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The effect of supplementation with selected micronutrients on dementia and osteoporosis

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Abstract

People had done everything they are capable of to stay young; however, aging is a definite process that could not be tricked. Aging results in cognitive memory impairments and osteoporosis which affects mobility. Memory impairment is the inability to encode information. It has been common to hear people complaining from this disease not only in the process of aging but also at young ages. That is why this topic has been grabbing the attention of not only concerned candidates but also scientists and media. In this study, we will tackle both dementia and osteoporosis, where in the first arm Alzheimer's disease was chemically induced in rats using aluminum chloride ($AlCl_3$) and in the second arm we used ovariectomized mice as an osteoporosis model. Rodents were divided into five groups in each study; group 1, the negative control group; where rats/mice were administered regular diet only, group 2; the positive control group not consuming any premixes were either $AlCl_3$ treated or ovariectomized rodents for dementia and osteoporosis studies, respectively. Group 3 were $AlCl_3$ treated or ovariectomized and fed with a premix of micronutrients with the European Food Safety Authority (EFSA) authorized claims of positively improving cognitive abilities or osteoporosis (ingredients that are authorized by the EFSA and supported by scientific research). Group 4 were $AlCl_3$ treated or ovariectomized and fed with a premix of micronutrients with content claim that possess health claims not authorized yet by EFSA, however it is based on potential scientific studies. Group 5 were $AlCl_3$ treated or ovariectomized and fed with a master mix, the combination of all ingredients consumed by groups 3 and 4. This research aimed to test the prevention and/or suppression of Alzheimer's Disease and Osteoporosis. For this purpose, dementia study tested results through Morris Water Maze (MWM), Enzyme-Linked Immunosorbent Assay (ELISA) and histopathological examination of the rats' brain, while for osteoporosis results were tested though detection of blood calcium (total & ionized) and Alkaline Phosphatase (ALP) and histopathological examination of mice femur bone. In this context, results for both studies demonstrated that supplementation with the selected vitamins and minerals improved prevention of AD and osteoporosis. In the dementia study, the three premixes given showed better results compared to positive control in the behavioral testing and Amyloid β-42 biomarker testing, specifically group 4 which showed the best performance. In the osteoporosis, the detection of blood calcium (total & ionized) and alkaline phosphatase (ALP) did not show indicative results, that is why more testing through histopathological examination of

mice femur bone was done showing good results by the consumption of the premixes. To conclude the premixes consumed in these two studies show potential in fighting dementia and osteoporosis, while some premixes have shown better results than others.

1. Background and Introduction

Time changes nearly everything, nations disappear, generations grow, technology evolves, but the human body deteriorates by time and negligence. Since early ages, exactly 700 BC, a Greek philosopher, *Solon*, has discovered that through time one's brain changes, its capacity to function properly diminishes, and human cognitive and intellectual ability are sort of shaken on average by the age of 56-63. In agreement a Roman poet calls time a thief for stealing our memory away, stealing the ability to perform in a normal and healthy way. While science, technology, and beauty products have managed to keep humans young and functional, they stay paralyzed in front of memory loss and humans' mobility. From 1965 till 2013 the number of articles published each year that has anything to do with memory is tremendously increasing, by the year 2013 it has been more than 3000 articles, which in fact shows the importance of exploring human's memory in all aspects, how it functions, how it is affected and how to enhance it (Park & Festini 2016).

Dementia is the general term including lots of various neurological diseases. The key symptom of dementia is deterioration in memory which result in mental unfitness. Other symptoms may include problems with speaking, impaired thinking and judgment, struggling in learning new skills, inability to solve problems and personality problems. All these symptoms make it inevitable to proceed with daily activities on their own; independence cannot be reached at this point; caregivers are a must. One of the most common causes of dementia is Alzheimer disease. The term dementia and Alzheimer disease (AD) are sometimes used equivalently which may create confusion of the comprehension of each term. Simply AD is the most common cause of dementia in many countries including the US, however there are other causes of dementia such as Parkinson disease (Boyer and Shapiro 2011). Dementia's memory impairments not only can affect the daily activities of people, it affects their whole life. Memory is affected through time differently; it differs in its intensity; it can be low degree memory impairments to extreme Alzheimer's disease cases. Aging is not only affecting memory it is also affecting the overall body, its reserve and its ability to function (Parikh et al. 2015).

In addition, memory is evaluated by many aspects other than one's birth certificate date (age) such as; the speed of recalling such events, the ability to learn anything new, the ability to be creative, the ability to remember words over images or images over words. Researches have pointed that older adults aged 60-80 have slower memory, cracked memory and use more images as a way to remember things, while younger aged 34-59 adults have slightly more vivid memory, are faster to recall specific events, rely more on words, and use their imagination more frequent than older adults, even if they are still slow. On the other hand, young people aged from 12-17 achieved the best performances on all of the above. While memory is indeed affected by many other aspects such as the availability of resources, the personal willingness to learn or to remember, the lifestyle and health factors of performers, and the method used for memory recovery whether it is the use of cues, images, words, etc, still age was one of the most dominant factors in affecting memory (Park & Festini 2016).

Mostly, researchers have found that older people have worse memory than younger people. Leaving this fact to be explained with more depths later on, dissimilar it is negotiable that aging may not be the most crucial factor manipulating memory. For example, in younger generations lifestyle may be a crucial factor affecting memory, people born during the millennium are more connected and exposed to technologies such as digital screens, prepped meals, and are more career oriented, and experience many social and political strains, which can easily and rapidly distort their memory over time (Park & Festini 2016).

Moreover, as years go on, the human body reserve of nutrients and vitamins is diminishing, most women over 60 years old have osteoporosis while a high percentage of men over 60 falls under the same trap of osteoporosis but still lower than women percentage. Osteoporosis is a disease that is slow to discover and tricky, it is the condition of having weakened bones over years, bones are becoming more delicate, and tend to break more frequent like hip and wrist fractures (Nguyen, Center & Eisman 2010).

One of the characteristics of osteoporosis is bone mass reduction and the microarchitectural structure of bone tissue disturbance that will be further explained in details. For instance, in the European Union, osteoporosis is considered one of the principal causes of illness and death in elderly it also known for being one of the main causes of having expensive medical care, due to all of these critical aspects caused by osteoporosis. In addition to the obvious deterioration in the

patients' quality of life and the high financial costs associated with the disease, it creates the need for further experimental research to understand pathogenesis and the action of pharmaceutical means in the avoidance or curing of the disease (Lelovas et al. 2008).

The objective of this research is to test the relationship between the supplementation of specific vitamins and minerals and the enhancement of people suffering from dementia and osteoporosis, in a preventive model where induction of either AD or osteoporosis is done simultaneously while administering the micronutrients premixes. We hypothesize that there is a positive relationship between both variables.

1.1 Hypotheses:

- Consuming authorized health claims ingredients has a positive relationship on dementia
- Consuming content health claims ingredients has a positive relationship on dementia
- Consuming master mix ingredients has a positive relationship on dementia
- Control groups not consuming any of the premixes will show severe symptoms of dementia compared to groups consuming any of the premixes.
- Consuming authorized health claims ingredients has a positive relationship on osteoporosis
- Consuming content health claims ingredients has a positive relationship on osteoporosis
- Consuming master mix ingredients has a positive relationship on osteoporosis
- Control groups not consuming any of the premixes will show severe symptoms of osteoporosis compared to groups consuming any of the premixes.

2. Literature Review

2.1 Introduction to literature review

Dementia is a cognitive disorder that grabbed the attention of many researchers. To fully understand cognitive disorder, it is crucial to start with simple definitions. As such, cognitive disorder is known to affect mental health affecting the ability to learn, perceive, remember and even solve problems it also includes amnesia, delirium and dementia. The medicine industry is struggling to deal safely and effectively with cognitive disorders such as ADD (attention deficit disorder), amnesia, and Alzheimer's disease (AD) (Thippeswamy et al. 2013).

Moreover, other studies showed that in many instances, effort and money are spent to discover a cure for AD and dementia other diseases, since aging nowadays is accelerated. Decades ago, people complained from aging symptoms starting a specific age bracket somewhere around 70 and 80 years+, however nowadays this age bracket is becoming lower, people with age brackets of 50+ are starting to complain about dementia symptoms. People age rapidly in this generation; age related diseases are hitting younger age groups which steps up the need for a cure to aging diseases typified by dementia (Shen and Ji 2015). Also, dementia is an overwhelming and devastating disease that requires significant and professional care, the disease leaves its patients totally depending on help and eventually leads to death mostly in later stages. Some drugs provide short term improvements of symptoms tied with such disease, lots of studies with substantial budget ultimately hoped for a definite drug, a way, or even a lead that would eventually build up to cure it but still no drug is known for preventing or curing dementia symptoms (Soni et al. 2012).

AD which is one of diseases that causes dementia is taking life out of its patients slowly by slowly, not being able to perform daily activity is one struggle but having your family becoming strangers to AD patients by robbing all memories associated together is a bigger challenge; even the faces do not bring a story to patient's mind anymore. Patients seem well, living with daily struggle but they are not well removing integrity out of the smallest activity they do, needing to be reminded to take a bath, wash their face, put their clothes on, these are examples of the extreme side of the seriousness of such disease, not to mention in intense cases patients are all washed out from everything they do care about. AD is a challenging disease and finding a cure to such disease is a human need (Fazio et al. 2018).

