EFFECTS OF THE ANTIEPILEPTIC MEDICATION VALPROIC ACID SODIUM SALT (VPA) ON THE HISTOLOGICAL BONE STRUCTURE IN *XENOPUS LAEVIS*

A Report of a Senior Study

by

Mallory AnnaLois Kirkland

Major: Biology

Minor: Mathematics

Maryville College

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Date Approved_____, by _____

Faculty Supervisor

Date Approved_____, by _____

Division Chair, Natural Sciences

ABSTRACT

Epilepsy and seizures affect approximately 2.2 million people in the United States with approximately 200,000 new cases diagnosed each year. Due to neither an effective prophylaxis nor an available cure, antiepileptic medications serve as the treatment of choice in reducing and suppressing seizure activity. Some of these antiepileptic medications exhibit adverse side effects. Although it is commonly acknowledged that enzyme-inducing antiepileptic drugs (e.g., carbamazepine, phenytoin, and phenobarbital) cause osteoporosis, valproic acid sodium salt is a well-known cytochrome P450 enzyme and estrogen inhibitor that has shown mixed results on affecting bone density. This study used an amphibian model (*Xenopus laevis*) to investigate the effects on bone density after thirty-day exposure to the anti-epileptic drug valproic acid sodium salt (VPA). Histological analysis of the femurs (n=4 exposed, n=4 controls) showed that VPA did not influence the number of osteoblasts or osteocytes within the diaphysis of the femur ($p = 0.4384$ and $p = 0.4799$, respectively). Similarly, the number of chondrocytes within epiphyses of femur was not affected by the drug ($p = 0.1335$). In conclusion, VPA exposure led to no significant affect on bone density in *X. laevis*. Future studies should examine the effect of chronic use of VPA and VPA combined administration with supplements containing both calcium and vitamin D, an osteopenia preventative method recommended by physicians.

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CHAPTER I

INTRODUCTION

Epilepsy

Epilepsy and seizures affect approximately 70 million people worldwide (~ 1-2 % of the population), with an estimated 2.2 million living in the United States.[1] With approximately 200,000 new cases each year,[1] research in this area is active.[2] Surprisingly, this disease is more common than most disorders of the nervous system such as autism, cerebral palsy, multiple sclerosis, and Parkinson disease combined; however, epilepsy falls second only to stroke and Alzheimer's disease, where stroke damaged areas exhibit pathological precursors of Alzheimer's.[2-3] The high incidence of epilepsy is due to the a significant number of causes: genetic abnormalities, malformation or degeneration of the brain, febrile convulsions, craniofacial trauma, central nervous system infections (i.e. meningitis), hypoxia, ischemia, and tumors.[2]

A seizure refers to a momentary change of behavior due to the disordered, synchronous, and rhythmic firing of populations of central nervous system neurons.[4] These impulses travel from the neuron along the axon, and then stimulate the release of neurotransmitters which flow across the synaptic cleft to the dendrites of the receiving cell. According to the Epilepsy Foundation of America (EFA), if there is a consistently higher level of the excitatory neurotransmitters, or too few inhibitory ones, the likelihood

of a seizure is increased.[5] Some of the newer medications, which will be discussed later, relate directly to this process and are designed to increase the level of inhibitory neurotransmitters, especially gamma-aminobutyric acid (GABA), or to decrease the amount of the excitatory ones, such as glutamate.[5] In comparison to a seizure, the term "epilepsy" simply refers to a disorder of brain function characterized by the periodic and unpredictable occurrences of seizures.[4] Due to the high incidence in epilepsy, various advancements in medicinal treatments are being derived in order to specially treat the various types of epileptic seizures such as primary generalized seizures and/or focal (partial) seizures (see Figure 1).[6]

Primary generalized seizures cause both cerebral hemispheres to be affected by dramatically changing the electrical activity of the neurons and causing unconsciousness.[7] Examples of generalized seizures include tonic-clonic, clonic, absence, myoclonic, and atonic seizures. Tonic-clonic seizures, or grand mal seizures, are the most common types associated with seizures in general due to their severity.[8] Injuries are usually not linked to tonic-clonic seizures; yet, they can potentially lead to biting of the tongue or muscle strain. The tonic, or stiffening phase, is the first physiological response as all the muscles stiffen. In addition, air that is being forced past the vocal cords tends to cause a cry or a groan. Lastly, the person quickly loses consciousness and due to inability to properly breathe, cyanosis (turning blue) occurs. After the tonic phase, the clonic phase enables rapid arm or leg jerking, bending and relaxing, which tends to last no more than a few minutes. As the body relaxes, bladder or bowel control sometimes is lost leading to incontinence. Consciousness returns slowly and the person may express a variety of sensations: drowsiness, confusion, agitation, or depression. As stated previously, seizures

do not always have tonic stiffening and clonic jerking sequences, but instead they can be categorized as tonic only or clonic only.[8] Other than tonic-clonic, tonic, and clonic seizures, absence seizures are another characterized generalized seizure. Absence seizures (petit mal seizures) involve a brief, sudden lapse of consciousness that resemble episodes of daydreaming.[9] During this type of generalized seizure, a person tends to stare absently for approximately 30 seconds. Once the seizure has stopped, the person resumes their normal activity as though nothing happened. Absence seizures are rather easy to characterize since they are induced or enhanced by hyperventilation in more than 90% of patients.[11] Next, myoclonic seizures, or "jumps", are rapid, brief contractions and relaxations of bodily muscles, which usually occur at the same time on both sides of the body.[8] Unlike the other types, they last only a few seconds and result in an increase in muscle tone. Contrarily, atonic seizures are characterized by sudden diminution of muscle tone as they cause the muscles to go limp. It is thought atonic seizures are caused by use of "intense inhibitory mechanisms involving negative motor areas, discharges from primary sensorimotor cortex-inhibiting spinal motor neurons, recurrent cortical inhibition that involves the thalamocortical pathways and brainstem pathways that activate the pontomedullary reticular formation." [11(p. 21)] Since the areas of the brain stem that are responsible for increasing and decreasing muscle tone are relatively close in proximity, it is assumed that myoclonic and atonic seizures stem from the same area of the brain.[8]

In comparison to generalized seizures, focal, or more commonly known as partial, seizures have an excessive electrical discharge coming from a specific portion of the brain (i.e. the frontal, temporal, parietal, or occipital lobe).[5] Although generalized

seizures are examined more due to their severity, partial seizures are the most common type of seizure experienced by people with epilepsy, initiating virtually any movement, sensory or emotional symptom to occur, including complex visual or auditory hallucinations. Partial seizures are further categorized as either simple partial seizures or complex partial seizures. A simple partial seizure lasts no longer than 30 to 60 seconds, with no loss of consciousness. Additionally, they lead to sudden movements (jerks), sensory phenomena, and transient weakness or loss of sensation. On the other hand, a complex partial seizure lasts roughly 1 to 2 minutes. It is known to include an aura (i.e. sensation in stomach), automatisms (i.e. lip smacking, picking at clothes, fumbling), and/or unawareness of the environment. Complex partial seizures differ most from simple partial seizures in that the individual loses consciousness and encounter amnesia for seizure events. Furthermore, partial seizures, whether simple or complex, may spread or progress to a generalized tonic-clonic seizure, in which case the classification category is partial seizures secondarily generalized.[5] Triggers that can cause these electrical spikes in both generalized and focal seizures include illness, fever, stress, excitement, menstruation, and photosensitivity.[7]

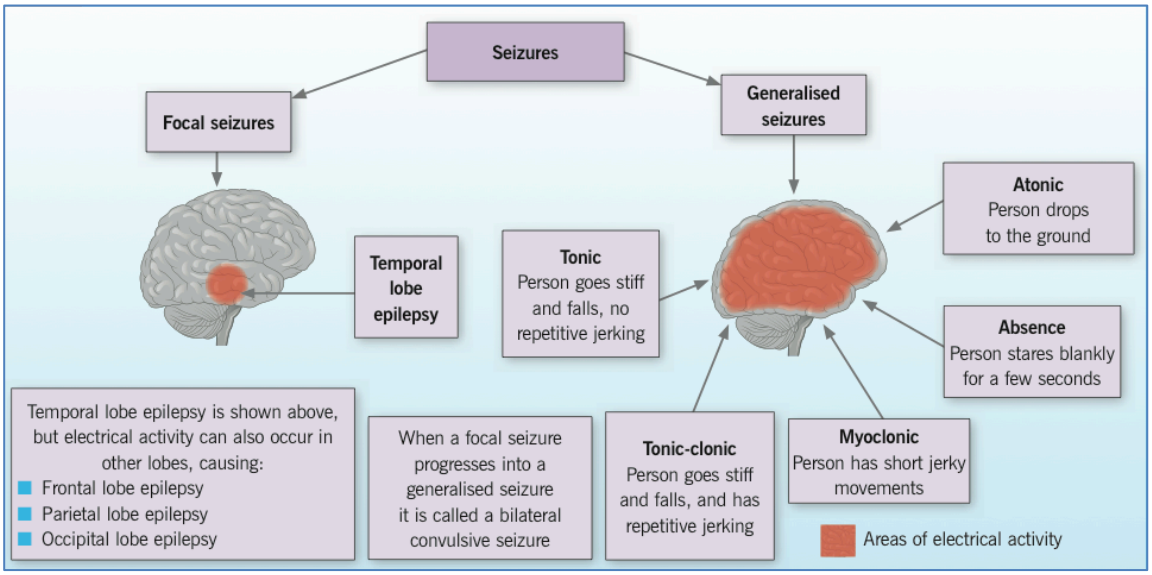


Figure 1. Pictorial representation of the activity and affects of generalized and focal seizures.[7(p. 29)]

Depending on the pattern of generalized or focal seizures, epilepsy is divided into four main categories: idiopathic, symptomatic, provoked, and cryptogenic. Idiopathic epilepsy is an epilepsy of predominantly genetic or presumed genetic origin, which consists of no gross neuroanatomical or neuropathological abnormality.[12] Next, symptomatic epilepsy is a type of epilepsy thought to be of an acquired disease of genetic cause, associated with gross anatomic or pathologic abnormalities. As a result, this category includes developmental and congenital disorders (genetic or acquired), in addition to single gene and other genetic disorders. Provoked epilepsy, instead, is defined as an epilepsy in which a specific environmental or systemic factor is the primary cause of the seizures with no neuroanatomical malformations. Finally, cryptogenic epilepsy accounts for epilepsy of presumed symptomatic nature in which the cause has not been identified. Although little is known about this category, it accounts for at least 40% of adult-onset cases of epilepsy.[12]

Treatments

The type of epilepsy (idiopathic, symptomatic, provoked, or cryptogenic) determines the treatment options for epileptic patients. Available drugs reduce the frequency of seizures in a large majority of patients; however, only 40% are free of seizures despite optimal treatment.[4] Currently, neither an effective prophylaxis nor a cure is available excluding neurosurgical resection of epileptic tissue in selected instances.[4] As a result of this limitation, antiepileptic medications serve as the treatment of choice.

