Achilles tendon. The signal from the recording electrode was fed into a differential amplifier, filtered, and monitored on an oscilloscope (Tektroniks Instruments). The analog signal was sampled in a 1401 data interface (CED 1401, Cambridge, UK) and analyzed using

Spike 2 software. The amplitude of the evoked MAPs from peak to peak was calculated and analyzed.

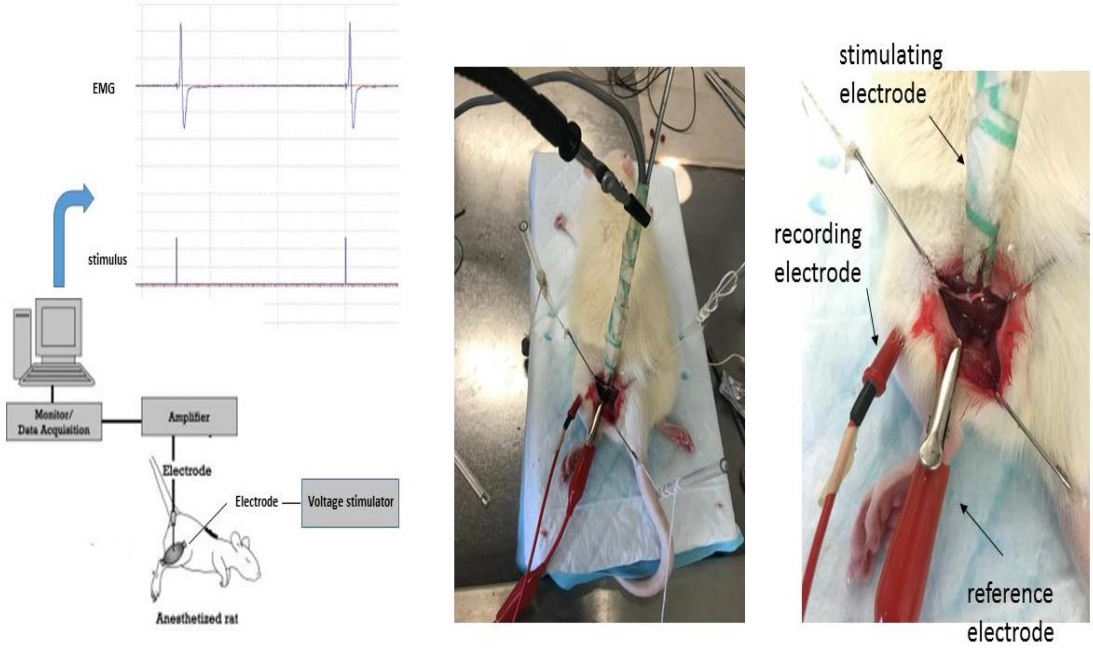


Figure 3: Setup of the electromyography carried out in the saline and Gadobutrol groups. A bipolar stimulating electrode is connected to the proximal left sciatic nerve and a recording electrode is inserted in the gastrocnemius muscle.

G. Quantification of Gadolinium using ICP-MS

In order to measure the concentration of Gadolinium in the nervous system, the brain, spinal cord, peripheral nerves (trigeminal and sciatic nerves) were extracted from all groups following perfusion. The extracted tissues were digested at 180°C for 30 minutes. Along with the samples, a blank, spiked blank, certified referenced sample, and a matrix spike were run as well. The concentration of Gd was measured by using ICP-MS (Agilent 7500ce; Agilent, Waldbronn, Germany).

H. LDH assay

The activity of LDH was measured in the saline group (n=4) and Gadobutrol group (n=4) one day after the last injection. The rats were anesthetized then decapitated to extract the right sciatic nerve and right hippocampus. The extracted tissues were kept on ice at all times to preserve enzyme activity. Prior to dissection, the tissues were rinsed in phosphate buffered saline (PBS) (pH=7.4) to remove all traces of blood. The sciatic nerve was carefully teased to remove connective tissue and the surrounding epineurium. Then, the tissues were homogenized, using an electric homogenizer, in 5ml buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA, per one gram of tissue. Following homogenization, the samples were centrifuged at 10,000 x g for 15 minutes at 4°C then the supernatant was collected. A working reagent was prepared from reagents provided by the LDH assay kit (QuantiChrom™ Lactate Dehydrogenase Kit, D2DH-100). More specifically, for one well, the working reagent was prepared by mixing 14 µL MTT Solution, 8 µL NAD Solution, 1 µL Diaphorase and 175 µL Substrate Buffer. Into a 96 well plate, 200µl of distilled water and 200µl of calibrator were transferred into separate wells along with 10µl of the sample supernatant and 190µl of the working reagent. Immediately, the OD565 was measured using a Microplate Fluorometer/Luminometer Fluoroskan Ascent FL (Thermoscientific) at t=0min and at t=25min. LDH activity was subsequently calculated according to the formula: $\text{LDH Activity} = \frac{\text{ODS25} - \text{ODS0}}{\varepsilon \text{mtt} \bullet \text{l}} \times \frac{\text{Reaction Vol (µL)}}{\text{Time} \bullet \text{Sample Vol (µL)}} \times \text{n} = 43.68 \times \frac{\text{ODS25} - \text{ODS0}}{\text{ODCAL} - \text{ODH20}} \times \text{n (IU/L)}$. For the right hippocampus only, the collected supernatant was diluted using a dilution factor of n=5.