Many studies researched the effect of certain medicines on treating AD (Soni et al. 2012) while other studies concentrated on researching individually and collaboratively the effect of vitamins to treat AD. However, the current study will be testing the ability to prevent the progression of dementia to AD with natural ways using vitamins and minerals (Boyer and Shapiro 2011).

2.2 Dementia and Alzheimer

Dementia, as the defined in the introduction, is the term combining many neurological diseases all including symptoms such as memory loss, inability to solve problem, judge events, and inability to think. Dementia is not a disease while Alzheimer Disease (AD) is a disease that is most common cause of dementia. Extreme memory impairments will cause AD. AD is developing in stages, permanent, and fatal. The disease is named after the neuropathologist and psychiatrist Alois Alzheimer who identified the disease as a neurological disorder that in fact will ultimately destroy the brain, such discovery was done during an autopsy. AD is not a normal part of aging; it is a disease that affect older victims; the older a person gets the more probable to be affected by such disease. The chances of getting AD disease is dramatically higher above the age of 85 years, this disease will occupy the victim's life; pieces of their life will vanish and will never be brought back. The course of such disease is on average from 2 to 22 years however the most common is an average of 10 years (Boyer and Shapiro 2011).

50 to 60% of dementia cases of elderly above the age of 65 are due to AD. AD is becoming a public health struggle. There are 35 million patients worldwide who are currently suffering from AD and the challenge is real if no profound medicines are discovered by 2050 as the number of patients will increase to reach 115 million patients worldwide (Wasiak et al. 2015). Moreover, the scientific characteristics of AD include memory damage and mental and behavioral impairments. AD is representing the main root of dementia, it is a neurodegenerative disorder, and is distinguished by loss of memory and behavioral and cognitive decline. The hallmarks of the pathology are the existence of senile plaques which are microscopic mass of decaying, decomposing and fragmented nerve terminals around amyloid core. Senile plaques are formed by amyloid-β (Aβ), and neurofibrillary tangles (NFTs), formed by aggregated hyperphosphorylated tau protein (Gugliandolo, Bramanti, and Mazzon 2017). Amyloid precursor protein (APP) undergoes a proteolytic cleavage by β- and γ-secretases to from the Aβ peptide, which is the

primary component of senile plaques, extracellular deposits that are present in the grey matter of the brain (Chen et al. 2017). Aβ peptide is an approximately 40-residue long peptide, where its presence has been proven to be directly related to the onset and development of Alzheimer's disease (AD), the most common form of dementia (Findeis, 2007). The accumulation of Aβ peptides in the brain is an early toxic indicator in the pathogenesis of Alzheimer's disease. One of the most important biomarkers for recognizing AD is the detection of Aβ peptides, specifically the longer form Aβ-42, which is assumed to be the initiator of a cascade of events leading to oxidative stress, neurotoxicity and inflammation (Hardy and Selkoe, 2002). The deposition of Aβ-42 in the brain occurs in early stages before the presence of other shorter forms of the Aβ peptides, like the Aβ-40 (Parvathy et al., 2001). Therefore, the ability to detect Aβ-42 in a suspected patient is a great tool to recognize AD presence before a diagnosis is possible. AD patients do not decease because of these changes in the brain, but rather due to the related symptoms such as pneumonia, malnutrition because of inability to swallow and immobility (Singh et al. 2018).

AD occurs in 10-30% of people over 65 years with a frequency of 1-3% of familial AD discovered in early age around 45 years. On the other hand, 95 % of AD patients are unpredictable forms that are discovered later around the age 80-90 years old (Gugliandolo, Bramanti, and Mazzon 2017). Dementia is a syndrome, it is known as the most common cause of AD, symptoms that are illustrating dementia include problems with cognitive skills that affects the quality of life; affecting the ability to perform daily activities, such as difficulties in speaking, memory and even thinking and problem solving (Fazio et al. 2018).

In addition, AD is not a particular disease, it is a term used to describe a collection of symptoms altogether defining AD. AD is not only about memory impairments as it leads into definite incapability and eventually death risks within 3-9 years from diagnosis. AD can be discovered in early stages starting at the age of 30, however it is more likely to acquire one or most of AD symptoms mid 60's. Like any disease AD has different stages: the early stage, the moderate stage, and the final stage where all symptoms are aggravated (Banerjee et al. 2015).

Dementia affects around 25 million people currently, of which the majority have AD, a healthy diet is a crucial variable that plays an important role in the advancement of AD and in the cognitive decay. Certain food rich in vitamins that are identified to delay cognitive decline. For example; consuming fresh fruits, and leafy greens are considered preventive to cognitive decline,

while reducing the consumption of saturated fats, processed food and refined carbs. Studies have shown that there is a relationship between the consumption of vitamin A, E, C and AD (Mehta et a. 2017).

Besides, one of the main causes of AD is oxidative stress, which means the imbalance between the production of free radicals and antioxidants. The free radicals are simply molecules containing uneven number of electrons. Antioxidants are the molecules that are able to provide free radicals with electrons without being jeopardized to being unstable which tend to stabilize free radicals by making them less reactive. The imbalance between reactive species and antioxidants defense permit the former to react causing oxidative damage, where the lack of antioxidants is not enough to fix (Halliwell 2007).

2.1 Methods to induce AD in rodents

There is more than one method to induce AD in animals, for instance genetically modified model is one method preferred by geneticists when using mice, since mice are smaller and, in a way, easier to be genetically manipulated. That's is why transgenic research use mice as the most prevailing animal for such genetical research. Mice can provide genetical insights while they lack physiological insight (Benedikz, Kloskowska, and Winblad 2009). Another possible way to test the cognitive research is chemically inducing rats with a substance that will result in having AD, since it was explained that environmental factors play a big role in neurological diseases. One of these factors is the long-term consumption and or continuous contact with aluminum. Aluminum as a substance that is not normally labelled as harmful, however people with neurological diseases such as Parkinson's disease, AD, and many more, have found to have a high concentration of aluminum. According to another study exploring the effect of some substance that may help limiting the symptoms of dementia specifically AD, the use of $AlCl_3$ result in a deterioration in the behavior and learning of rats (Thippeswamy et al. 2013).

One the most frequently used metals in the food industry is aluminum since it is used in storage, packaging, and transportation of food. The average consumption of an adult is 3-12 mg of aluminum whether it is directly consumed or in an indirect way (Thippeswamy et al. 2013). On the long-run consuming aluminum will cause an accumulation in the kidney, bone, muscle and brain which in turn will result in many disorders to the body such as neurodegenerative disorders

(AD). Not to mention that it may also reduce acetylcholine levels in the hippocampal area, which is accelerating and empowering the development of cognitive and memory disorders (Thippeswamy et al. 2013).

Over 180 years ago, the rat was the first mammalian species trained for scientific research, since then it has been the most popular studied model creature specifically for cancer, cardiovascular, toxicology, neurodegeneration, and last but not least aging research. It cannot be overestimated what rats contribute to human health; for decades it has been the number one pick for most behavioral and physiological research. Rats are favored when it comes to behavioral scientists, since rats are considered quick learners also physiologists favor the fact that physiological mechanisms are similar in both humans and rats. Not to mention that rats being considerably large which is convenient for physiological measures (Benedikz, Kloskowska, and Winblad 2009).

That is why in this study using an oral gavage, rats will be consuming Aluminum Chloride $AlCl_3$ for the cognition research.

2.2 Morris Water Maze as a Memory Testing Experiment

The Morris Water Maze (MWM) was recognized and proven by neurologist Richard G. Morris in 1981 to be able to examine hippocampal-dependent learning which contains the attainment of spatial and long-term memory. It is a quite basic and simple test consisting of a fixed number of days trial to be able to distinguish between a hidden and a visible platform. The purpose of the test is to prove that rats can identify and memorize the place of an object (a platform) that they are visualizing, hearing, or even sensing, giving fixed area and surroundings. The test includes many detailed procedures to follow to be able to perform the trial; these procedures will be explained in detail later in this paper (Morris 1981).

The Morris water maze is an examination of spatial ability. It necessitates the animal to navigate to an escape platform that is not seen, hidden under water, and find its location (Kennard and Woodruff-Pak 2011). To explain the process of the MWM, it is a simple process where it includes a round pool; rats go through acquisition phase; when they are trained to reach a visible platform visibly located above water. Second phase is retention; where the platform is below water using a safe water dye so the pool water can be opaque. Simply it is a maze that helps to study the

behavior of rats when it comes to memory. Rats are trained in a way to memorize every angle of the pool wherever their starting point they are trained to reach the visible platform. So, when the platform is no longer visible, they can rely on their memory to reach it, it also helps that the pool is divided equally into four quadrants to keep track where was their starting point (Thippeswamy et al. 2013).

MWM is the most used in labs when it comes to investigating spatial learning, memory, and/or behavioral neuroscience. The process is really simple however it has to be precisely implemented because of the sensitivity of subjects it is evaluating. Many researches have shown that the sensitivity of results of MWM is affected by different training techniques done in the training phase and the rats characteristics (D'Hooge and De Deyn 2001).

Many factors are affecting the behavior of rats in MWM such as age, body weight, gender, nutrition, and stress level endurance and sickness. It was discovered that rats' gender may affect the results of MWM; male rats perform better in spatial learning and memory research, while the hormones in female rats may affect their behavior unless female rats used are ovariectomized (D'Hooge and De Deyn 2001). Hormones in female rats alter their behavior, while male rats have more steady behaviors. Also, mice and rats have very different behaviors in MWM. For mice; their ability to swim and float is not as good as rats. Additionally, rats have more endurance to stress than mice; when mice are exposed to any kind of stress they freeze or it impairs their behavior rather than searching for escape strategy, their reactivity to stress is higher and more sensitive than rats (D'Hooge and De Deyn 2001).