Antiepileptic medicinal treatments can result in three primary responses: "spontaneous" remission, remission with treatment, and continuing seizures. Approximately 20-30% of patients enter into long-term remission after a variable period of time and activity with antiepileptic drug treatment.[13] These drugs suppress seizures until "spontaneous" remission occurs. Epilepsy symptoms such as benign neonatal seizures, benign rolandic epilepsy, and childhood absence epilepsy fall into this category. In addition to spontaneous remission, antiepileptic drug therapy can lead to complete remission with continuous usage. This also occurs in 20-30% of epileptic patients and may require more than one antiepileptic drug treatment. Examples of this group include juvenile myoclonic epilepsy in addition to the bulk of the localization related (partial) epilepsies. Lastly, antiepileptic medications cause 30-40% of patients to experience continuing seizures. Even with constant treatment and multiple medications, seizures are only somewhat inhibited due to their severity and frequency. Conditions in this category include many symptomatic/cryptogenic localization related epilepsies (i.e. mesial

temporal sclerosis, cortical dysplasia, gross structural brain lesions, the progressive myoclonic epilepsies, and West syndrome).[13]

Although medicinal treatments suppress seizures, they also can have adverse effects on a patient. Table 1 shows the most common conventional antiepileptic drugs (AEDs) and the seizure types and epilepsy syndromes they are primarily used to treat/suppress among specific age groups. More importantly, both the minor and severe effects of each AED are presented.

Table 1. Specified treatment options and side effects of conventional antiepileptic medications.

Generic Name [14-17]	*Brand Name* [14]	*Seizure Type* [14,15,17]	*Epilepsy Syndrome* [14,17]	*Age (Years)* [14]	*Common Adverse Effects of AEDs* [14-16]		*Severe Adverse Effects of AEDs* [14-16]	
Carbamazepine	Carbatrol Epito Equetro Tegretol (X)	Complex Partial Simple Partial Generalized Tonic-Clonic Mixed (Both)		Any	Dizziness Diplopia Blurred Vision Ataxia	Sedation Neutropenia Rash Hyponatremia	Agrangulocytosis Aplastic anemia Hepatic failure Stevens-Johnson Syndrome Congestive heart failure	Jaundice Arrhythmia Hyponatremia Unusual bruising or bleeding Osteopenia
Clonazepam	Klonopin Klonopin Wafer	Atonic Absence Myoclinic	Lennox-Gastaut Syndrome	Any	Drowsiness Decrease in Cognitivity Rash Blurred Vision	Headache Nausea Insomnia Weight Changes	Atrial flutter Breathing problems Hallucinations Worsening seizures	Painful urination Jaundice Behavioral Changes
Clorazepate	Tranxene SD Tranxene T-Tab	Complex Partial Simple Partial		> 9	Drowsiness Amnesia Dizziness Insomnia	Blurred vision Headache Nausea Rash	Tremor Depression	Confusion Painful urination
Diazepam	Valium	Any Convulsive Disorder		> 6	Cognitive problems Drowsiness Dizziness Nausea	Blurred vision Rash Muscle weakness	Breathing problems Worsening seizures Tremor	Hyperactivity Hallucinations Depression
Ethosuximide	Emeside Zarontin	Absence	Childhood Absence Epilepsy Juvenile Absence Epilepsy	> 5	Headache Dizziness Drowsiness	Nausea Weight loss Lack in coordination	Atrial flutter Atrial fibrillation Hallucinations	Swelling Flu symptoms Rapid weight gain
Felbamate	Felbatol	Partial Generalized	Lennox-Gastaut Syndrome	Any	Weight change Headache Dizziness Drowsiness	Insomnia Nausea Blurred vision Rash	Worsening seizures Painful urination Breathing problems	Atrial flutter Flu symptoms
Gabapentin	Gralise Horizant Neurontin Gabarone	Partial Generalized Seizures		> 3	Dizziness Drowsiness Blurred vision	Nausea Rash Irritability	Worsening seizures Jaundice Flu symptoms Confusion	Swelling Breathing problems Behavioral changes
Lacosamide	Vimpat	Partial		> 16	Dizziness Diplopia Blurred Vision	Headache Nausea	PR interval prolongation Atrial fibrillation	Atrial flutter Multiorgan hypersensitivity
Lamotrigine	Lamictal	Partial Absence Generalized Tonic-Clonic Tonic Clonic Myoclonic	Lennox-Gastaut Syndrome Juvenile Myoclonic Epilepsy Childhood Absence Epilepsy Juvenile Absence Epilepsy	> 1	Dizziness Diplopia Blurred Vision	Headache Insomnia Rash	Stevens-Johnson Syndrome Toxic epidermal necrolysis	Multiorgan failure Hepatic failure
Levetiracetem	Keppra	Partial Myoclonic Generalized Tonic-Clonic Tonic Clonic	Childhood Absence Epilepsy Juvenile Absence Epilepsy	> 4	Fatigue Dizziness Somnolence	Irritability Mood swings	Psychosis	
Oxcarbazepine	Trileptal	Partial	Benign Rolandic Epilepsy	> 2	Dizziness Diplopia Blurred Vision	Headache Nausea Hyponatremia	Stevens-Johnson Syndrome	Toxic epidermal necrolysis

Generic Name [14-17]	*Brand Name* [14]	*Seizure Type* [14,15,17]	*Epilepsy Syndrome* [14,17]	*Age (Years* [14]	*Common Adverse Effects of AEDs* [14-16]		*Severe Adverse Effects of AEDs* [14-16]	
Phenobarbital	Solfoton Luminal	Partial Generalized Tonic-Clonic		Any	Cognitive problems Drowsiness Dizziness	Nausea Blurred vision Irritability	Broken blood vessels Breathing problems Restlessness	Fever/Sore throat Atrial flutter Osteopenia
Phenytoin sodium	Dilantin Kapseals Phenytek	Complex Partial Generalized Tonic-Clonic		Any	Fatigue Dizziness Ataxia Confusion	Gingival hyperplasia Hirsutism Osteopenia Rash	Stevens-Johnson Syndrome Toxic epidermal necrolysis Blood dyscrasia	Pseudolymphoma Lupus-like Syndrome Osteopenia
Phenytoin acid	Dilantin Infatabs Dilantin Suspension	Complex Partial Generalized Tonic-Clonic		Any	Fatigue Dizziness Ataxia Confusion	Gingival hyperplasia Hirsutism Osteopenia Rash	Stevens-Johnson Syndrome Toxic epidermal necrolysis Blood dyscrasia	Pseudolymphoma Lupus-like Syndrome Osteopenia
Pregabalin	Lyrica	Partial Onset		> 18	Fatigue Dizziness Ataxia	Diplopia Weight gain Edema	None reported	
Primidone	Mysoline	Complex Partial Simple Partial Generalized Tonic-Clonic Atonic Myoclonic		Any	Dizziness Drowsiness Blurred vision	Nausea Rash Irritability	Loss of coordination Communicative difficulties	Unusual weakness
Rufinamide	Banzel	Partial Generalized	Lennox-Gastaut Syndrome	> 3	Somnolence Headache Dizziness	Diplopia Fatigue Nausea	Shortened QT interval	Multiorgan hypersensitivity
Tiagabine	Gabitril	Partial		> 12	Dizziness Drowsiness Decrease in Cognitivity Weight change	Blurred Vision Headache Nausea Insomnia	Rash Confusion Hallucination Communicative difficulties	Increase heart rate Flu symptoms Tremor
Topiramate	Topomax	Partial Generalized Tonic-Clonic Tonic Clonic Myoclonic Atonic Absence	Lennox-Gastaut Syndrome Juvenile Myoclonic Epilepsy Childhood Absence Epilepsy Juvenile Absence Epilepsy Benign Rolandic Epilepsy	> 2	Drowsiness Ataxia Communicative difficulties Difficulty concentrating Anorexia	Weight loss Paresthesias Metabolic acidosis Oligohydrosis Nephrolithiasis	Acute close angle glaucoma	Heat stroke
* Valproic acid	Depakene (Syrup) Depakote (Sprinkles)	Atonic Myoclonic Absence Complex Partial	Lennox-Gastaut Syndrome Childhood Absence Epilepsy Juvenile Absence Epilepsy	> 2	Drowsiness Ataxia Tremor Hair loss	Weight gain Thrombocytopenia Hyperammonemia	Hepatic failure Aplastic anemia Pancreatitis Osteopenia	Lupus-like Syndrome Stevens-Johnson Syndrome Toxic epidermal necrolysis
Vigabatrin	Sabril	Infantile Spasms		< 18	Dizziness Drowsiness Decrease in Cognitivity Weight change	Blurred Vision Headache Nausea Insomnia	Vision changes Tremors Mood changes	Worsening seizures Difficulty concentrating Increased heart rate
Zonisamide	Zonegran	Partial	Juvenile Myoclonic Epilepsy Childhood Absence Epilepsy	> 18	Drowsiness Ataxia Difficulty concentrating Anorexia	Weight loss Nausea Nephrolithiasis Oligohydrosis	Aplastic anemia Rash Stevens-Johnson Syndrome	Toxic epidermal necrolysis Heat stroke

The sodium salt of valproic acid ($C_8H_{15}NaO_2$), more commonly referred to as sodium valproate (VPA), has long been used as a non-induced enzymatic antiepileptic medication to prevent many types of seizures. It is a commonly used broad-spectrum drug that shows efficacy in partial and generalized seizure types (i.e. atonic, myoclonic, absence, or partial).[18] VPA is available in different names, strengths, and formulations (liquid, delayed tablets or extended release tablets, or sprinkle capsules).[19]

Due to its effectiveness, it appears to involve several mechanisms and act on a variety of targets. According to the EFA, it is thought to block high-frequency, repetitive neuronal firing by blocking voltage-dependent sodium channels.[20] In addition, it may enhance the action of glutamic acid decarboxylase (GAD), a GABA-synthesizing enzyme, and at high concentrations, it can impede GABA-T (GABA transaminase), which is known to hasten the degradation of GABA.[20-21] Because of its affect on the function of the neurotransmitter GABA in the human brain, it also acts as a common treatment of bipolar disorder, in place of lithium salts. VPA is a proven broad spectrum anticonvulsant since it blocks the voltage-gated sodium channels in addition to blocking T-type calcium currents existent in absence seizures.[20] Furthermore, VPA acts as an inhibitor of histone deacetylases (HDACs), glycogen synthase kinase 3 (GSK3), and cytochrome P450 and depletes cellular inositol-1,4,5-triphoshphate (1,4,5-IP_3).[18,22-23] Because of its general actions, VPA shows potential in combination therapy for cancer and Alzheimer's disease treatment.[24-25]

Because epilepsy and bipolar disease require lifelong treatment, the continuous and long-term use of VPA becomes a major concern to patients due to the potential adverse effects, which are summarized in Table 1. Among potential side effects are low

bone mineral density (BMD) causing the increased chance of osteoporosis, osteopenia, and/or fracture risk.[26] As can be inferred from other studies, it is more common that the enzyme-inducing antiepileptic drugs (i.e. carbamazepine, phenytoin, and phenobarbital) cause osteoporosis since they accelerate the metabolism of vitamin D by inducing the cytochrome P450 enzymatic system.[18] However, VPA is a well-known cytochrome P450 enzyme inhibitor which has shown multiple affects on bone.