I. Statistical analysis

All results were statistically analyzed using GraphPad Prism 6 (GraphPad Software, Inc., CA, United States). The data were represented as average ± standard error of the mean (SEM). For the behavioral tests, one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison test were carried out to compare the averages of all the tested groups at each time point. Also, multiple t-tests within each group were carried out to compare the results at different time points with the baseline as a reference point. An unpaired t-test was used to analyze statistical significance in the results obtained from the ICP-MS and LDH activity assay while multiple t-tests were used in the EMG results to compare the mean amplitudes at each voltage. Statistical significance was indicated by a P value less than 0.05.

CHAPTER III

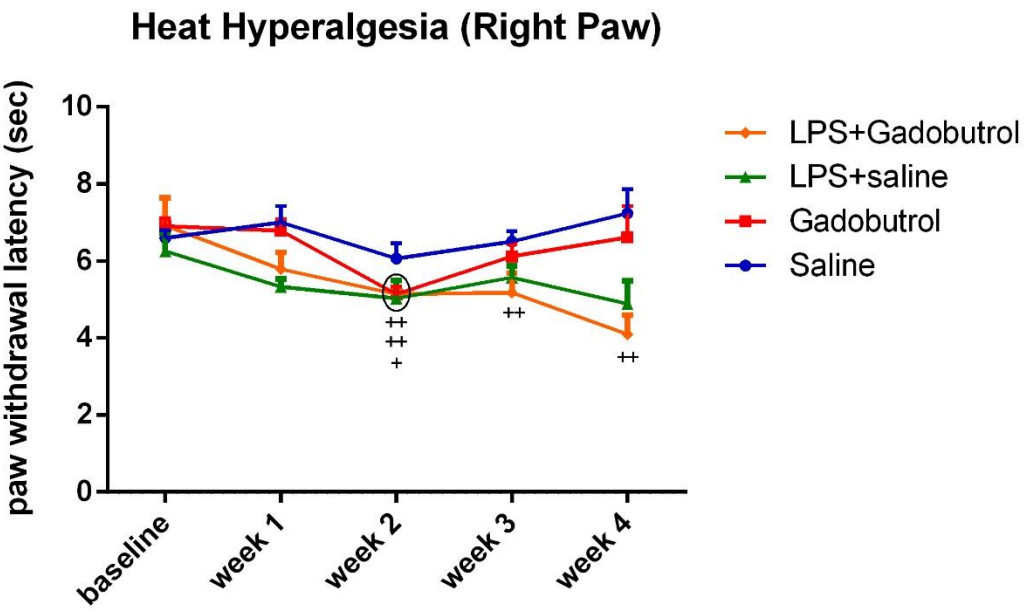
RESULTS

A. Heat Hyperalgesia

Heat hyperalgesia tests were conducted on all rats once a week over a period of 4 weeks to assess their response to a noxious heat stimulus. A reduction in paw withdrawal response indicates an increased sensitivity to noxious thermal stimulation. For the right paw, the LPS + saline group (5.33±0.2113) showed a statistically significant reduction in paw withdrawal latency when compared to the saline group (7.005±0.4178; P=0.0265) at week 1. A similar decrease in latency was observed for the LPS + Gadobutrol group at week 4. Compared to baseline, the withdrawal latency of the LPS + saline, Gadobutrol and LPS + Gadobutrol groups declined significantly at week 2 (P=0.0039; P=0.0044; P=0.004). On the other hand, the withdrawal latency of only the Gadobutrol group decreased significantly compared to baseline at week 3 and week 4 (P=0.0061; P=0.0015).

For the left paw, at the end of week 3, the LPS + saline group (5.14±0.3275) and LPS + Gadobutrol group (4.755±0.3163) demonstrated significantly lower withdrawal latency than the saline group (7.025±0.28; P=0.0193; P=0.0055). Also, the LPS + Gadobutrol (4.755±0.3163) has significantly lower withdrawal latency than the Gadobutrol group (6.545±0.538; P=0.0263). Compared to baseline, the LPS + Gadobutrol group exhibited a statistically significant decline in withdrawal latency at for the duration of the experiment (P=0.0318; P=0.0175; P=0.001; P=0.0005).