In accordance, it was shown that severe impairments related to mice's age were detected by the age of 24-27 months, however other studies have shown impairments in learning or acquiring new skills by the age of 18-22 months. Also, mice take more time in training than rats, their acquisition phase is longer and usually their performance is poorer when it comes to MWM, in addition to their sensitivity to cold water and weaker swimming skills. Mice will be struggling with swimming which may weaken its ability to orient to indicators thus reducing its performance capacity in the water maze. Mice impairments can be detected as early as 12 months. That is why rats are more likely to be used in studies with high stress level and in MWM (Kennard and Woodruff-Pak 2011).

Besides, the age of the animal used in the study is a crucial decision; it was recognized that like anything else and like human's life the behavior of rats weakens by time, their performance in MWM also declines due not only to its worsen swimming capability but also to changes in cognitive performance. Aging alters the brain behaviors due to physical and physiological alterations in brain functions responsible to steady cognitive behavior. Young rats are a very suitable pairing when it comes to MWM. Furthermore, nutrition is an important factor affecting the behavior of rats. Malnutrition negatively affects the behavior of rats to concentrate and find the hidden platform, their training results are poorer than rats that receive balanced nutrition and commonly the same with sick and infected rats and stressed rats (D'Hooge and De Deyn 2001).

So, any failed attempt for the animal to reach the platform may be due to the age of the animal; aged rats may have poorer performance, problems in the vision, or any environmental variable; any change in the environment may burden animal and affect their performance, any change in sound, temperature, and light will cause stress leaving them incapable of fully performing that is why these variable should stay constant during the experiment to obtain accurate positive results (Bromley-Brits, Deng & Song 2011).

2.3 Osteoporosis

When it comes to osteoporosis, it is very important to know that osteoporosis is very common; it is a disease in which 53 million in the United States have been diagnosed with or are in jeopardy to have it. Osteoporosis causes low bone mass which in turn leaves the body prone to many bone fractures. This disease affects all races; men and women however women after menopause are at higher jeopardy to osteoporosis (Randolph and FAAOHN 2016). It is a devastating disease that is related to the skeleton; a bone disease leaving the body with low bone strength. This disease is very common in the elderly population in 2011 affecting 10 million American with a ratio one in two women and one in five men, in Canada it is affecting only 2 million with a ratio of one in five women and one in eight men over the age of 50 (Karaplis et al. 2011).

This disease causes the bone to be fragile and delicate. Bone is a living tissue; such tissue is continuously in the process of breaking down and then being replaced. When this process is not balanced and equal here is when osteoporosis occurs; simply when creating new bone tissue is not

equal to the elimination of the old one. In early phases of osteoporosis, no symptoms of bone loss are identified. However, in later stages symptoms include ruptured or weakened vertebra which lead to back pain, shortened body height over time, bad body posture such as being bowed or round shouldered, and body bones are being fractured without strong body traumas; these fractures may include hip, wrist, and spine fractures (Randolph and FAAOHN 2016).

In the light of the 21th century comes the knowledge that joint and bone diseases are of major cause of not only pain but also physical disability worldwide. There are wide range of diseases that can cause pain and loss of mobility. These diseases are becoming more common; that is why the WHO declared the new century as 'The Joint and Bone Decade 2000-2010' such declaration was the reason behind the overwhelming attention whether it was media or academic regarding diseases that affects the bones. The higher the number of the elderly population the higher the number of people suffering from bone diseases. The National Osteoporosis Foundation NOF announced that the case of hip fracture is predicted to a 240% increase in women and 310% increase in men by 2050 if no scientifical prevention methods took place. On the optimistic view, already there is lots of researchers and studies that contributed to a greater understanding about the bone disorders (Bartl and Frisch 2009)

Osteoporosis is affected by factors such as health conditions lifestyle, race and age. For example, weaker bones can be due to low sex hormone, also menopause play a big role for osteoporosis disease since at menopause is when the ovaries' decrease the production of the hormone estrogen (Randolph and FAAOHN 2016). Lately it has been recognized that osteoporosis demonstrates no gender discrimination with the exception of only affecting men in later stage than women, it begins in time of andropause which is similar to menopause in women. Andropause is when the male hormones start to decline and a deficiency in androgen take place, in which reproductive activity decline, it all take place from the age of 50 to 60. That is why calculations have shown a 13% risk of fracture affect men over 50 years old (Bartl and Frisch 2009).

Osteoporosis is connected to injuries and death with considerable economic costs. It is assumed that homocysteine amino acid is connected to osteoporosis. Homocystinuria is a disease caused by high levels of homocysteine in plasma, an amino acid formed by protein breakdown in the blood. Homocystinuria or high level of homocysteine is theorized and proved to be connected with osteoporosis fractures in elderly with both men and women (Van Meurs et al. 2004). Collagen

crosslinks are part of the bone matrix upon which minerals are deposited, it is crucial for building a strong and stable connective tissue. Homocysteine was proven to interfere with collagen cross-linking, and therefore homocystinuria patients with high levels of plasma homocysteine are likely to develop a weak bone protein matrix, resulting in fragile bones and developing osteoporosis at early age. In a study, folic acid (vitamin B9) and cobalamin (vitamin B12) consumption dramatically decreased hip fractures in particular (Gaby 2015)

Diet plan is a crucial factor as well, for example low calcium consumption and or some surgeries such as the reduction of stomach size or removing a piece of the intestine affect the body and can build weak defense system for the body when it comes to osteoporosis. However, osteoporosis can be slightly prevented when healthy lifestyle is adopted; with an appropriate diet plan and exercising the body can prevent farther bone loss and the possibility of bone fracture or crack. A well-balanced exercise can reduce the possibility of falling; smoking should be prohibited to people with low bone density as it increases the percentage of bone loss (Randolph and FAAOHN 2016).

2.3.1 Osteoporotic Ovariectomized Mice

Osteoporosis is a silent and dangerous disease, a silent disease that is only heard with the first fracture. To study the prevention and handling of such disease, animals' models are on top of the list for the first step to understanding and treating osteoporosis. The most commonly used for this study is ovariectomized rodent model, quoting the FDA Food and Drug Administration guidelines, that the best preclinical model used for post-menopause osteoporosis is the ovariectomized rodent model. The reason behind such statement is that such model succeeds to mimic the bone loss, estrogen deficiency and also demonstrates the clinical indicators of postmenopausal osteoporosis. Primary osteoporosis is whether age related or postmenopausal osteoporosis, this form is the most common form of osteoporosis. The age-related osteoporosis is a primary form of osteoporosis that tempts bone loss in a gradual way and affects cortical bone. While postmenopausal osteoporosis affects the trabecular bone and bone loss in this case it is not gradual it is somehow rapid because of increases osteoclast activity (Yousefzadeh et al. 2020).

Postmenopausal osteoporosis is known for decreasing bone mass and a decline in the whole bone architecture. 30-40% of women worldwide are at risk for having osteoporosis fractures such

percentage has a significant economic and social impact. The ovariectomy rat model (OVX) is the most used model when it comes to menopausal osteoporosis however mice might be seen as more effective since lower drug doses are required for treatment and results are reflected in shorter period of time. The ovariectomized mouse model has been widely approved to be appropriate for the study of osteoporosis. In this study, in order to induce osteoporosis, the ovariectomized (OVX) mouse model was used (Zhou et al. 2018), post ovariectomy bone loss can reach 30% in three to 4 weeks (Sophocleous and Idris 2014).

2.4 The EFSA Health Claims

Due to the food crisis of earlier decades The European Food Safety Authorization EFSA was established in 2002, it was known for providing scientific guidance and assistance. Since it has expert panels who were serving as experts for EFSA's own initiative and/or answering to specific demand from European Commissions. All this was with particular order of interactive communication on risks tied with the food chain. The establishment of EFSA main's aim was to regain the trust of consumers in EU food safety, ensuring the public that any risk associated with food supply is carefully evaluated. It is also its role to evaluate and verify the rationality behind any health claims submitted, its main concern is to break the walls between scientists and consumers. The power and beauty of such authorization is its association to European Commission's Laws which means that any denied claims are banned; claims are only approved once they get the EFSA's approval (Vero & Gassbarrini 2012)

EFSA has emphasized on providing scientific proof for health claims, from this point, everything was under profound investigation: health claims in ads, in labels and in any piece of paper associated with a product. Every piece of information is evaluated in terms of its clarity and its accordance to supported scientific proof, some claims may be effective on the short-term and totally deceitful on the long run. One of the challenges EFSA is facing is to keep the balance between innovation and safety without being a rigid and traditional system; lots of entrepreneurs in the scientific and medicine fields claim to being shut down by EFSA for not having enough scientific proofs to be approved; which slowed the path of innovation (Vero & Gassbarrini 2012).