The effects of chronic (8 weeks) VPA treatment (0, 2, 4, and 6 g/kg food) on the total bone mineral content (BMC) by dual energy x-ray absorptiometry in C3H/HeJ mice showed a 9.1% ($p < 0.01$) reduction in BMC.[27] In comparison, VPA has also displayed a direct effect on bone cultured bone cells, resulting in increased bone turnover with osteoblastic (bone formation) and osteoclastic (bone resorption).[26,28] Additionally, it has been proposed that enzyme-inducing anticonvulsants, excluding VPA, reduce vitamin K levels since interaction with vitamin K, which acts as a cofactor in the synthesis of osteocalcin (marker of bone formation) acts as a possible mechanism of osteoporosis in epileptic patients.[26] VPA also increases testosterone levels in patients, thereby causing a decrease in estrogen levels, which normally reduces bone resorption affecting osteoclast purpose and origin.[26]

Furthermore, 40 adults with epilepsy were placed on long-term VPA monotherapy.[29] The BMD of the second metacarpal was determined as T- and Z- scores. From control, the BMD reduced approximately 14% with the use of VPA. To elaborate, nine patients had T-scores below -2.5 SD (standard deviation) suggesting osteoporosis and fifteen had T-scores between -1.0 and -2.5 SD, which was an implication of osteopenia (decrease in the amount of calcium and phosphorus in the bone resulting in

low bone density).[29] Moreover, serum concentrations of calcium and bone Gla protein (bone formation marker) and pyridinoline cross-linked carboxy-terminal telopeptide of type-I collagen (ICTP – bone resorption marker) were significantly higher with VPA administration in comparison to the control values. Z-scores for BMD in patients taking VPA resulted in a negative correlation between calcium and ICTP; therefore, it was inferred that long-term VPA monotherapy increases bone resorption leading to an overall decrease in BMD.[29]

Lastly, a relationship between the bone mineral statuses in ambulatory pediatric patients that were on long-term antiepileptic drug therapy was confirmed.[30] For at least two years, 18 patients within the age range of rapid bone mineralization having primary epilepsy were exposed to a variety of anti-epileptic medications including VPA (n=9), carbamazepine (n=3), phenobarbital, ethosuximide, and clonazepam.[30] Three of the remaining six patients were placed on polytherapy. During this instance, the lumbar spine bone mineral density was measured by DXA (dual-energy X-ray absorption) and showed that five of the eighteen patients showed osteopenia, with three of these five taking VPA.[30] The results also showed that males had a higher state of decreased bone turnover resulting in osteopenia.[30]

Bone Development and Growth

During embryonic development, a cartilage frame produced by fibroblast cells is replaced by bone to add stringency and strength when forming the skeleton. Bone ossifies in two different embryonic manners: intramembranous ossification and endochondral ossification.[31] The intramembranous bones develop into flat bones (e.g., bones of the skull), whereas the endochondral bones develop into long, short, or irregular bones.[31] Since the two categories develop into different bone types, they somewhat vary in mechanisms of bone formation and development.

In intramembranous ossification, connective tissue forms in sheets highly invested with blood vessels at sites where flat bones will eventually be causing fibroblasts to differentiate into osteoblasts.[31] The osteoblasts form an extracellular matrix of collagen and protein polysaccharides called an osteoid causing the formation of spongy bone.[31-32] Eventually, the osteoblasts get trapped within the hard matrix developing into osteocytes. The osteocytes instigate the connective tissue sheets to become the bone's periosteum, and after formation of the periosteum, the osteoblasts change function and begin to accumulate on the edges of spongy bone laying down harder matrix as compact bone.[31]

In contrast, endochondral ossification involves bone formation by replacement of hyaline cartilage. Initially, chondrocytes within the cartilaginous model begin to die in the primary ossification center located in middle of diaphysis before they move onto the secondary ossification center in the epiphyses.[31,33] Due to the loss of chondrocytes, the cartilaginous model becomes highly susceptible to blood vessels, nerves, and osteoblasts, which secrete hard extracellular matrix material until they get trapped and transform into osteocytes.[31] The cartilage of the diaphysis and epiphyses gets replaced by spongy during

the initial ossification. Meanwhile, periosteum, which supplies the needed osteoblasts for bone development, eventually becomes incapable of entering the spongy bone to form osteoclasts; instead, the osteoblasts start building up on the periphery of the spongy bone forming compact bone.[31]

Because bone ossification is a recurring process throughout life, perichondral bone is constantly added around the periphery of the cartilage skeleton causing the increasing thickness of the bone shaft. This process, known as the postembryonic development of bone, allows the bone to be constantly remodeled in order for growth and metabolism (see Figure 2).[31] In order for downregulation, osteoblasts orchestrate bone resorption through activation signals from some of the following systemic factors: growth hormone (GH), interleukins (IL-1,IL-6), parathyroid hormone (PTH), and/or withdrawal of estrogen (-E2).[33] In addition, M-CSF and RANKL (Receptor Activator of Nuclear Factor-KappaBeta Ligand) are the two major osteoblast mediated factors that regulate the recruitment and differentiation of the osteoclasts.[33] Osteoclasts resorb the bone by secreting collagenase and other enzymes and releasing calcium into the blood.

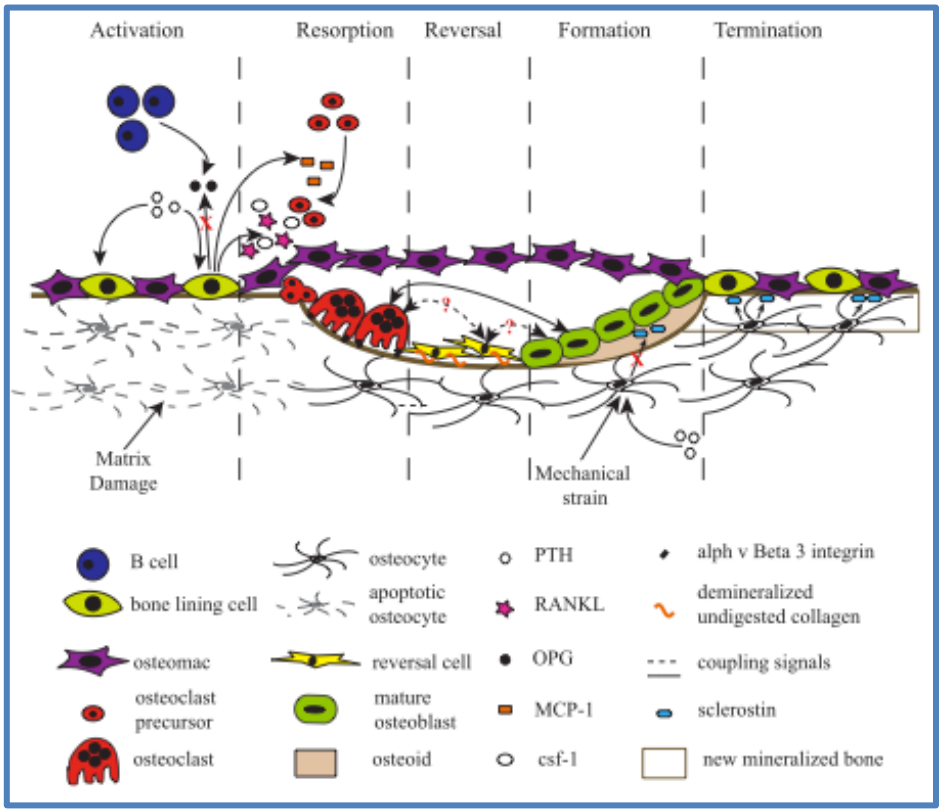


Figure 2. Pictorial representation of the bone remodeling cycle during postembryonic development.[34(p.25205)]

The inhibition of any of these hormonal signals from osteoblasts or osteoclasts results in reduction in bone resorption. The entire life cycle (i.e. recruitment, proliferation, apoptosis) of osteocytes, osteoblasts, and osteoclasts along with their regulatory factors are vitally important for understanding the pathogenesis of osteoporosis. More specifically, a known key regulator of osteoblast and osteoclast activity in bone is mechanical strain. The excessive amount of osteocytes, or mature bone cells, distributed throughout the bone matrix "are thought to be the major cell type responsible for sensing mechanical strain and translating that strain according to the intensity of the strain signals."[35] Hence, alterations or inhibiting factors acting on any

stage along the process of recruitment, activation, differentiation or cell death can lead to imbalances in remodeling, eventually resulting in not only a reduction in bone resorption, but also a decrease in bone mineral density.[33] Current techniques used to study bone growth and development are limited since the study of endochondral ossification requires the use of appropriate experimental models (i.e. embryo, post-natal, and *in vivo*).[36]

As stated earlier, bone formation continues until the peak bone mass (maximum solidness and strength), or bone density is reached around age 30.[37] If osteoclasts respond with excessive bone resorption, a loss in bone density will result. Factors that contribute to bone loss include having a low calcium diet, not exercising, smoking, age, and taking certain medications (i.e. corticosteroids or antiepileptics). When bone resorption becomes accelerated, the bone is broken down much faster than it can be renewed and diseases such as osteoporosis can cause increase in porosity and fragility.[37]

Question

The purpose of this study is to evaluate if *Xenopus laevis* frogs can be used as a model organism to examine the effects of one antiepileptic drug, valproic acid sodium salt, on bone mineral density. The number of osteoblasts and osteocytes will be recorded to determine the effects the drug has on bone loss. It is expected that the drug will reduce the amount of osteoblasts and osteocytes on the bone surface in the hindlimbs (femur-long bones) of *X. laevis* resulting in the remaining osteoclasts causing excessive bone resorption, hence, a decrease in bone density through bone loss.

CHAPTER II

MATERIALS AND METHODS

Specimen Collection and Care

Xenopus laevis frogs (n=10; 5-6.25 cm) were obtained from Nasco (enasco.com). Prior to and during the treatment, the frogs were each kept in separate 3.8 L plastic containers of 1.89 L of water treated with Kordon AmQuel® Plus to detoxify chloramines (2.5 ml per 5 gallons). The frogs were fed Nasco Frog Brittle (44% protein, 6% crude fat, 2% crude fiber, and 15% ash) every two days. All procedures were approved by MC IACUC (see Appendix).

Dosing

The ten *X. laevis* frogs were staged and separated into two groups: control (n=5) and experimental (n=5). Individuals in the experimental group were exposed to an antiepileptic medication, valproic acid sodium salt (Sigma Aldrich, P4543), based on the maximum recommended dosage for humans (60 mg/kg/day). We assumed a relatively low absorption rate of the drug into the frog (1%). Dosing calculations for each frog are shown in Table 2.