A


Heat Hyperalgesia (Right Paw)
paw withdrawal latency (sec)
10
8
6
4
2
0
baseline
week 1
week 2
week 3
week 4
LPS+Gadobutrol
LPS+saline
Gadobutrol
Saline

B

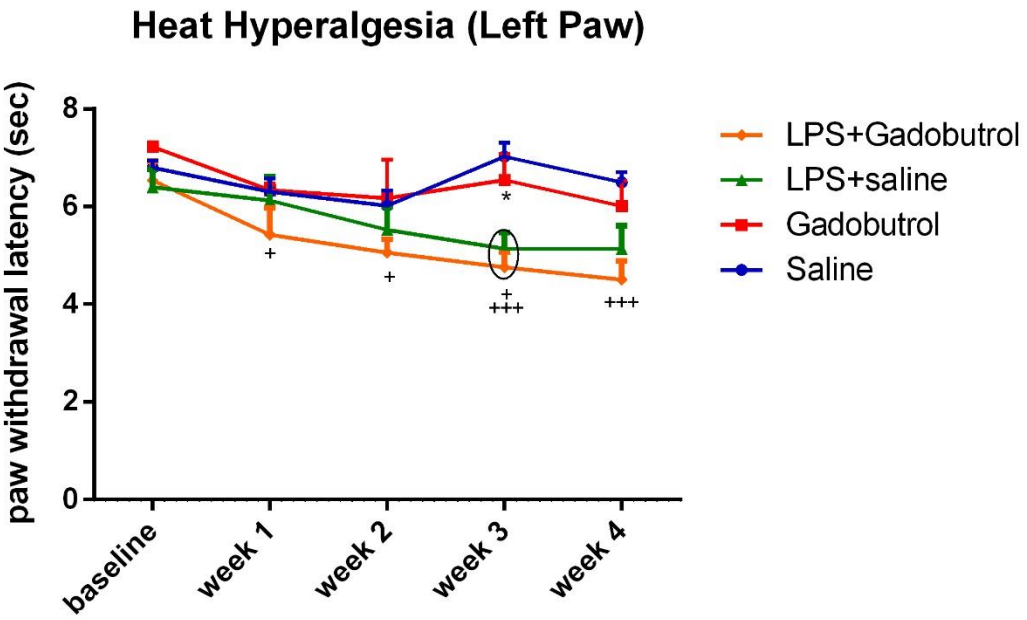

Heat Hyperalgesia (Left Paw)
paw withdrawal latency (sec)
8
6
4
2
0
baseline
week 1
week 2
week 3
week 4
LPS+Gadobutrol
LPS+saline
Gadobutrol
Saline

Figure 4: A- Effect of Gadobutrol administration on heat hyperalgesia in the right hindpaw. Data are represented as mean ± SEM. Multiple t-tests were used to compare the mean at each time point with the baseline (++: P <0.01). One-way ANOVA test followed by Tukey's test were used to compare the means at each time point between groups.

B- Effect of Gadobutrol administration on heat hyperalgesia in the left hindpaw. Data are represented as mean ± SEM. Multiple t-tests were used to compare the mean at each time point with the baseline (+: P<0.05; +++: P <0.001). One-way ANOVA test followed by Tukey's test were used to compare the means at each time point between group (*: P <0.05).

*at week 3: significant difference between the LPS + Gadobutrol and Gadobutrol

B. Beam Walking

Beam walking test was conducted to assess balance and coordination. Statistical analysis results showed that only the LPS + Gadobutrol group (2.697±0.3511) had a significantly longer beam crossing duration than the saline group (1.648±0.1435; P=0.0231) during week 3. On the other hand, compared to baseline, The LPS and LPS + Gadobutrol groups required a longer time to cross the beam when tested at week 2 (P=0.0188, P=0.0282), week 3 (P=0.0076, P=0.0011), and week 4 (P=0.0038, P=0015).

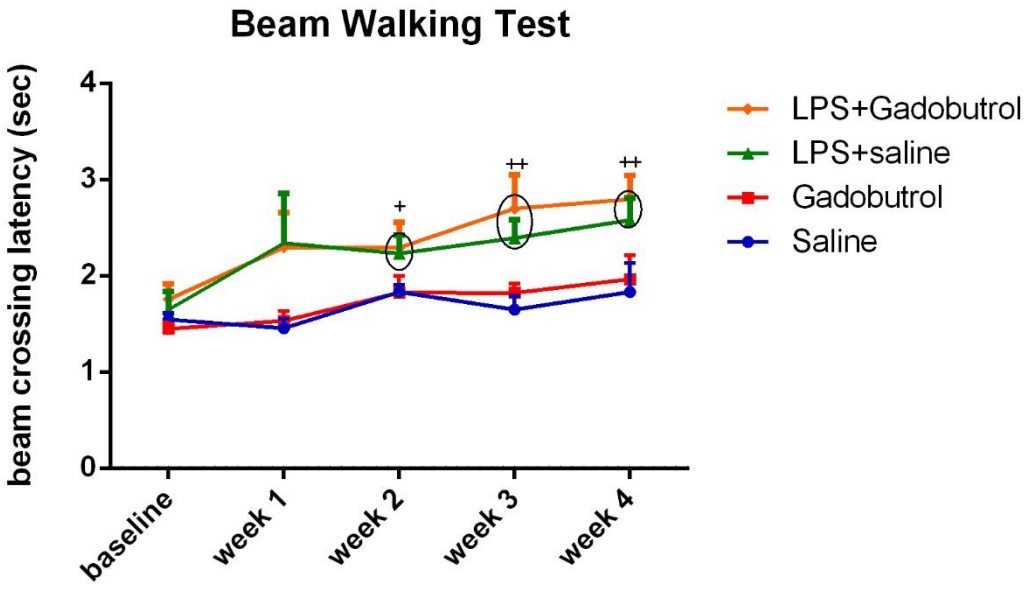


Figure 5: Effect of Gadobutrol administration on motor performance in the beam walking test. Data are represented as mean ± SEM. Multiple t-tests were used to compare the mean at each time point with the baseline (+: P<0.05; ++: P <0.01). One-way ANOVA test followed by Tukey's test were used to compare the means at each time point between groups.