On the other hand, health claims are any announcements about the relationship between food and health, such statements should be clear and comprehensible by the consumers. Any claim

that has direct physiological effect is considered. Claims may have instant or short-term effects; these claims are called acute claims. For instance, a medicine may have direct effect on specific disease but only short-term effect, while on the long-run such effect may vary or differ. Acute claims may be approved claims as well if supported with scientific evidence and it must be investigated on humans, tested and documenting results. A cognitive function has many domains such as attention, memory, sleep, anxiety etc, and any supported claim regarding one or more of the previous domains is considered an improvement on cognitive function (EFSA 2012)

Ingredient		**Memory / Cognition Study**	
Premix 1	Authorized Health Claim	Vitamin B1	Thiamine
		Vitamin B2	Riboflavin
		Vitamin B3	Niacin
		Vitamin B5	Pantothenic Acid
		Vitamin B6	Pyridoxine
		Vitamin B7	Biotin
		Vitamin B9	Folate
		Vitamin B12	Cyanocobalamin
		Vitamin C	Ascorbic Acid
		Magnesium	
		Zinc	
		Iron	
		Iodine	
		Calcium	
Premix 2	Content Claim	Vitamin E	Tocopherol
		Phosphorus	
Premix 3	Master Mix	Vitamin B1	Thiamine
	(All Ingredients)	Vitamin B2	Riboflavin
		Vitamin B3	Niacin
		Vitamin B5	Pantothenic Acid
		Vitamin B6	Pyridoxine
		Vitamin B7	Biotin
		Vitamin B9	Folate
		Vitamin B12	Cyanocobalamin
		Vitamin C	Ascorbic Acid
		Vitamin E	Tocopherol
		Magnesium	
		Zinc	
		Iron	
		Iodine	

		Calcium	
		Phosphorus	

Table 1: Illustration of the composition of the premixes used in the dementia study

Ingredient		**Osteoporosis Study**	
Premix 4	Authorized Health Claim	Vitamin B6	Pyridoxine
		Vitamin C	Ascorbic Acid
		Calcium	
		Magnesium	
		Manganese	
		Zinc	
Premix 5	Content Claim	Vitamin E	Tocopherol
		Vitamin B9	Folate
		Vitamin B12	Cyanocobalamin
Premix 6	Master Mix	Vitamin B6	Pyridoxine
	(All Ingredients)	Vitamin C	Ascorbic Acid
		Vitamin E	Tocopherol
		Vitamin B9	Folate
		Vitamin B12	Cyanocobalamin
		Calcium	
		Magnesium	
		Manganese	
		Zinc	

Table 2: Illustration of the composition of the premixes used in the osteoporosis study

2.4.1 Authorized claim premix

Authorized claim premix is a premix approved by EFSA; each vitamin of the premix has scientific evidence to positively affect dementia. It includes Vitamin B1, Vitamin B2, Vitamin B3, Vitamin B5, Vitamin B6, Vitamin B7, Vitamin B9, Vitamin B12, Vitamin C, Magnesium, Zinc, Iron, Iodine and Calcium.

A lot of studies have examined the relationship between magnesium and AD, not to mention examining the relationship between high calcium, potassium and magnesium intake with dementia, this study was conducted on a sample of Japanese men and women. Results have shown a negative relationship between dementia and these three substances. Also, other studies examined these same results backwards by testing AD patients to identify the presence of ionized magnesium

for example within these patients compared to healthy groups, and it was always lower for the AD patients (EFSA, 2014).

2.4.2 Content claim premixes ingredients

Content claim premix contains vitamins and minerals that are not authorized by EFSA to have a health claim, yet studies suggest that the ingredients have particular beneficial nutritional properties. This premix includes Vitamin E and Phosphorus. The study initially included Vitamin A, Vitamin D and Vitamin K, but due to obstacles that are stated in research limitations, we were not able to include those micronutrients. Vitamin K was excluded due to non-availability at the premix supplier. Vitamin A and Vitamin D were excluded because of weighing challenges, the very low concentration of both ingredients in the premix was below the minimum weighing limit.

2.4.2.1 Vitamin E

Vitamin E is found in a variety of foods which include vegetable oil, seeds and nuts. Vitamin E is debatable in terms of benefits; many previous studies have shown little or no benefits of the consumption of vitamin E when it comes to its effect on AD and cognitive improvements. While other studies have demonstrated the harm associated with consuming high amounts of vitamin E to even tie it with death. What we know for sure is that vitamin E is associated with many question marks when it comes to memory impairments. Yet surprisingly recent studies have shown a little improvement in the quality of life of people suffering from dementia in terms of dealing with their daily life like dressing up, bathing, etc. but again, the improvement was minor and the study had many limitations in terms of measuring the harms associated by such consumption. A study also suggested that consuming vitamin E has no harm nor benefit when it comes to dementia, its effect is neutral (Farina. Llewellyn, Isaac and Tabet 2018).

Moreover, a good consumption of fresh vegetables, fruits, whole grains, low fat dairy and reducing the consumption of; sweets, fried food, food high in fat and processed food is the natural way to reducing the risk of AD. In a study of 5395 people, between all the antioxidants used results have shown that the most considerable amount of protection against AD and dementia was thanks to vitamin E and its role (Mehta et al. 2017). Vitamin E dosage can vary from 3 mg to 15 mg per 100 g body weight varying according to different countries and different age groups, each age group has its own dosage. One of its main roles is acting as antioxidant in the body, vitamin E

plays a crucial role when it comes to the brain since it has an important role in the development of tissue and organs. Vitamin E deficiency causes a lack of antioxidant role and its protection to the body as well as damage of the cells and cognitive disorder. Vitamin E was proved to reduce oxidative stress which is the main cause of AD (Gugliandolo, Bramanti, and Mazzon 2017). Vitamin E plays an important role as an antioxidant, and its deficiency is high likely to cause destruction of neurons. As vitamin E has many forms, the best forms in guarding against AD are α-tocopherols and γ-tocopherols (Mehta et al. 2017).

In agreement to this previous debate carried decades through decades; whether or not vitamin E is associated with cognitive health; it was stated that many other factors can affect vitamin E performance. These factors include; which form of vitamin E was used, taking into consideration that it has eight natural forms, also the amalgamation of treatment which can affect the efficiency of vitamin E (Cervantes and Ulatowski 2017).

Another study showed that taking vitamin E as a prevention to AD was insignificant in preventing patients with hereditary AD from developing AD; patients with family history of AD still developed AD even if they were on vitamin E supplements for years, however in the case of patients with no hereditary history further research is needed (Gugliandolo, Bramanti, and Mazzon 2017).

Only lately evidence has been researched that vitamin E is valuable when it comes to osteoporosis. Researches have shown that vitamin E has been beneficial in increasing bone density. Also, it was reported that vitamin E stops bone calcium loss by neutralizing antioxidants; it reduces calcium bone loss in rats who have been ovariectomized. Vitamin E supplements has been able to guard bones from oxidative damage by collecting free radicals. It protects the bone from any harm caused from oxidative stress; which is created by shortage of sex hormone. Vitamin E supplements reinstated bone strength even in aging rats and rats who have been ovariectomized. When it comes to bone remodeling and bone metabolism calcium has a crucial effect for these two. Vitamin E deficiency, possibly due to reduced calcium absorption, will cause bone damage that will eventually result in calcium deficiency and will augment the activity of free radicals (Mohamed et al. 2012).

2.4.2.2 Vitamin D

Vitamin D is commonly known as good for bones, it is known for most people, even people with no scientific background, that vitamin D is beneficial for bones. However, Vitamin D is beneficial for a lot more than that. It is beneficial for fighting against diseases related to aging such as type 2 diabetes, cardiovascular disease and even cancer (Soni et al. 2012). In addition, vitamin D has an antioxidant effect and it also plays a role in the regulation of neurotrophic factors which in turn help the endurance, growth and function of neurons. It was also shown that vitamin D creates a form of protection against calcium toxicity. When it comes to cognitive diseases it was demonstrated that there is a relationship between vitamin D deficiencies and the presence of cognitive diseases. The risk of cognitive disease was four times higher in case of a present vitamin D deficiency (Soni et al. 2012).

According to Banerjee et al. (2015) there is a relationship between vitamin D3 and AD whether it is a direct relationship or indirect; meaning that vitamin D deficiency may lead to AD or severe symptoms rather than mild symptoms or no development of AD while having enough vitamin D intake. On the other hand, few studies showed no relation at all between vitamin D intake and AD. It is crucial to be aware that vitamin D intake should be monitored on studies of longer periods, since its effect takes longer to show results.

Additionally, many studies have focused on finding more insight on the relationship between vitamin D deficiency and dementia. Results have shown a positive relationship between them; the majority of people suffering from dementia tested positive for vitamin D deficiency, and even people with no vitamin D deficiency suffering from dementia certainly had lower vitamin D in their blood than control groups with no dementia at all (Shen and Ji 2015). However, a deeper relationship between vitamin D and dementia need further research to identify whether its consumption can prevent dementia or even fight against symptoms of AD, research have shown that people regularly taking vitamin D had lower chances of getting dementia than people not taking it (Shen and Ji 2015).

When it comes to osteoporosis, vitamin D is crucial to having strong bones, daily intake of vitamin D can prevent having weakened bones thus can prevent osteoporosis, not to mention vitamin D role in improving calcium absorption and improving bone health. For adults at the age of 50 or older need at least 800-1,000 international units IU of vitamin D if they do not have any

vitamin D deficiencies, this dosage can be consumed through healthy food or supplements (Randolph and FAAOHN 2016).

While evaluating the relationship between osteoporosis and calcium and vitamin D, these two together are crucial for improving the strength of bones, between the age of 18 to 50, men and women need 1,000 milligrams of calcium per day for strong bones, however such amount will increase to 1,200 milligrams for women when they turn 50 and will also increase for men who turn 70. Also, a healthy dietary plan containing healthy sources of calcium such as low-fat dairy products, soy products such as tofu, orange, salmon, sardine, and cereals that are calcium fortified (Randolph and FAAOHN 2016).