Table 2. The original dosing technique for experimental *X. laevis* administered the maximum dosage (60 mg/kg/day) for humans, while assuming 1% (X 100) absorption, directly to the water.

Frog	Mass (kg)	X 60 (mg/day)	X 100
Exp. 1	0.02202	1.3212	132.12
Exp. 2	0.02510	1.5060	150.60
Exp. 3	0.03000	1.8000	180.00
Exp. 4	0.02124	1.2744	127.44
Exp. 5	0.02602	1.5612	156.12
Cont. 1	0.02688		
Cont. 2	0.03288		
Cont. 3	0.02963		
Cont. 4	0.02674		
Cont. 5	0.02502		

Four days after the first dosing, the experimental frogs died due to the increased acidity (pH ~ 4.02) of the water, which had an original pH of approximately 6.56; as a result, a new dosing regime was created. Four of the control frogs were combined with four juvenile *Xenopus laevis* frogs from a breeding colony at Maryville College (courtesy Dr. Drew Crain). Frogs were separated into the two distinct groups again (control and experimental) distributing based on mass. Instead of assuming 1% absorption of valproic acid, the second treatment regime assumed 50% (see Table 3). Half of the water from each container (~ 0.94 L) was removed every three days and replaced with fresh water treated with Kordon AmQuel® Plus. For the rest of the experiment, each tank was then dosed again with half of the diluted dosage (5 mg/mL). Although the control group received no valproic acid sodium salt, all motions and handling of the dosing were

replicated (i.e., thorough stirring of the water to account for the drug being mixed in the experimental groups). Treatment was continued for thirty days.

Table 3. Revised dosing techniques for experimental *X. laevis* administered the maximum dosage (60 mg/kg/day) for humans, while assuming 50% (X 2) absorption, after appropriate dilutions.

Frogs	Mass (kg)	X 60 (mg/day)	X 2
Exp. 1	0.00480	0.2880	0.576
Exp. 2	0.01130	0.6780	1.356
Exp. 3	0.02960	1.7760	3.552
Exp. 4	0.02818	1.6908	3.382
Cont. 1	0.00632		
Cont. 2	0.00824		
Cont. 3	0.02654		
Cont. 4	0.02796		

Euthanasia and Microdissection

Following thirty days of treatment, frogs were placed in an anesthetic, tricaine methanesulfonate (400 mg/L MS-222 with 1g/L sodium bicarbonate added as a pH neutralizer) (Sigma Aldrich, E1052). After the frogs were fully anesthetized, the femurs of each frog were removed under a dissection microscope. The aforementioned limbs were then placed in Bouin's fixative (Sigma Aldrich, HT10132) until histology could be performed. Frogs were then frozen at -20°C.

Decalcification and Histology of X. laevis limbs

Before the tissues were dehydrated, one of the two femurs extracted from each frog was decalcified after fixation (at least 24 hours) by being immersed in 11% formic

acid (for 24 hours). Both decalcified and calcified bone were transferred into fresh 70% ethanol and continually refreshed until all Bouin's was removed. The bones were stored 3 months in 70% ethanol prior to wax embedding.

The tissues were dehydrated by placing each tissue in a plastic cassette and transferring it to 80% ethanol, 95% ethanol, 100% ethanol, and a second 100% ethanol for 2 hours, 1.5 hours, 1 hour, and 1 hour, respectively. The tissue was cleared in CitriSolv for 1 hour. The femurs were embedded individually in wax blocks by soaking in four series of paraffin waxes, each for an hour, under increasing pressures (12, 15, 21, then 25 psi). Lastly, the tissue was carefully placed and arranged in a mold filled with paraffin wax and given overnight to set. Once the block properly hardened, it was trimmed using a blade and then sectioned on the microtome at 12 micrometers. The wax strips were gently placed in a warm water bath with a pinch of gelatin. After a few seconds of soaking, the strips were then gently oriented onto a microscope slide and set out to dry. Once dry, the slides were stained as outlined in Humason's Hematoxylin and Eosin staining protocol (Figure 3) and a cover slide was then glued on using Permount.[37]

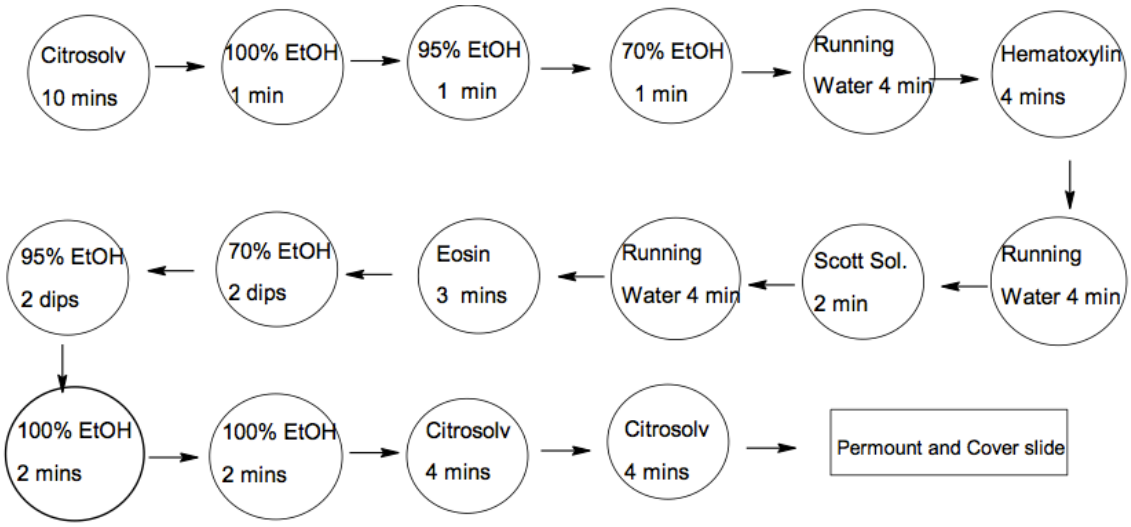


Figure 3. Hematoxylin and Eosin staining protocol used for histological analysis of *Xenopus laevis* femurs.[38]

Analysis of X. laevis Femurs

Slides were photographed to show bone structures (diaphysis, periosteum, osteoblasts, osteocytes, etc.). Figure 4 below shows these structures viewed at the various magnifications (100X and 400X). At 400x magnification, osteoblasts and osteocytes of three different sections of the diaphysis of a single femur were counted and recorded within the field of view. Two separate t-tests were conducted: one comparing the decalcified femurs treated with 11% formic acid and one comparing undecalcified femurs treated with 70% ethanol. Once it was determined that the two groups had no statistical difference ($\alpha > 0.05$), they were combined into two main groups (Experimental an Control) and another t-test assuming equal variance was conducted to determine whether VPA had reduced the overall number of osteoblasts and/or osteocytes potentially leading to early-onset osteoporosis.

After quantification of osteocytes and osteoblasts, the number of chondrocytes in the epiphyseal plate was measured. The same overall statistical procedure was used to analyze the chondrocytes.

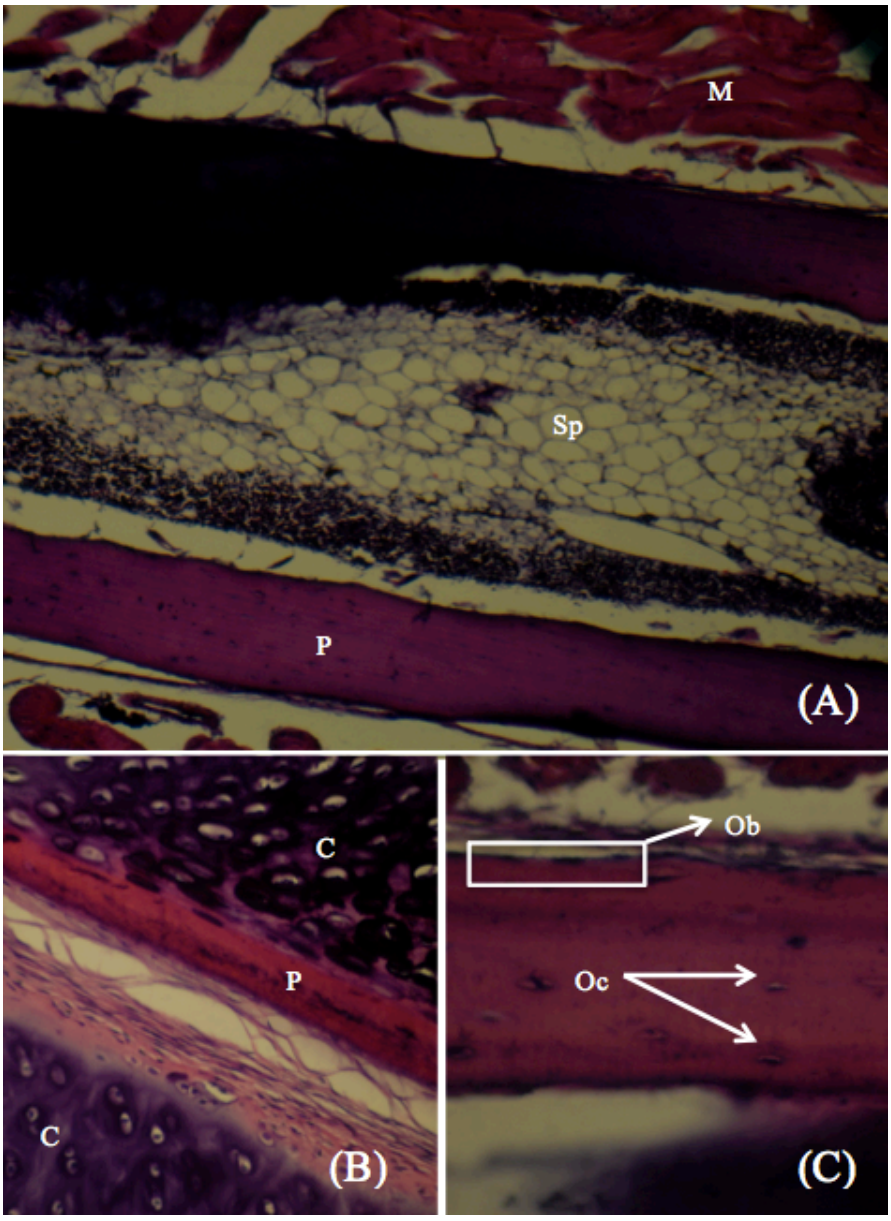


Figure 4. A longitudinal section of *X. laevis* femur at 100X (A) and 400X (B and C). Muscle (M) is present around the diaphysis as well as spicules (Sp) of bone, inner layer of periosteum (P), chondrocytes (C), osteoblasts (Ob), and osteocytes (Oc).