C. Spontaneous alternation T- Maze test

In order to evaluate the cognitive performance of the tested groups, the spontaneous alternation T-maze test was performed once a week for 4 weeks. Statistical significance was only detected between the means of the saline (100±0) and LPS + Gadobutrol (58.34±8.335) groups during week 3 (P=0.0103). Also, compared to baseline, no significant decrease in working memory was detected.

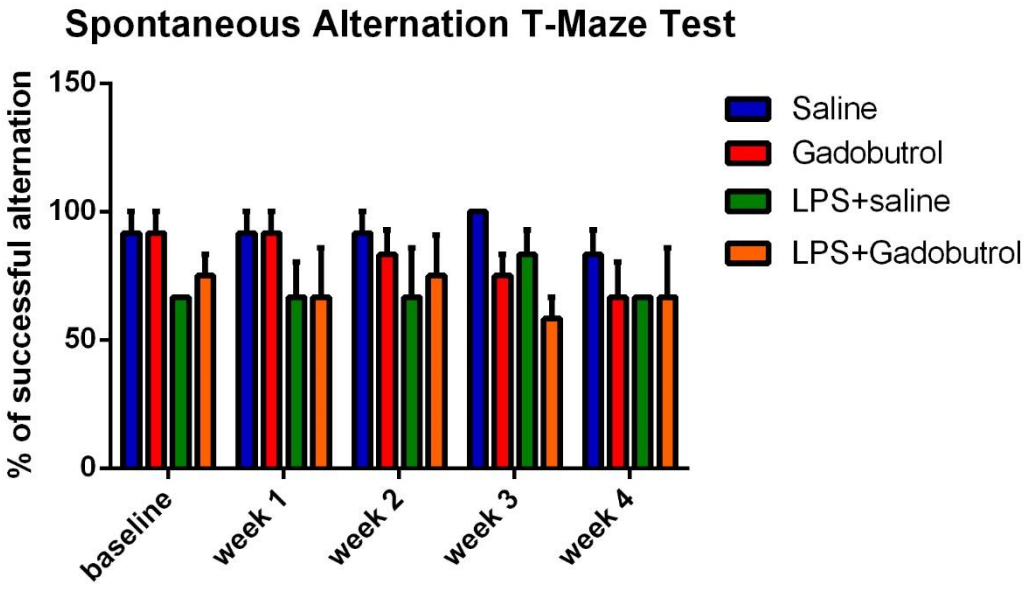


Figure 6: Effect of Gadobutrol administration on spatial working memory in the T-maze test. Data are represented as mean ± SEM. Multiple t-tests were used to compare the mean at each time point with the baseline. One-way ANOVA test followed by Tukey's test were used to compare the means at each time point.

D. Electrophysiological Assessment

Electromyographic recording did not reveal significant difference between the mean amplitude of the evoked CMAPs of the saline and Gadobutrol groups. However, the Gadobutrol group exhibited muscle activation at a stimulating voltage of 1V (0.6887 ± 0.1084) while the saline group required a higher voltage of 5V for activation (1.49 ± 0.4264).