2.4.2.3 Vitamin B9 and vitamin B12

In the light of this research it was highlighted that any nutrient deficiency rushes bone loss and increases the tendency to fall, which is a main cause in hip falls and fracture for elder people. Studying the bone's physiology vitamin D and calcium have been the most crucial vitamins for bone health, however some reviews have shown that other nutrients may be as important for bone health such as the B vitamins. In general, the B vitamins are cofactors for the enzymes involved in the energy producing metabolic pathways for fats, carbohydrates and proteins not to mention its important role in sustaining functions of the nervous system. Studies have shown that bone health is related to the B vitamins in particular vitamin B9 (folate) and vitamin B12 (cobalamin), however it has been determined that there is a relationship between B2, B6, B9, B12 intake and low risk of osteoporosis (Dai and Koh 2015).

It is important to know that vitamin B12 is acquired from animals for example in meat, dairy and fish. For a healthy individual a daily consumption 5-30 µg of vitamin B12 is required such dosage is not absorbed in full only 1-5 µg is considered to be absorbed. There is a slight difference between the recommendation of vitamin B12; the United Kingdom is recommending 1.5 µg, the U.S. 2.4 µg and the European Union recommending 1 µg. Vitamin B12 deficiency will not show immediately by reduced daily intake or reduced body absorption however it will show in several years after the stores' depletion. Deficiency occurs with the rise of requirements, for example; in pregnancy, or through growth in adolescence and children, also people with a poor diet and mal-nutrition or even a vegetarian diet. In the US 6% of people under 60 years old and

almost 20% of people over 60 years old have experienced a vitamin B12 deficiency. Vitamin B12 deficiency is common but cannot be taken lightly it is a grave condition when ignored (Hunt et al. 2014).

Vitamin B9 (folate) can be found in food such as fruits that are citrus, dark leafy greens, brussels sprouts, asparagus, and broccoli. It also has a big role in the one-carbon metabolism in the nucleotide synthesis, as well as the methylation of RNA, DNA phospholipids and DNA, not to mention its role in the metabolism of homocysteine. In the methylation pathway, the synthesis of methionine depends on both folate and B12 for re-methylation. Deficiency of any of those two B vitamins will result in major changes in the bone marrow and other tissues (Dai and Koh 2015).

When it comes to osteoporosis the B vitamins indeed play an important role in the prevention of fractures caused by osteoporosis. In a study of 2919 persons with age group above 65 years old who were proved of having high homocysteine level living in the Netherlands, individuals who received a fair dose of vitamin B12, vitamin B9 and vitamin D had lower osteoporosis fracture injuries by 16% compared to the group with placebo effect. For example, hip fractures are demonstrating a considerable decrease by 78% when consuming folic acid and vitamin B12 (Gaby 2015).

2.4.2.4 Vitamin A

Vitamin A plays a crucial role in the development of the Central Nervous System CNS. Vitamin A has been usually considered as an anti-oxidant compound, but when it comes to older people it plays a role in maintaining higher CNS as well. Vitamin A and Beta-carotene both have revealed their anti-oxidative form, also being known as cell protective. It was also proved that higher concentration of Beta-carotene was linked to better memory performance. An association between vitamin A deficiency and inadequate cognitive function in older adults, however evidence that vitamin A is related to poor cognitive performance and thus negatively related to dementia is a grey area and need further deeper research in order to generalize such results on vitamin A and not any other substances (Ona and Yamada 2011).

In accordance vitamin A is crucial for the development of the Central Nervous System through growth for children, adolescents and adults. It does not only guard the neuronal cells but it also supports the rejuvenation of neuronal cells while recovering from neurodegeneration. An

analysis has demonstrated that AD patients have shown lower serum levels of vitamin A, folic acid, vitamin B12, vitamin C and vitamin E. It has been also demonstrated that beta-carotene and vitamin A have been tied to memory performance and spatial learning (Mehta et al. 2017). Vitamin E, D, and A have shown positive deficiencies in patients with AD and have antioxidant components, also have shown possible relationship in improving cognitive functions. That is why it is important to properly investigate their potential effect on AD and dementia.

2.4.2.4 Phosphorus

Phosphorus is mostly found in the form of phosphate minerals since it's a highly reactive mineral. Phosphorus is essential for bones, one of the minerals that are rich in the body; the presence of phosphorus in the body of an adult is around 400-800 g. Phosphorus can be found in eggs, nuts, meat, fish, dairy and grains. Also, phosphate additives are used as flavoring agent, preservative and acidity controller, that is why processed food is rich in phosphorus (EFSA, 2014).

It is physiologically useful to maintain healthy bones throughout one's life span, great bone formation will result to an increase in BMD bone mass density and in bone mineral content BMC which is a measurement of the amount of bone mineral in bone tissue. BMD and BMC can be treated as biomarkers for the indication of bone health. A good diet habit can be crucial when it comes to bone health. For example, consuming Calcium supplements can result to an increase in BMD or even prevent the loss of BMD for old adults and women post menopause. When it comes to phosphorus previous studies had controversial findings, some studies have shown that phosphorus when consumed in high dosage can be harmful to the overall bone health, however these findings were for specific subjects whose calcium to phosphorus level was reported to be very low. However, it has been reported that subjects with normal balance of Ca to P showed no harmful impact on bone health. Not only that but maintaining such balance lead to improving overall bone health and reducing osteoporosis effect (Lee and Cho 2015).

According to recent studies, studying the effect of phosphorous intake, there were 2 controversial results, first one that high serum phosphorous is positively related to dementia and AD, however, this is specifically to the age bracket lower than 60 years old. On the other hand, results have shown that low serum phosphorous, which turns to be a phosphorus deficiency due to malnutrition, for the age bracket over 60 years, is positively related to dementia and AD. This

means that phosphorus deficiency can be one of the issues causing dementia and AD (Li et al. 2017).

2.5 Dosing premixes to rodents

Administration of substance to the laboratory animal is a crucial part of any study, since the nature of the substance may differ and so the importance and sensitivity of how to do it. The substance may include infectious disease mediators; many therapeutics as injections, antimicrobials, pharmacologic mediators, sedatives, chemical and radiocontrast test agents (Turner et al. 2011). For the dosage calculations of ingredients, the human RDA's can be used as a basis, but the doses in rats or mice need to be converted to mg per kg body weight per day (mg/kg/day) and the species differences in metabolism need to be accounted for. Knowing the weight of the rodent and how many grams are consumed per day, we can calculate the dosing. (Reagon-Shaw et al. 2007). It is equally crucial to choose the vehicle used to administer it. There are many ways to do so, it could be done by giving it orally, using gastric gavage to directly deliver it into the stomach, injections which can be muscle injection, blood injection, under the skin, injecting the brain, injecting the eye, etc. Every study has its way to deliver the substance to the laboratory animal (Turner et al. 2011).

Gastric gavage or also called oral gavage is a means of delivering the substance directly into the stomach using a small tube. This process does require high technical ability in order to be performed, it is important to have proper training, before performing this process on laboratory rats to minimize the disadvantages tied with poorly conducting this process. Training may be as easy as following simple clues and directions given by technical personnel on inserting the gavage and choosing the best size for the animal. This training is important to guarantee that the process is performed in an accurate, not time consuming and human way. The adverse effect, of not performing oral gavage properly, may be over filling the stomach which may lead to reflux, injury and/or gastric irritation or rupture, also stressing the rats. In addition, it can cause distention in rats which means that they basically get swollen since rats are not able to vomit, that is why it is crucial to choose the proper size of gavage to perform this process. However, these associated risks may still occur with professionally trained personnel and may cause sudden death to the animal in addition to the distress and health risks, it is important to be able to identify and to report any of these risks once observed. (Turner et al. 2011).

Also, administration of substance is an important technique for any experiment; even if the preferred way is consumption in a voluntary way since it is the most natural way but oral gavage guarantees that all the substance is administered inside the animal's stomach. The process is described as a stainless steel, hard and ball tipped needles. To reduce the risks associated with this process it is required to gently pass the gavage tube, it is also important to note that the best timing to insert the tube from pharynx to esophagus is when the rat is already performing the swallowing act. It is easy to identify that the tube is not within the trachea through simple observation. However, robust restrain is the key to an effective gavage; not to rush into inserting the tube, restraining one's self by observing the cues and clues for proper installments. Starting with the alignment of the needle and the animal's body as a far as reaching the oropharynx, then start lightly moving the tip so that the animal start the swallowing act which facilitates the process, finally counting into three before withdrawing the tube to ensure the dose is fully delivered (Talcott, Akers and Marini 2015).

Testing is the major part of any study, using the right tests in crucial to the success or failure of a study. For the dementia study, testing was done through three mediums, the first medium is the behavioral testing which was done using Morris Water Maze MWM for better understanding of the behaviors of rats, the second was the blood biomarkers through ELISA test (enzyme-linked immunosorbent assay). Finally the third was the histopathological examination of the brain in order to observe neurons and how affected to the whole study and process. On the other hand, for the osteoporosis study, testing will take place through two mediums, first the blood biomarkers through the detection of calcium in blood (total & ionized) and Alkaline Phosphatase (ALP). The second medium is the histopathology examination of femur bone.