CHAPTER III

RESULTS

Valproic acid sodium salt exposure did not have any significant effects on the bone development of *X. laevis*. There was no difference in number of osteoblasts that were either decalcified or undecalcified in both the control (Figure 5, $p = 0.1110$) and experimental (Figure 6, $p = 0.2178$) groups. As a result, the decalcified and undecalcified bones were grouped together. The test revealed that the number of osteoblasts of frogs placed under a month long treatment with antiepileptic medication versus those unexposed control groups showed no statistical difference ($p = 0.4384$).

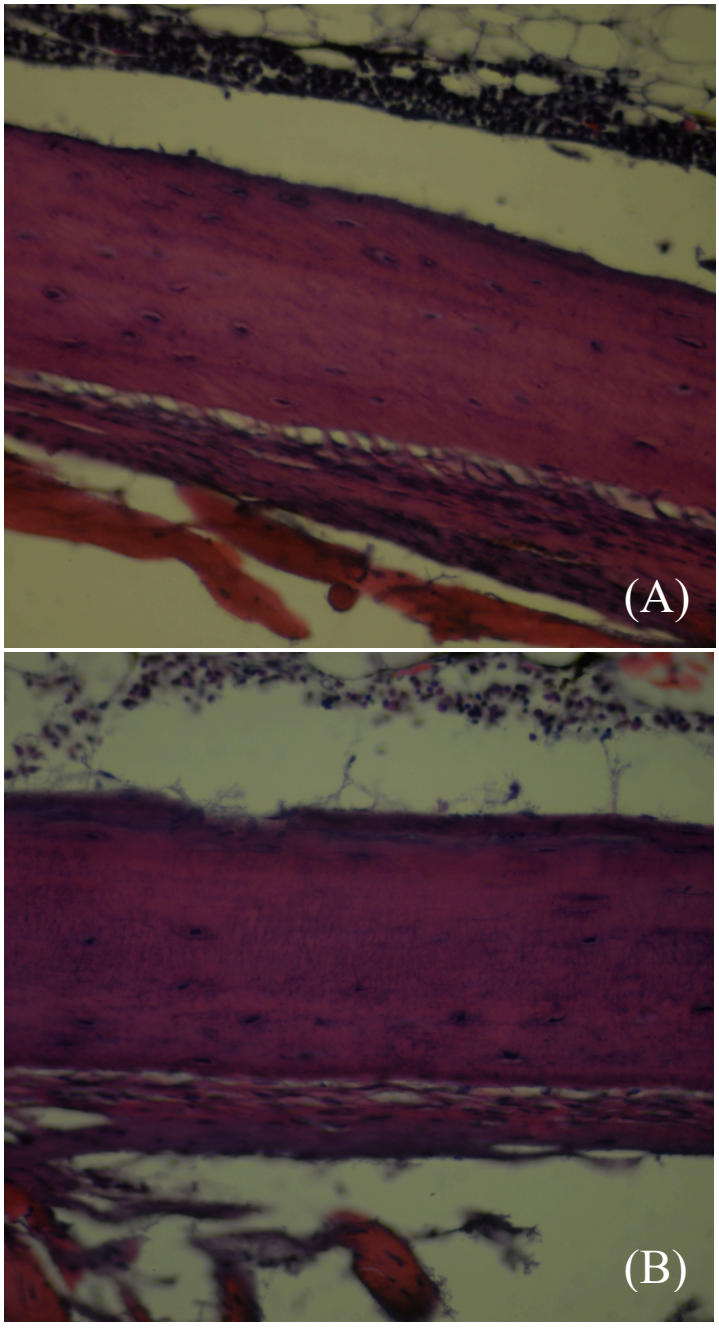


Figure 5. Undecalcified (A) and decalcified (B) longitudinal section of control *X. laevis* femur (400X).

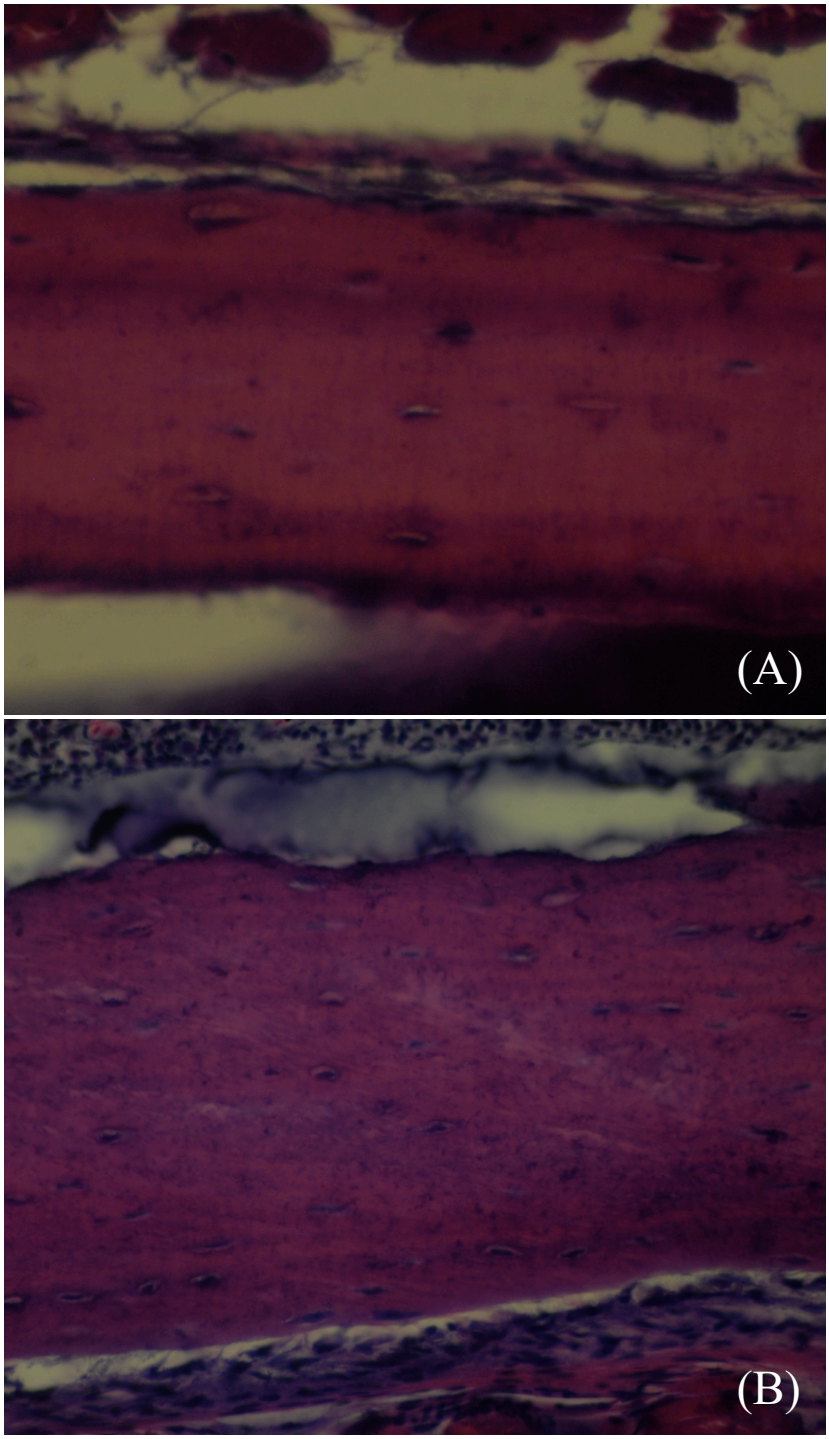


Figure 6. Undecalcified (A) and decalcified (B) longitudinal section of experimental *X. laevis* femur (400X).

Based off appearance, the femurs treated with 11% formic acid seem to have little differentiation in comparison to the femurs left undecalcified; however, as stated earlier, a t-test was conducted between the controls and another between the experimental groups revealing no significant difference (Figure 7).

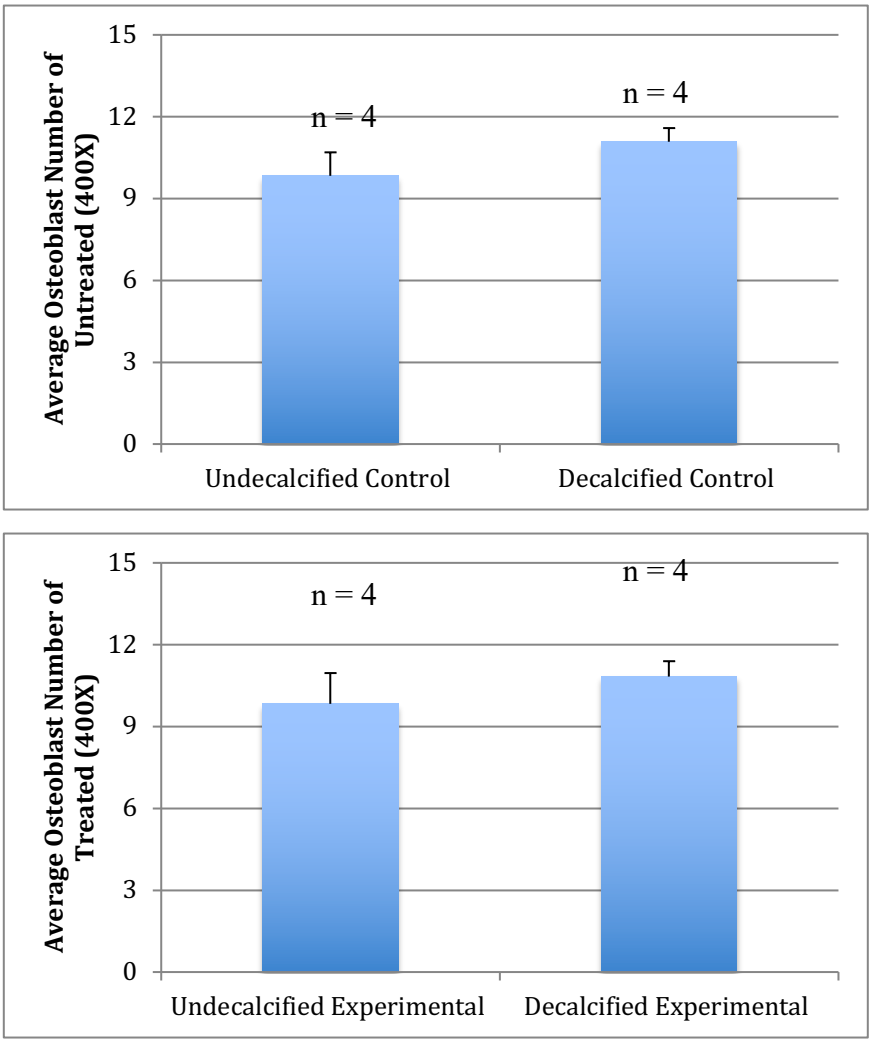


Figure 7. A comparison of mean osteoblast number (+ 1SE) of the control groups (undecalcified versus decalcified) and the experimental groups (undecalcified versus decalcified) of *X. laevis*.