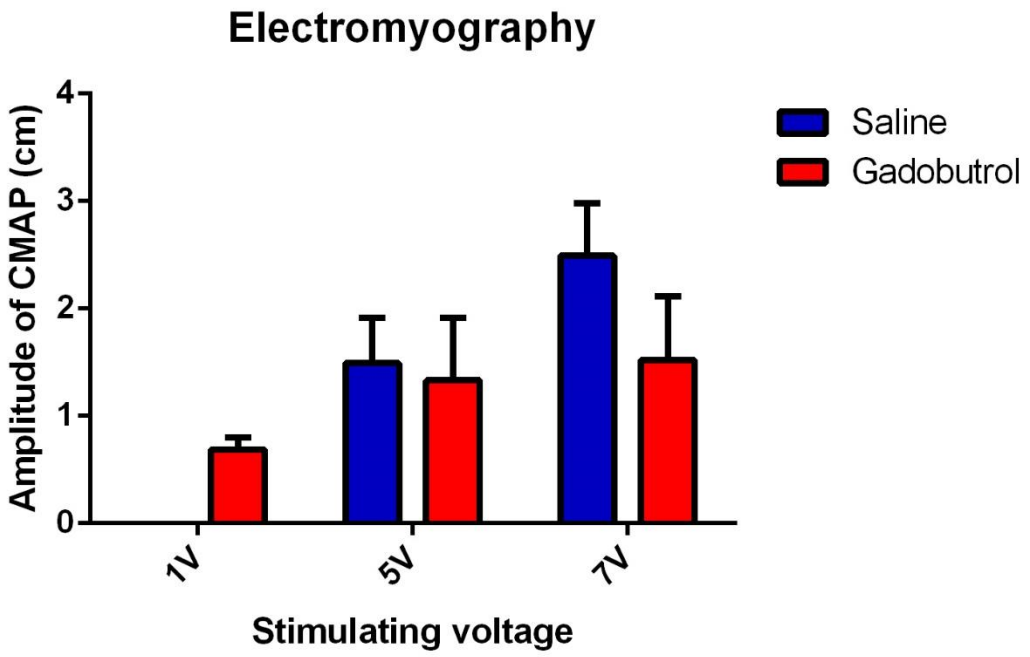


Figure 7: Amplitude of the evoked compound muscle action potentials recorded at a voltage of 1V, 5V, and 7V in the saline and Gadobutrol groups. Data are represented as mean ± SEM. For statistical analysis, multiple t-tests were done at each voltage.

E. Quantification of Gadolinium using ICP-MS

In the cerebrum, the concentration of Gd was significantly greater in the Gadobutrol group (0.4465±0.0394) compared to the saline group (0.0560±0.0213; P= 0.0001). Moreover, the LPS + Gadobutrol group (1.715) showed greater Gd deposition than the Gadobutrol group (0.4465±0.0394). Similarly, in the peripheral nerves, the Gadobutrol group (9.253±1.069) showed a significantly greater concentration of Gd compared to the saline group (0.4245±0.1811; P=0.0031). However, the LPS + Gadobutrol group (3.935) showed greater Gd deposition than the Gadobutrol group (9.253±1.069).

	Cerebrum	Peripheral Nerves
Saline group (n=4)	0.0560±0.0213	0.4245±0.1811
Gadobutrol group (n=4)	0.4465±0.0394	9.253±1.069
LPS + Gadobutrol group (n=2)	1.715	3.935

Table 2: The concentration of Gadolinium in the cerebrum and peripheral nerves of the saline group, Gadobutrol group, and the LPS + Gadobutrol group. Data are represented as mean ± SEM.

1

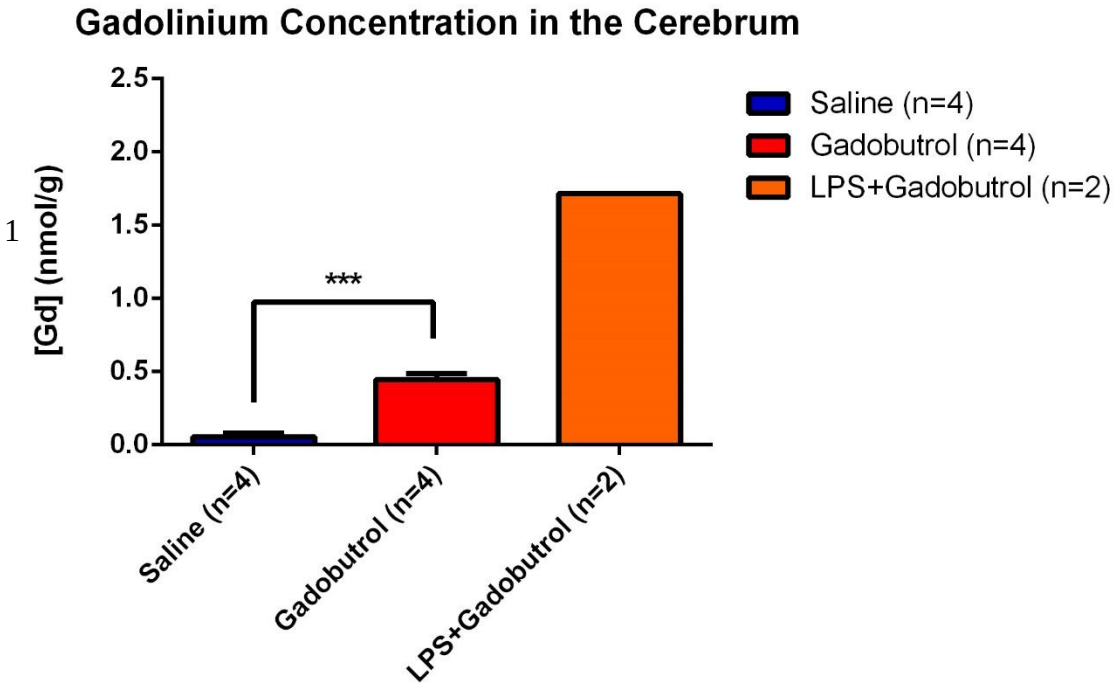


Figure 8: Concentration of Gadolinium in the cerebrum (area between the optic chiasm and the midbrain) of the saline group, Gadobutrol group, and LPS + Gadobutrol group. Data are represented as mean ± SEM. For statistical analysis, an unpaired t-test was done (***: P <0.001).