3. Materials and Methods

3.1 Animals:

For dementia experiments Sprague-Dawley male rat model was used. Rats were purchased from Misr University for Science and Technology, Giza Governorate. Average weight per rat was 150-170 g at the time of the purchase and reached 250 g by the end of the rehabilitation phase with average age of 52 to 60 days old. For osteoporosis experiments C57BL/6J female mouse model was used. Mice were purchased from Theodor Bilharz Research Institute, Giza Governorate. Mice average weight at the time of the purchase was 20 g and the age was 70 to 84 days. The animals were relocated to the Medical Experimental Research Center at Mansoura University (MERC), where they were kept on the rehabilitation phase for 30 days at standard environmental conditions. They were housed in standard metallic cages (6 animals per cage) with a room temperature of 25± 2°C on a 12/12 hours light/dark cycle and had ad libitum access to rat/mice chow and water. The experimental protocol of this study was approved by the Local Ethical Committee, Faculty of Medicine, Mansoura University in accordance with the ethics committee of the Egyptian National Research Center with registration number (09/189).

3.2 Chemicals and Nutrients:

Aluminum Chloride $AlCl_3$ was administered to rats to chemically induce dementia. $AlCl_3$ was purchased from Alpha Chemika, Batch No: AC198. Dosage of $AlCl_3$: 100 mg per kg body weight. $AlCl_3$ was dissolved in distilled water. Rats' average weight at the beginning of the experiment was 250 g, rats exposed to $AlCl_3$ were administered with 25 mg $AlCl_3$ dissolved in 1

mL distilled water by oral gavage. After 26 days, at the middle of the experiment, average weight for rats reached 300 g and $AlCl_3$ dosage was adjusted accordingly to 30 mg $AlCl_3$ in 1 mL distilled water per rat. Administration of Aluminum Chloride for relevant study groups was prolonged for 52 days.

Premix is a blend of micronutrients, specifically vitamins and minerals. In the present study we used three premixes in the dementia arm and three premixes in the osteoporosis arm of the study. Premixes were purchased from DSM Nutritional Products. The premixes used in the dementia and osteoporosis studies were:

- Premix-1 (KOD: E3208): 1 kg of premix contains 377 mg Vitamin B1, 595 mg Vitamin B2, 5382 mg Vitamin B3, 2059 mg Vitamin B5, 476 mg Vitamin B6, 164 mg Vitamin B7 10%, 82 mg Vitamin B9 80%, 164 mg B12 1%, 27598 mg Vitamin C, 252283 mg Magnesium Oxide 50%, 4672 mg Zinc Oxide 72%, 15697 mg Iron Sulphate Monohydrate 30%, 492 mg Iodine 10% and 689959 mg Calcium Carbonate. Those ingredients are approved by EFSA with an authorized health claim directly related to improving cognitive functions. Dosage of 76.75 mg premix per 250 g rat per day were dissolved in 1 mL distilled water to deliver the micronutrients recommended daily allowance RDA. After 26 days, dosage was adjusted to meet increased average rats' weight to be 92 mg per 300 g rat per day dissolved in 1 mL distilled water.
- Premix-2 (KOD: E3209): 1 kg of premix contains 6134 mg Vitamin E 50% and 993866 mg Dicalcium Phosphate Phosphorus. Those ingredients do not have an EFSA authorized health claim relating to cognitive functions. They do have a nutritional content claim. Dosage of 100 mg premix per 250 g rat per day were dissolved in 1 mL distilled water to deliver the micronutrients RDA. After 26 days, dosage was adjusted to meet increased average rats' weight to be 120 mg per 300 g rat per day dissolved in 1 mL distilled water.
- Premix-3 (KOD: E3210) is a master mix with all ingredients from premix-1 and premix-2. 1 kg of premix contains 4964 mg Vitamin E 50%, 232 mg Vitamin B1, 366 mg Vitamin B2, 3309 mg Vitamin B3, 1266 mg Vitamin B5, 293 mg Vitamin B6, 101 mg Vitamin B7 10%, 50 mg Vitamin B9 80%, 101 mg B12 1%, 16971 mg Vitamin C, 155140 mg Magnesium Oxide 50%, 2873 mg Zinc Oxide 72%, 9653 mg Iron Sulphate Monohydrate 30%, 303 mg Iodine 10% and 804378 mg Dicalcium Phosphate. Dosage of 125 mg premix per 250 g rat per day

were dissolved in 1 mL distilled water to deliver the micronutrients RDA. After 26 days, dosage was adjusted to meet increased average rats' weight to be 150 mg per 300 g rat per day dissolved in 1 mL distilled water.

- Premix-4 (KOD: E3211): 1 kg premix contains 298 mg Vitamin B6, 17312 mg Vitamin C, 158254 mg Magnesium Oxide 50%, 681 mg Manganese Oxide 62%, 2930 mg Zinc Oxide 72% and 820524 Dicalcium Phosphate. Those ingredients are approved by EFSA with an authorized health claim directly correlated with enhancing mobility. Dosage of 19.4 mg premix per 20 g mouse per day were dissolved in 0.5 mL distilled water to deliver the micronutrients RDA.
- Premix-5 (KOD: E3212): 1 kg premix contains 9862 mg Vitamin B9 80%, 19724 mg Vitamin B12 % and 970414 Vitamin E 50%. Those ingredients do not have an EFSA authorized health claim related to mobility functions. They do have a nutritional content claim. Dosage of 0.15 mg premix per 20 g mouse per day were dissolved in 0.5 mL distilled water to deliver the micronutrients RDA.
- Premix-6 (KOD: E3213) is a master mix with all ingredients from premix-4 and premix-5. 1 kg premix contains 5038 mg Vitamin E 50%, 297 mg Vitamin B6, 102 mg Vitamin B12 1 %, 51 mg Vitamin B9 80%, 17222 mg Vitamin C, 157433 mg Magnesium Oxide 50%, 677 mg Manganese Oxide 62%, 2915 mg Zinc Oxide 72% and 816265 Dicalcium Phosphate. Dosage of 19.5 mg premix per 20 g mouse per day were dissolved in 0.5 mL distilled water to deliver the micronutrients RDA.

3.3 Administration:

In this study the administration includes the different premixes delivered to rats and the Aluminum Chloride induced to rats. In this study oral gavage was the mean used to administer the premix and the AlCl3. Proper training for this step was admitted in order to perform this step right, rats were held in a way where they couldn't move their heads and a thin tube was inserted through their mouths right into their stomach to deliver the different premixes for each rodent's group and also to deliver the AlCl3.

Figure 1: Rats Oral Gavage with $AlCl_3$

Figure 2: Mice Oral Gavage with premix-4

3.4 Experiment Protocol

3.4.1 Dementia Supplementation Protocol:

In the dementia study, forty-two Sprague-Dawley male rats are randomly divided into five groups, where two of the groups are negative and positive control groups, respectively. Animals were kept thirty days to acclimatize after being transported to MERC, the administration of premixes and $AlCl_3$ started on the 31st day, administration route was oral gavage for both $AlCl_3$ and premix. Group A was administered 2 mL distilled water (vehicle control). Group B was administered 1 mL distilled water and 1 mL $AlCl_3$. Group C, D and E were administered with 1 mL $AlCl_3$ and 1 mL premix-1, premix-2 and premix-3 consecutively for 52 days.

Group 1: Negative control group, containing 10 rats were administered 2 mL distilled water via oral gavage to be exposed to same external conditions as other groups.

Group 2: Positive control group, containing 8 rats were administered $AlCl_3$ by oral gavage for 52 days. Days 1 to 26, animals were administered 25 mg $AlCl_3$ dissolved in 1 mL distilled water. Day 27 to 52, animals were administered 30 mg $AlCl_3$ dissolved in 1 mL distilled water. One rat died during second week of supplementation.

Group 3: Eight rats were administered premix-1 and $AlCl_3$ by oral gavage. Days 1 to 26, animals were administered 25 mg $AlCl_3$ dissolved in 1 mL distilled water and 76.75 mg premix-1 dissolved in 1 mL distilled water. Days 27 to 52, animals were administered 30 mg $AlCl_3$ dissolved in 1 mL distilled water and 92 mg premix-1 dissolved in 1 mL distilled water.

Group 4: Eight rats were administered premix-2 and $AlCl_3$ by oral gavage for 52 days. Days 1 to 26, animals were administered 25 mg $AlCl_3$ dissolved in 1 mL distilled water and 100 mg premix-2 dissolved in 1 mL distilled water. Days 27 to 52, animals were administered 30 mg $AlCl_3$ dissolved in 1 mL distilled water and 120 mg premix-2 dissolved in 1 mL distilled water. One rat died during the 6^{th} week of the supplementation.