The two controls (decalcified and undecalcified) were combined, as were the two experimental groups (decalcified and undecalcified). A final t-test was conducted on the combined groups and the results were shown on a column graph again using Excel and StatPlus (Figure 8).

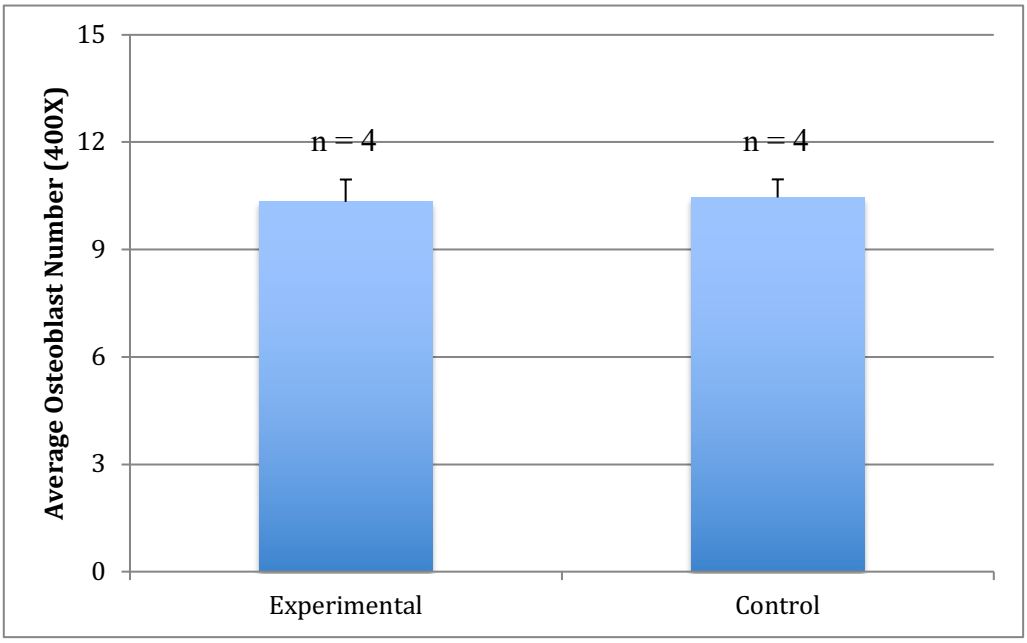


Figure 8. A comparison of mean osteoblast number of the femur diaphysis (+ 1SE) of the combined experimental and control groups of *X. laevis*.

Using the same technique, the osteocytes of the same region were also counted and recorded in order for analysis. Figure 9 below shows the comparison between the femurs treated with 11% formic acid and those left undecalcified (control: $p = 0.0817$; experimental: $p = 0.2440$). Due to no significant difference, the groups were combined (Figure 10) and descriptive tests were run revealing that in addition to no significant change in osteoblast count, the number of osteocytes varied little ($p = 0.4799$).

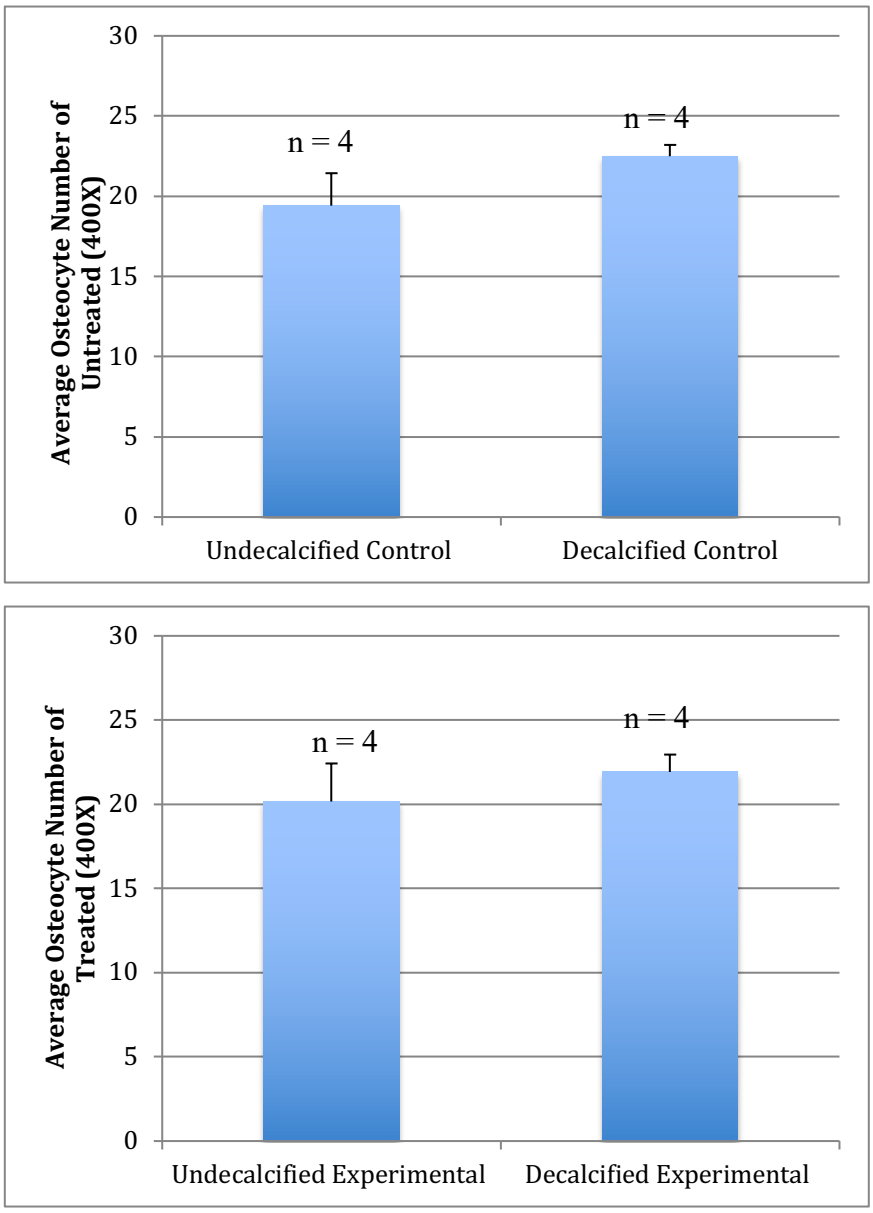


Figure 9. A comparison of mean osteocyte number (+ 1SE) of the control groups (undecalcified versus decalcified) and the experimental groups (undecalcified versus decalcified) of *X. laevis*.

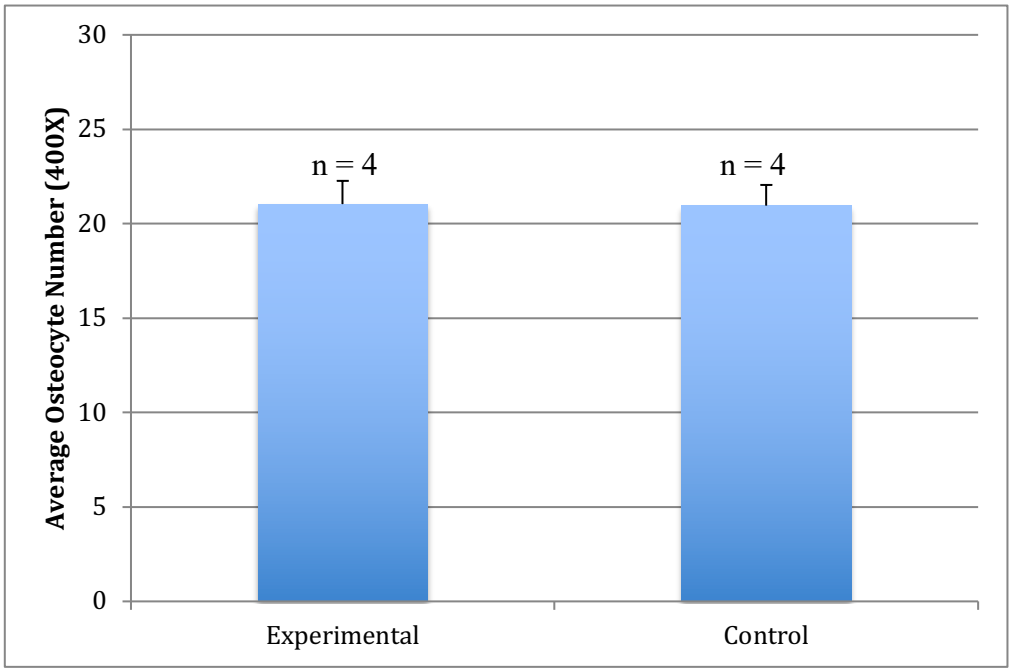


Figure 10. A comparison of mean osteocyte number of the femur diaphysis (+ 1SE) of the combined experimental and control groups of *X. laevis*.

A visual comparison of the VPA affects on the chondrocytes of both the decalcified and undecalcified femurs are presented for both the experimental (Figure 11) and control (Figure 12) groups.