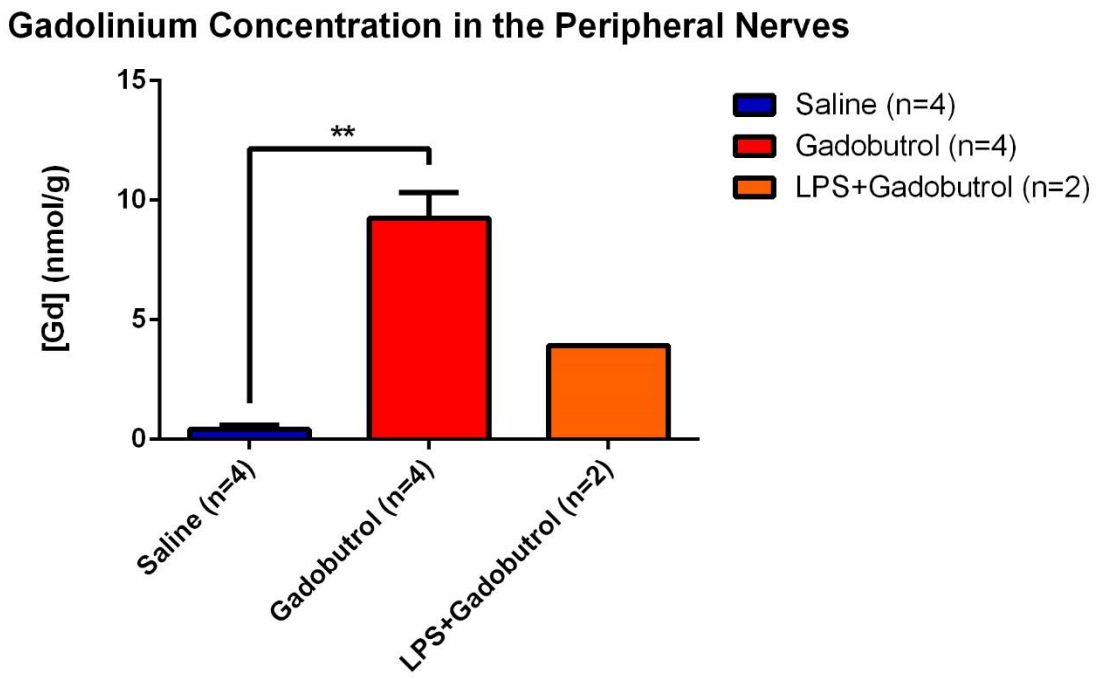


Figure 9: Concentration of Gadolinium in the peripheral nerves (sciatic and trigeminal nerves) of the saline group, Gadobutrol group, and LPS + Gadobutrol group. Data are represented as mean ± SEM. For statistical analysis, an unpaired t-test was done (**: P <0.01).

F. LDH assay

In the right hippocampus, LDH activity was significantly greater in the Gadobutrol group (1661±75.46) compared to the saline group (1260±59.90; P= 0.0059). However, no significant difference was detected between the two groups in the right sciatic nerve (Gadobutrol: 209.7±21.50; saline: 234.8±42.26).

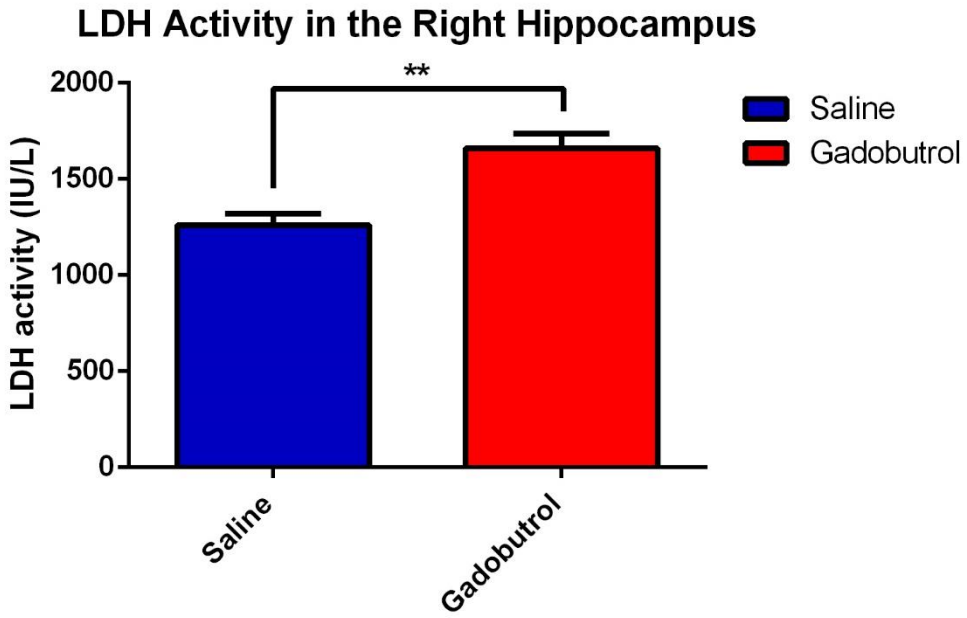


Figure 10: Lactate dehydrogenase activity in the right hippocampus of the saline group and the Gadobutrol group. Data are represented as mean ± SEM. For statistical analysis, an unpaired t-test was done (**: P <0.01).