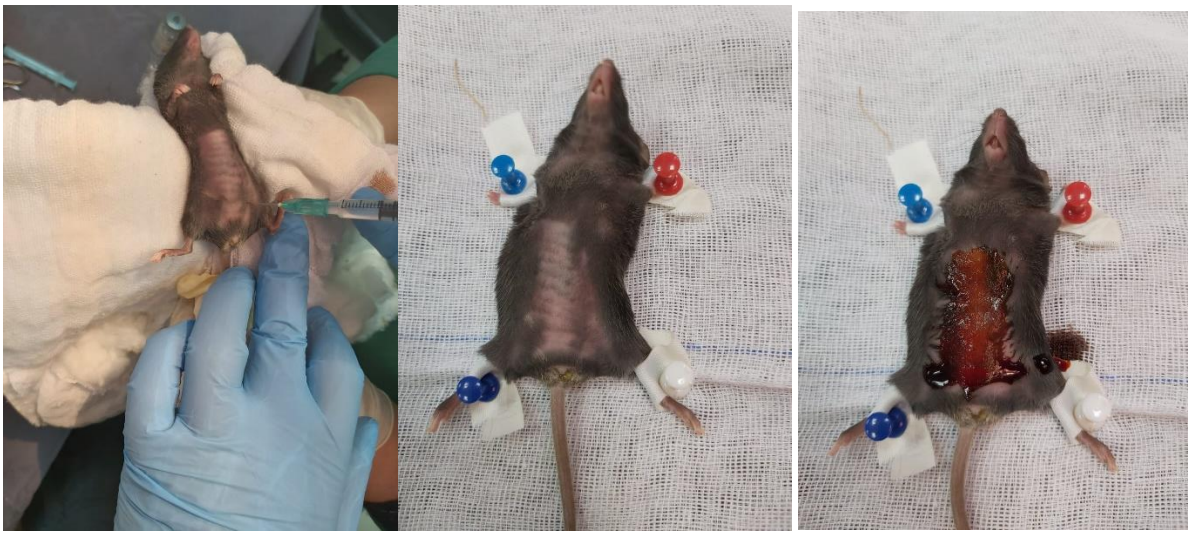
Group 5: Eight rats were administered premix-3 with $AlCl_3$ by oral gavage for 52 days. Days 1 to 26, animals were administered 25 mg $AlCl_3$ dissolved in 1 mL distilled water and 125 mg premix-3 dissolved in 1 mL distilled water. Days 27 to 52, animals were administered 30 mg $AlCl_3$ dissolved in 1 mL distilled water and 150 mg premix-3 dissolved in 1 mL distilled water. One rat died during the second week of supplementation and a second rat during the fifth week of supplementation.

3.4.2 Osteoporosis Supplementation Protocol:

In the osteoporosis study, forty-seven female C57BL/6J mice were also divided randomly into 5 groups, average nine mice per group, including two control groups. Also, a SHAM procedure was done to group 1 the negative control group, where mice went into the same operation to ensure that all mice went into the same incidental effects of the operations as all other rats. Ovaries where identified without having any ovariectomy. During and following the ovariectomy, a significant number of mice died affected by the operation, changing the final number of mice per group.

Animals were kept thirty days to acclimatize after being transported to MERC, on the 31st day ovariectomy procedure was performed on eight mice per day followed by 2 weeks post-surgery recovery.

Ovariectomy was performed using the following procedure, after general anesthesia was obtained by injecting the mice intraperitoneal injection (I.P.) with ketamine-xylazine mixture (ketamine: 75 mg/kg and xylazine: 15 mg/kg). After that, hair at the pelvic area was clipped and shaved followed by disinfecting the shaved area with povidone iodine 7.5% and alcohol 70%. A midline ventral incision was made at the pelvic area. Viscera was pushed on the wet sterile gauge using sterile warm saline to avoid dehydration of abdominal viscera. Ovaries were located and fine dissection was made from the surrounding fat tissue. Ovaries were then ligated by non-absorbable surgical knots and then removed. Muscle, subcutaneous tissue and skin were sutured individually by 5.0 absorbable simple interrupted suturing material. Post-operative care was applied by sterilizing the incision line by povidone-iodine solution 7.5%. Mice were kept in cages heated electrically to avoid hypothermia. Intraperitoneal injection (I.P.) was done for 3 days by antibiotic and 1 mL warm saline in addition to sub-cutaneous injection with diclofenac sodium.



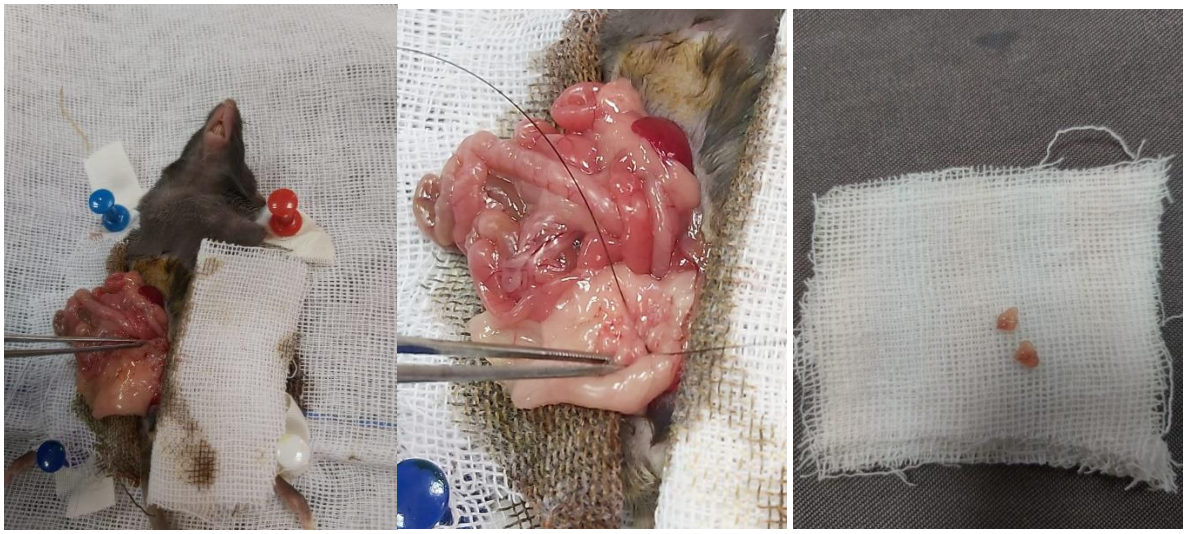


Figure 3: Snapshots of the ovariectomy procedure

After the post-surgery recovery, groups A (SHAM control) and B (ovariectomized mice) were administered 0.5 mL distilled water daily (vehicle controls). Groups C and D were administered 0.5 mL of premix-4 and premix-5, respectively for 90 days.

Group 1: Negative control group, containing 6 mice not taking premix supplementation and are SHAM operated, where ovaries were located but remained intact in the abdominal cavity, and were administered 0.5 mL distilled water by oral gavage for 90 days.

Group 2: Positive control group, containing 9 mice not taking premix supplementation but are ovariectomized to induce osteoporosis and were administered 0.5 mL distilled water by oral gavage for 90 days.

Group 3: Five mice are administered premix-4 and are ovariectomized. Mice were administered 19.4 mg of premix-4 dissolved in 0.5 mL distilled water by oral gavage for 90 days.

Group 4: Six mice are administered premix-5 and are ovariectomized. Mice were administered 0.15 mg of premix-5 dissolved in 0.5 mL distilled water by oral gavage for 90 days.

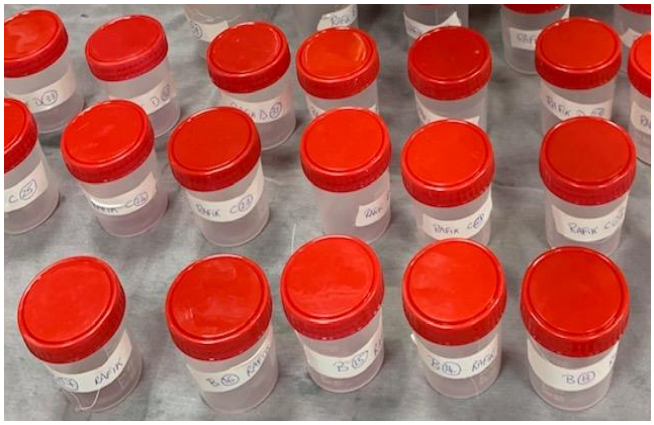
Group 5: Mice were supposed to be administered with premix-6 and are ovariectomized. No mice survived to reach the supplementation start-point. This group was eliminated.

3.5 Organs and blood isolation:

3.5.1 Rat blood and brain sample collection (Dementia):

Rats were anaesthetized by overdosed halothane inhalation to cause cardiovascular and respiratory depression as it is a direct myocardial depressant. Halothane was purchased from Arab Caps company, Batch No: 521637.

Pre-operative preparation and operation, the rats were clipped and shaved from the xiphoid cartilage to the pelvic region, the site of incision was disinfected with alcohol & povidone iodine (Betadine), rats were fixed in dorsal recumbency on a plate. Then the abdomen skin was opened in mid line incision from xiphoid cartilage to pectin of the pubis, blood sample was collected directly from the right ventricle of the heart using a needle syringe, blood was centrifuged to separate the serum, which was transferred to a separate vial and stored at -20°C to be ready for the ELISA tests. Animals were then sacrificed by cervical dislocation. In order to remove the intact brain for histological analysis, craniotomy was carried out, brains were removed and preserved in formalin. Each brain was kept in a separate sterile labeled container.