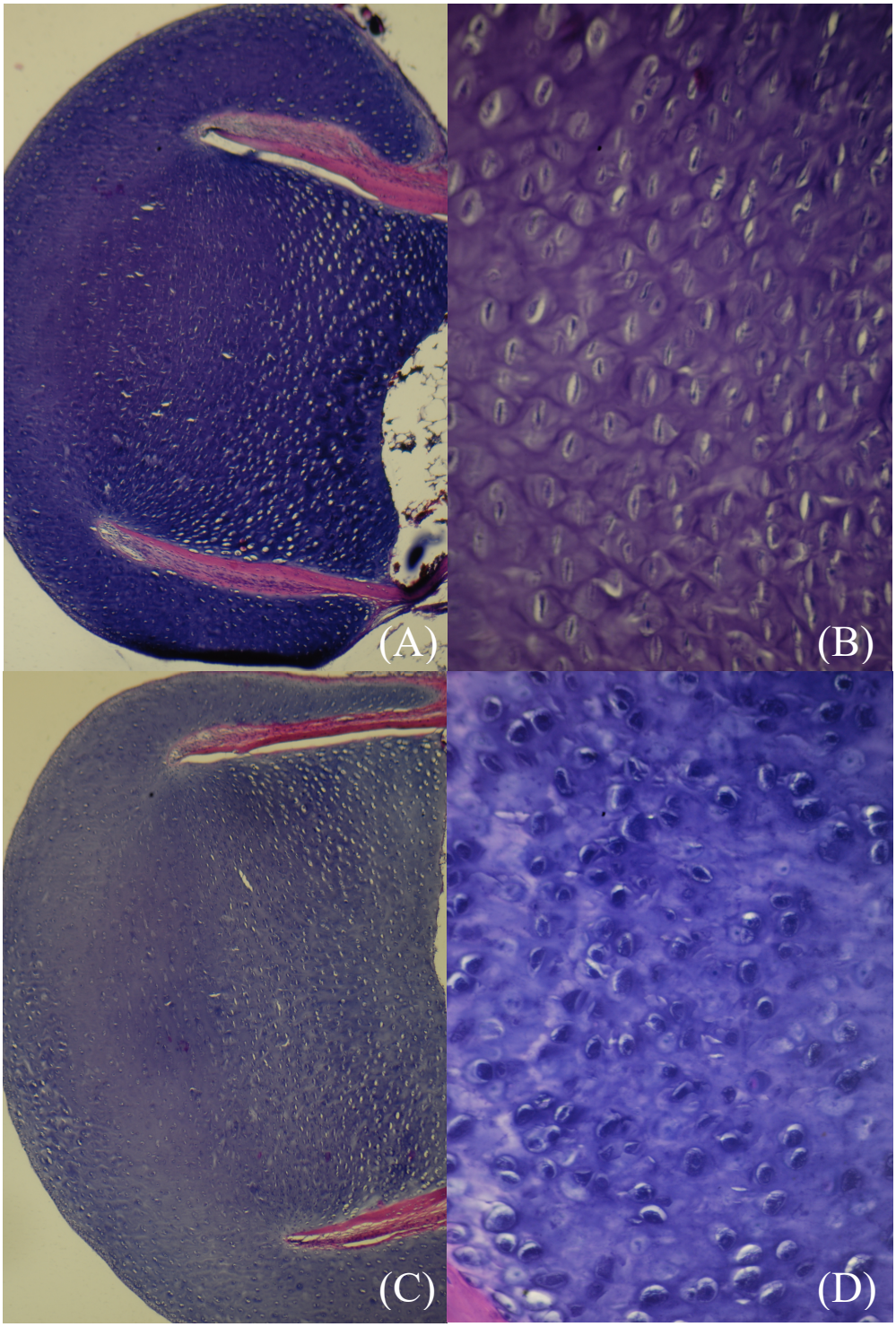


Figure 11. Undecalcified femur at 100X (A) and 400X (B) and decalcified longitudinal section of experimental *X. laevis* femur at 100X (C) and 400X (D).

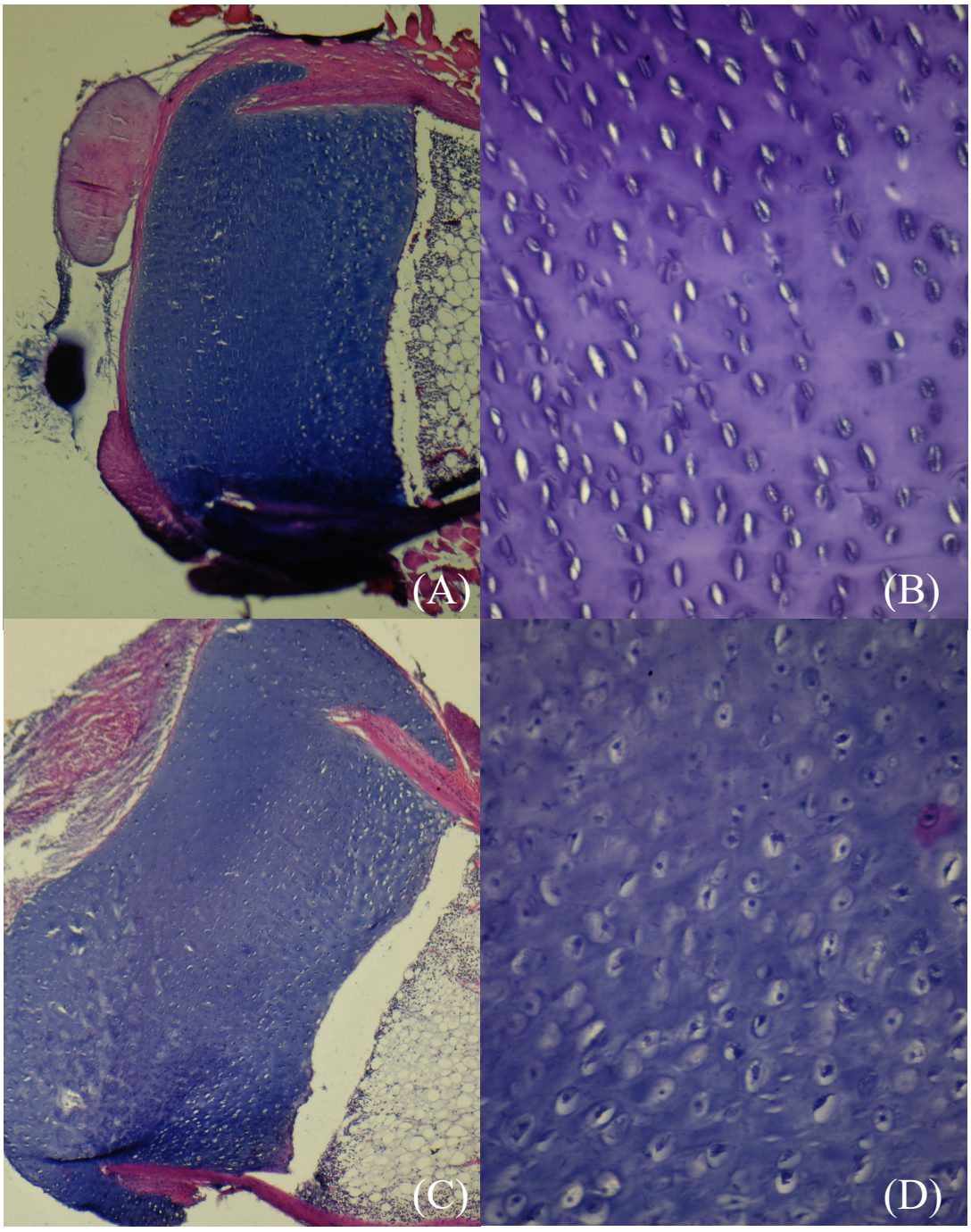


Figure 12. Undecalcified femur at 100X (A) and 400X (B) and decalcified longitudinal section of control *X. laevis* femur at 100X (C) and 400X (D).

Visually, decalcifying the bones appeared to have no affect on the amount of chondrocytes; however, to support this assumption, a t-test was conducted between the controls (decalcified versus undecalcified) and another between the experimental groups (decalcified versus undecalcified) revealing no significant difference (experimental: $p = 0.3162$; control: $p = 0.3682$). Figure 13 below displays the results of the comparison.

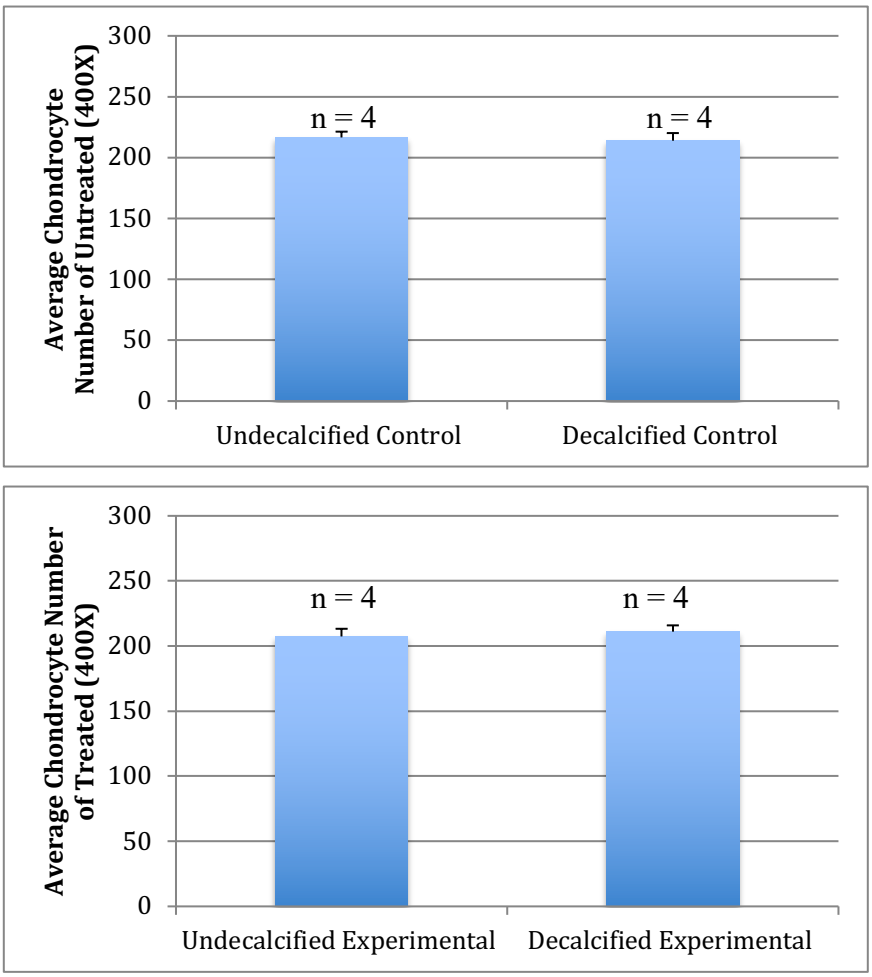


Figure 13. A comparison of mean chondrocyte number (+ 1SE) of the control groups (undecalcified versus decalcified) and the experimental groups (undecalcified versus decalcified) of *X. laevis* epiphysis.

Due to no significant difference among the decalcified and undecalcified groups, they were combined and descriptive tests were run shown in Figure 14. Similar to the results of the osteoblasts and osteocytes, VPA had no significant effect on chondrocyte number ($p = 0.1335$).

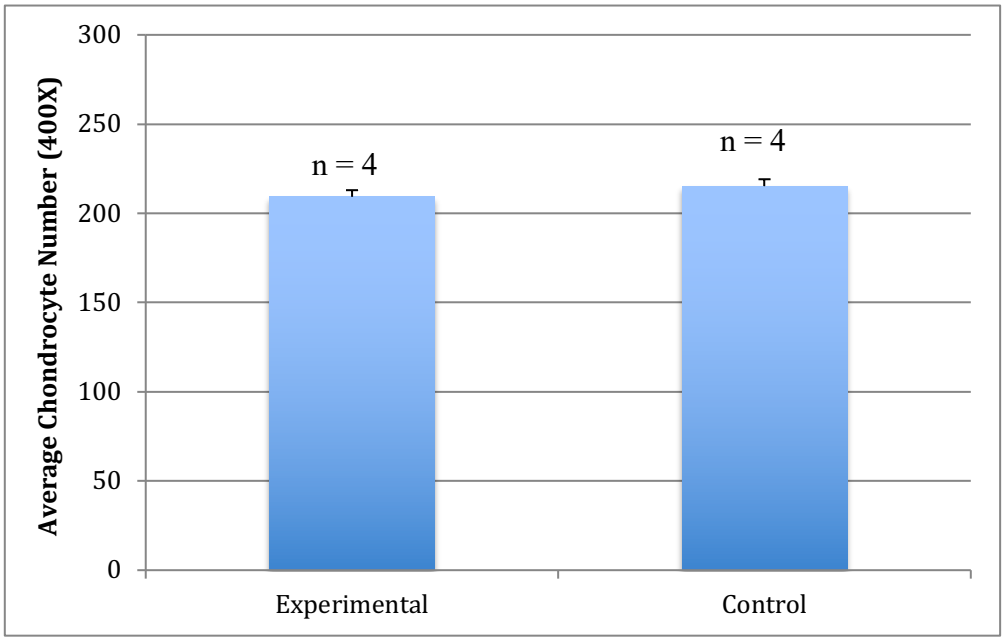


Figure 14. A comparison of mean chondrocyte number of the femur diaphysis (+ 1SE) of the combined experimental and control groups of *X. laevis*.