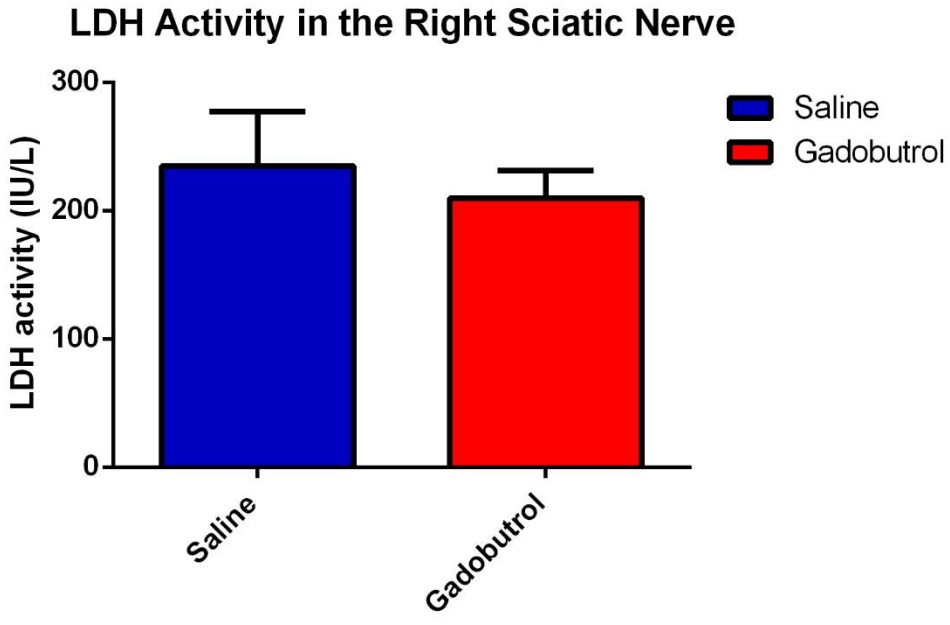


Figure 11: Lactate dehydrogenase activity in the right sciatic nerve of the saline group and Gadobutrol group. Data are represented as mean ± SEM. For statistical analysis, an unpaired t-test was done.

CHAPTER IV

DISCUSSION

This study was conducted to evaluate the safety profile of Gadobutrol by assessing its effect on sensorimotor and cognitive functions under normal and inflammatory conditions. Our findings have shown that repeated injections of Gadobutrol, under normal conditions, do not cause cognitive, motor, or sensory impairment. However, the cumulative effect of LPS-induced inflammation and Gadobutrol can result over time in increased sensitivity to noxious stimulation. Repeated injections of a high dose (2.5mmol/kg) of Gadobutrol resulted in Gd deposition in the cerebrum and peripheral nerves along with cellular damage in the hippocampus but not in peripheral nerves. Nevertheless, Gadobutrol led to a decreased activation threshold in the sciatic nerve. More importantly, our preliminary data showed that an LPS- induced inflammation resulted in greater Gd deposition in the cerebrum but lower deposition in peripheral nerves.

Results of the behavioral tests are consistent with previous studies demonstrating the safe use of macrocyclic GBCAs. Indeed, many clinical studies showed that the rate of adverse reactions following Gadobutrol injection was very low and multiple injections did not result in hyperintensities in the dentate nucleus or globus pallidus (40-42). Moreover, absence of pain sensitivity following gadobutrol injection is in agreement with the findings of the study by Alkhunizi et al., (2020), showing that macrocyclic GBCAs do not induce heat hyperalgesia in rats even at high dosages (49). Interestingly, our results showed that Gadobutrol, like Gadodiamide and Gadoterate Meglumine, caused no spatial working memory impairment in rats (49). Although no

assessment of hippocampal neurogenesis and cell proliferation was carried out in the present study, hippocampal cellular degeneration following Gadobutrol was evident by an increase in the LDH activity. Until present, there are no studies on the impact of Gadobutrol on the hippocampus, so our results are the first to provide evidence for cellular damage in this brain area. Previous studies have shown that cell death and oxidative stress are one of the mechanisms through which GBCAs could exert their effect (56, 57). Even though no markers of oxidative stress were measured in this study, the increase in hippocampal LDH activity lends support to this mechanism. Nonetheless, the present results are in line with those of an in vivo study showing that Gadobutrol increases LDH activity in cells with the effect being more pronounced using linear GBCAs (58).