CHAPTER IV

DISCUSSION & CONCLUSION

Bone density and development of *Xenopus laevis* femurs were not significantly affected after one-month exposure to antiepileptic medication valproic acid sodium salt (VPA). When osteoblasts were quantified, it was discovered that the experimental groups were not significantly affected by exposure, leading to no potential change in ossification. Similarly, neither the number of osteocytes nor the density of chondrocytes was affected by valproic acid. Overall, VPA did not affect the bone density and development of the exposed *X. laevis,* refuting the original hypothesis. Thus, the antiepileptic medication valproic acid sodium salt does not contribute to osteoporosis-like effects in *Xenopus laevis*.

Previous studies have conflicted in their conclusions as to whether or not VPA causes a reduction in bone density, with the majority of studies suggesting that VPA reduces bone density. Studies in humans have shown that VPA exposure causes increased serum calcium and decreased calcitriol.[29,39-40] In addition, other studies revealed the affect that osteoporotic bones have on other variable (i.e. chondrocytes). Ovariectomized rats with osteoporosis have a delayed fracture healing process due to increased chondrocyte proliferation without a change in apoptosis.[41]

Although the mechanism by which VPA may reduce bone mineral density is unknown, it has been suggested that the drug has direct effects on bone cells causing changes in calcium homeostasis by reducing calcium absorption, causing hyperparathyroidism, and calcitonin deficiency.[42] It is known, however, that the drug increases the testosterone/estradiol ratio, usually by decreasing the level of estrogen in the body. Because estrogen reduces bone resorption directly affecting osteoclast function and genesis, individuals have an increased risk of developing osteoporosis.[26].

Whereas the majority of studies support VPA therapy as a precursor to osteoporosis, other studies have shown no effect. One such study evaluated the cross-sectional relationship of duration and dosage of valproate monotherapy on bone mineral density (BMD) in 41 adult patients with epilepsy.[43] The BMD at lumbar level was measured by DXA in adult epileptic patients receiving long-term (at least 2 years) valproate monotherapy and blood samples were collected for total serum calcium, phosphorus, magnesium, 25-hydroxyvitamin D3 and parathormone.[43] Although osteopenia was present in 24% of subjects, no case of osteoporosis was documented meaning no correlation existed between the VPA monotherapy and BMD.[43] Additionally, another study displayed no significant correlation by using an alternative model (Wistar rats). The 28-day experiment was divided into the following groups: control rats, rats receiving the relatively new antiepileptic drug vigabatrin (250 mg/kg p.o. daily), phenytoin (20 mg/kg p.o. daily), or valproic acid (250 mg/kg p.o. daily).[44] Various parameters (ex. bone length, mass, diameter, mineral content, etc.) of the tibia and femur were examined and compared among the four groups. Although vigabatrin showed significant variation in bone parameters, BMD of phenytoin and valproic acid following

monotherapy did not significantly differ from the control rats.[44] Using an amphibian model, another study showed VPA as a class I/II HDAC inhibitor, which has been found to inhibit tail regeneration in the embryonic, post-refractory, post-amputation, and pre-blastema stage of *Xenopus laevis* and *Ambystoma mexicanum*.[45] Although it affects early regeneration of both species, just as in this study, the inhibition of HDACs did not significantly affect development of the limb or tail, meaning HDAC activity might be required to re-activate and stabilize developmental patterning genes.[45] Lastly, recent research using the appropriate model (*Homo sapiens*) has negated the possible assumption that VPA affects species differently, hence, supporting the results of the current study. In this recent study, 62 epileptic patients underwent chronic valproate therapy (758 ± 29 mg/day) for at least 6 months, without any vitamin D or calcium supplementation.[46] Serum markers of bone turnover, calcium, phosphorus, total alkaline phosphatase, and parathyroid hormone levels were measured in both groups revealing no statistical difference. As a result, it was deduced that because VPA monotherapy does not cause bone turnover it does not lead to early onset osteoporosis.[46]

A summary of the conflicting results of VPAs influence on bone density (see Table 4) suggests that the distinctive variable inconsistent among the various studies is the model species used. Because of funds, convenience, and efficacy as an aquatic model, *X. laevis* were used as a representative model in comparison to previous human case studies and rat models. As a result, calcium homeostasis was compared between amphibians and mammals. Mammals only use their parathyroid for calcium control, whereas amphibians retain some control of calcium levels through the pituitary gland and

the paraphysis[47]; therefore, bone ossification in animals of the current study was regulated by parathyroid gland, pituitary, and paraphysis.

Table 4. Results summary of the effects of valproic acid sodium salt (VPA) on bone structure and development of various organisms.

Organism	Endpoint	Effect	Reference
Homo sapiens	Long-term VPA monotherapy increases bone resorption, leading to decreased BMD	Yes	29
	Long-term VPA monotherapy decreases BMD by increased bone turnover	Yes	30
	Long-term chronic VPA monotherapy decreases BMD	No	43
	VPA alters biochemical bone parameters causing bone turnover	No	51
Rattus norvegicus	Chronic VPA treatment decreases total BMC and trabecular volumetric density	Yes	27
	Osteoporosis causes increased chondrocyte proliferation	Yes	41
Ambystoma mexicanum	Histone deacetylases inhibitors (VPA) prevent proper bone development	No	45
Xenopus laevis	Histone deacetylases inhibitors (VPA) prevent proper bone development	No	45
	VPA effects histological bone structures causing early onset osteoporosis	No	Current Study

The extracellular fluid calcium concentration is tightly controlled by a complex homeostatic mechanism involving fluxes of calcium between the extracellular fluid and bone.[47] Three major hormones – parathyroid hormone (PTH), calcitonin, and 1,25-

dihydroxyvitamin D – regulate these fluxes carefully. Any disruptions of this system can lead to disorders of calcium metabolism that have predictable effects (i.e. osteoporosis). PTH is an 84-amino acid peptide that is produced in the parathyroid gland. One of the main biological actions of PTH includes stimulation of osteoclastic bone resorption and release of calcium and phosphate from bone once the amino-terminal end of the PTH molecule binds to the PTH receptor.[47] Furthermore, the 32-amino acid peptide calcitonin is synthesized and secreted by the parafollicular cells of the thyroid gland. It directly inhibits osteoclastic bone resorption and is usually accompanied by the production of cAMP.[48-49] Also, an increase in cytosolic calcium can result in the osteoclast leading to the contraction of the osteoclast cell membrane. These effects are momentary and likely have little role in calcium homeostasis chronically. Lastly, 1,25-dihydroxyvitamin D has been found to increase plasma calcium and phosphate concentrations by increasing the absorption from the gastrointestinal tract.[47] It is more commonly known for increasing bone resorption and enhancing the effects of PTH.[48] It can also differentiate osteoclast precursors into multinucleated cells that are capable of resorbing bone.[49] By these actions, 1,25-dihydroxyvitamin D provides a supply of calcium and phosphate available at bone surfaces for the formation of normal mineralized bone.[47-48] Any deviation in any of the three major hormones involved in fluxes of calcium can cause chronic hypocalcemia eventually leading to osteoporosis.[49] Overall, because of the variation in calcium homeostasis, it was discovered that results of the study are somewhat inaccurate in predicting reactions that may occur in humans.

Likewise, because amphibians absorb most nutrients through their permeable skin, they differ from the digestive absorption technique used in mammals.[50-51] Due to

their physiological engagement in respiration and regulation of internal concentrations (ex. water and ions), the skin of amphibians is thought to be much more permeable than that of mammals. One study confirmed these assumptions by measuring the flux of five substances through the skin of a frog and pig.[51] Higher permeability values were measured in the frog in comparison to the pig, evidently caused by the differences in skin. For instance, the skin in most species is a specialized epithelium that consists of four layers: the hypodermis, dermis, viable epidermis and stratum corneum.[51] The stratum corneum is the layer that represents the barrier to percutaneous absorption. Therefore, it comes to no surprise that the stratum corneum is roughly 10 times thicker in pigs than in frogs (20 μm and ≈ 2 μm, respectively). Furthermore, the stratum corneum of aquatic amphibians consists of only one or two cell layers, whereas mammals contain a multilayered stratum corneum that seals lipids to function as a water barrier.[51] As a result, it is unfair to compare drug affects on varying species considering the different administration techniques (percutaneous versus oral) and absorption rates.[50-51]

In summary, it was discovered that valproic acid sodium salt does not affect bone cells (osteoblasts, osteocytes, and chondrocytes) in an amphibian model. Because calcium control and absorption techniques differed between the two classes, amphibians are not an appropriate model for examining VPA effects. In addition, the study may have provided differing results if placed under long-term exposure. Although the possible disadvantages of VPA were analyzed, it appears that short-term use is not problematic. The beneficial effects of VPA[6,22] appear to supersede any negative side effects.

APPENDIX: IACUC

MARYVILLE COLLEGE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE
Application for Use of Vertebrate Animals in Student Research

Provide information after each bold item

Student Name: **Mallory Kirkland**

Student Email Address: **mallory.kirkland@my.maryvillecollege.edu**

Date: **2/14/2013**

Senior Study Advisor: **Dr. Drew Crain**

Species to be used: ***Xenopus laevis***

Age of animals: **Juvenile Adult**

Number of animals in study: **10**

Duration of study: **~ 1-2 months**

Location of animals during the study (building and room): **SSC Room 114**

List personnel to call if problems with animals develop:

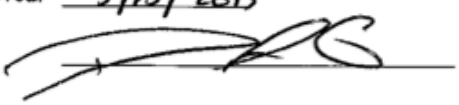
Name	Daytime Phone	Nighttime Phone	Emergency No.
Mallory Kirkland	(256)558-3573	(256)558-3573	(256)558-3573

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.

Five of the ten frogs will be exposed to valproic acid (anti-epileptic medication) to discover its effects on the bone mineral density. Histological slides will be made in order for analysis. Therefore, frogs will be euthanized with 400 mg/L MS-222.

(Do not write below line: For MC IACUC Use)

Maryville College IACUC Approval Number: 2013 07
Date Approved: 3/13/2013
Signed:



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