By comparing the ICP-MS results to a previous study that utilized the same dose of Gadobutrol, regimen of injection, and duration before tissue collection, Gd concentration is lower in the cerebrum than the dentate nucleus (19). It is possible that the large difference in the weights of the collected tissues may be attributed to the differences in Gd concentrations. With regard to other types of GBCAs, the ICP-MS results show that intraperitoneal injection of Gadobutrol leads to lower Gd deposition than Gadodiamide and Gadoterate Meglumine in the cerebrum (49). However, in peripheral nerves, Gadobutrol resulted in greater Gd deposition than Gadoterate Meglumine even though it was still less than that of Gadodiamide (49). Thus, Gd deposition does not only differ according to whether a GBCA is linear or macrocyclic but also depends on the nature of the GBCA administered. It is well known that linear GBCAs deposit more Gd than macrocyclic GBCAs; however, our understanding of the mechanisms underlying its toxic effect in peripheral nervous system is still preliminary,

and the clinical significance of such deposits remains undetermined. Although our behavioral results did not reveal any sensory changes or motor deficits in rats exposed to Gadobutrol, the EMG recordings clearly indicate that these rats exhibited enhanced motor unit recruitment in response to low stimulus threshold as compared to control, suggesting that Gd^{3+} deposited in nerves could interfere with nerve conduction and induce this increased response. Whether Gd^{3+} deposition has a differential effect on sensory and motor fibers remains to be determined. These findings possibly support the modulatory effect of Gd on voltage gated channels as a mechanism of action leading to impaired signal transduction in the peripheral nerves (75).

When LPS was injected prior to Gadobutrol, the amount of Gd deposited in the nervous system was altered. This is supported by previous studies which showed that 8 injections of 2.5 mmol/kg of Gadodiamide in rats with neuroinflammation lead to an increase in Gd deposition in the brain (68). LPS is known to induce a state of chronic neuroinflammation through activating a network of pro-inflammatory factors (TNFα and IL-18) (84). The onset of this activation is rapid as levels of proinflammatory cytokines were shown to increase 30 minutes following LPS administration (85). Furthermore, TNFα levels peak in the brain 60 minutes after an intraperitoneal injection of 5mg/kg LPS in mice (86). This study demonstrated for the first time that repeated exposure to Gadobutrol, coupled with inflammation, could lead to enhanced neural changes resulting in increased sensitivity to sensory stimulation. Moreover, the interplay between inflammation and Gadobutrol is supported by a previous finding that noted an increase in the anti-inflammatory cytokine, IL-10, levels following Gadobutrol exposure, but not as much as other GBCAs (80). The effect of Gd on levels of inflammatory cytokines is a possible mechanism of action for Gd neurotoxicity that

requires more investigation. On the other hand, inflammation induced by LPS could have possibly compromised the integrity of the BBB and allowed more Gd to reach the brain given the increase in Gd deposition in the LPS + Gadobutrol group. The compromised BBB could also explain the drop in Gd deposition in the peripheral nerves of the aforementioned group supporting the notion that Gd deposition increases near inflammatory sites. More importantly, the development of heat hyperalgesia in the LPS + Gadobutrol group may be the result of central or peripheral sensitization induced by the neuroinflammation, and enhanced by the Gd deposition in both areas (49).

Despite the novel findings of the study, it had several limitations. The small sample size per group might have skewed the results making the outcome insignificant. Thus, increasing the sample size is recommended to ensure more reliable results. Moreover, the levels of pro-inflammatory and anti-inflammatory cytokines prior to and following LPS injection were not measured to confirm the presence of inflammation in the CNS, and to determine the time at which inflammation is maximal to administer Gadobutrol. The levels of cytokines should be measured after 20 days of Gadobutrol to account for any changes. The high dosage of Gadobutrol used in the present study was another limitation because it does not correlate with the clinically administered dose. However, it would be interesting to investigate whether a lower dosage (0.8mmol/kg) could result in an increase in hippocampal LDH activity. Markers of oxidative stress should also be measured to better understand the mechanism of action of GBCAs. Due to time limitations, the LDH activity in the hippocampus and the sciatic nerves was not measured in tissues of rats injected with LPS, which is vital to perform in light of working memory decline exhibited by this group on week 3. Lastly, the cognitive test

used in this study measures the exploratory behavior in animals; hence, for a more robust evaluation of cognitive functions, additional memory tests should be performed.

CHAPTER V

CONCLUSION

This study examined the safety profile of repeated high dose injections of Gadobutrol by evaluating its effect on the CNS and PNS in normal and inflammatory states. The findings demonstrate evident Gd deposition in peripheral nerves and the cerebrum with a high hippocampal LDH activity indicating neuronal damage. Under inflammatory conditions, repeated injections of Gadobutrol induced the development of heat hyperalgesia and increased Gd deposition in the cerebrum, suggesting that inflammation could serve as a trigger factor for Gadobutrol neurotoxicity. It is worth noting that this study is the first to show not only hippocampal cell damage following a high dose of Gadobutrol but also altered sensory responses when Gadobutrol was given following LPS. These results shed the light on the role of inflammation in GBCA neurotoxicity and pave the way for clinicians and scientists to conduct intensive research into the role of inflammatory mediators in exacerbating the neurotoxic effect of retained Gd. Understanding the mechanisms of action of GBCAs requires further investigation, as they appear to employ different pathways resulting in central and peripheral neurological manifestations.

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