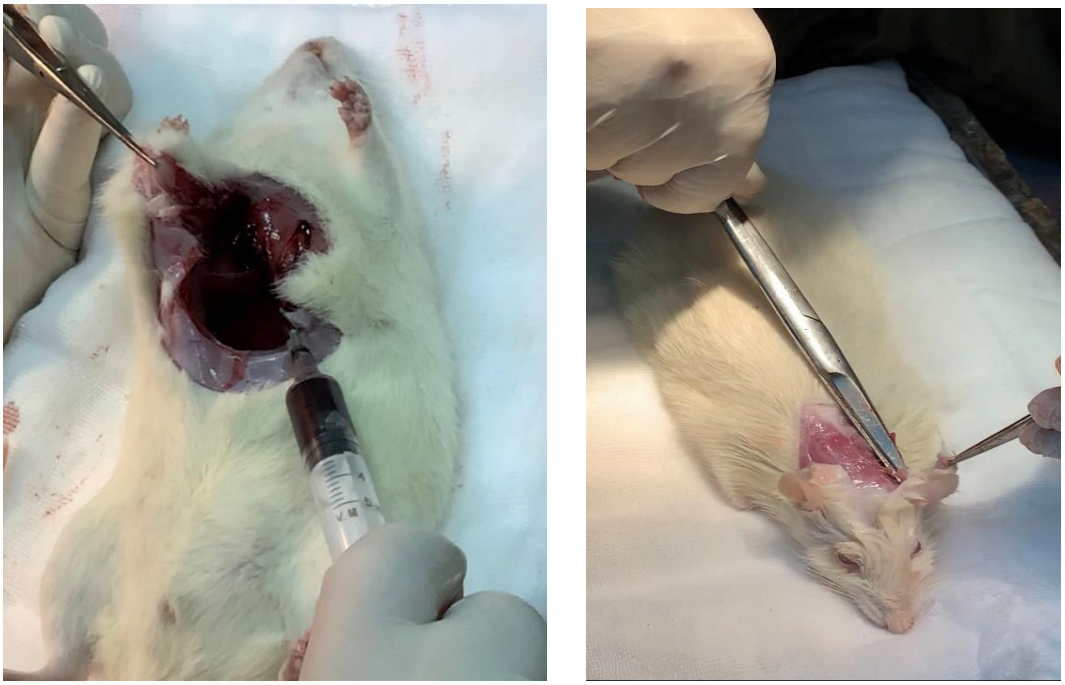


Figure 4: Snapshots during the blood and brain tissue collection

3.5.2 Mice blood and femur bone sample collection (Osteoporosis):

Mice euthanasia was done by overdose of sodium phenobarbital 60 mg/kg body weight via i.p. injection. Blood sample was collected from the heart by direct syringe puncture in the right heart ventricle. Serum was separated after blood centrifugation and transferred to a separate vial and stored at -20°C to be ready for the bio-markers tests. Femur bone was collected by cutting at the high limb and dissecting the surrounding muscles, skin and subcutaneous tissue.

3.6 Mounting and Staining:

Brain and femur bone samples collected were prepared for the histopathological examination each following their specific procedures.

3.6.1 Rat brain sample (Dementia):

Using a sharp blade, a cross section was carefully cut in the brain. The paraffin sections of the brain tissue were then prepared for light microscopy. The fixative used is 10% neutral buffered formalin for 24 h. After fixation, the tissue was dehydrated by serial ascending series of ethyl alcohol 70%, 80%, 90% and 95% for 30 minutes each. Tissues were cleared in xylene for 20 minutes (two changes) then embedded in paraffin wax (three changes) at 60°C for three hours and embedded in paraffin wax. The paraffin blocks were sectioned at 3-5 μm with Reichert Jung 2030 microtome (West Germany) using disposable blades, mounted on glass slide to be ready for hematoxylin and eosin staining (H&E) and cresyl violet staining (Nissl Staining).

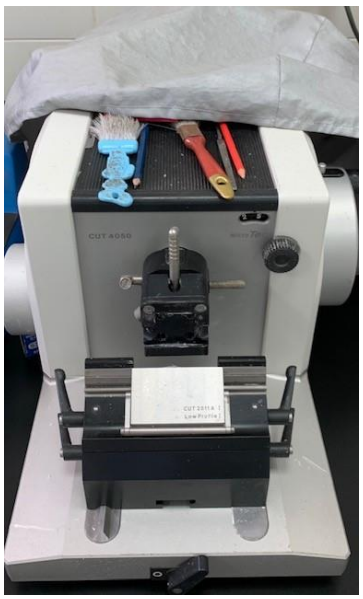


Figure 5: Reichert Jung 2030 microtome

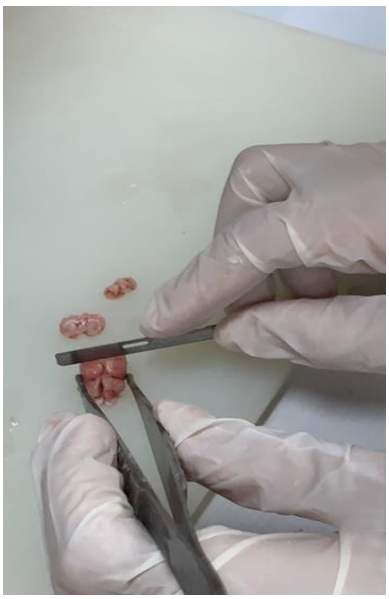
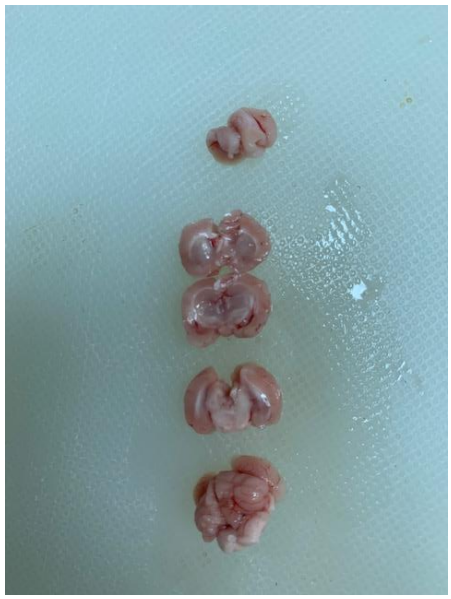


Figure 6: Cutting cross sections in the brain tissue



3.6.2 Mice femur sample (Osteoporosis):

After femur bone was collected from the mice, decalcification procedure was done to soften the bones by removing the calcium, and allowing to cut in the bones to prepare the mice femur for the histological examination. Formic acid 5% was used. Specimens were decalcified in this solution during one week following these procedures, bones were dissected and we removed as much soft tissue as possible. After appropriate fixation, tissue was washed in distilled water. Bone was placed in the 5% formic acid solution with shaking, formic acid solution was changed every two days. Decalcification was known to be complete when bone was soft and pliable. Specimen was rinsed with distilled water three times. Following decalcification, a longitudinal section was cut in the femur bone, and the paraffin section were prepared for light microscopy using the same protocol followed in the rats' brain. The tissue was fixed using 10% neutral buffered formalin for 24 hours. Following fixation, serial ascending series of ethyl alcohol 70%, 80%, 90% and 95% were done (30 mins each) to dehydrated the bone tissue. Xylene was applied for two changes (20 mins each) to clear the tissues and then embedded in paraffin wax at 60°C for three hours (three changes were done). Using the Reichert Jung 2030 microtome with disposable blades, the paraffin blocks were sectioned at 3-5 μm and then mounted on glass slide to be ready for staining using the hematoxylin and eosin staining protocol (H&E stain).

4. Testing

4.1 Dementia:

4.1.1 Behavioral Testing - Morris Water Maze (MWM):

The Morris Water Maze (MWM) was recognized and proven by neurologist Richard G. Morris in 1981 to be able to examine hippocampal-dependent learning which contains the attainment of spatial and long-term memory (Morris 1981). The principle of this test is that a rat is constantly trying to escape from a round closed pool by climbing on a platform based in a fixed place. By time, the rat can recognize the spatial location of the platform whatever his position in the pool is.

MWM Preparation:

The water maze is a round pool filled with a sufficient volume of water which is maintained at a temperature of approximately 26°C, the pool was divided equally into four quadrants to keep track of their starting point. An escape platform placed in the center of the maze was exposed 1 cm above the water level during the training phase, it teaches the rat that there is way to get out of the water. During the testing phase, the escape platform is hidden below the surface of the water and was made invisible using starch. Everything is constant; meaning that the lighting and water temperature of the pool are precisely documented to make sure that the training period and retention period have no other variables affecting the results, the environmental factors are constant for all three phases. In order to monitor the behavior of the rat and the path it undergoes; all trials were videotaped (Nunez 2008).

a. Maze Acquisition phase:

The maze acquisition phase took place on the 25th day of the supplementation. The rats were acclimated to the maze by being allowed to swim in it the day before testing begins. We performed four consecutive trials for each rat where four starting positions are indicated in the maze: north, east, south or west. Preceding the trial, the choice of order of the starting directions was set, the same start direction and starting points order will be used for all rats. Placing the animal gently in the water at one of those start points, the rat started swimming in the maze trying to reach the platform that is located at the middle of the pool. We recorded the time that the animal

took to reach the platform and if it fails to reach it in 90 seconds then the trial time is recorded as 90 seconds. The rat might swim around the pool edge trying to look for a way out but ultimately it will find the platform and climb it. If a rat fails to reach the platform, we did not take it out of the maze but rather guided it with our hand to the correct location. Rats that reach the platform are kept there for 15 seconds and if it tries to jump, we gently bring it back to be trained that the platform is available to rescue it from the pool. We then repeated for three more trials the same steps but starting at a different location. When the four trials are completed, animal was carefully dried with a towel and process was repeated for all forty rats consecutively, keeping the same group and rat order. Once animals are trained, we are ready to move to the testing phase, which takes place on the following day.

b. Maze Testing Phase – Short Memory Retention Latency:

This trial took place on the 26th day of the supplementation. The platform was hidden using corn starch which makes the water cloudy and by increasing the water level in the pool above the platform by 1 cm. Consequently, the rat will not be able to see the platform while swimming and will have to remember its location.

Each animal undertook two trials at two different starting points (North and south). Rats were placed in the water facing the pool's wall. Each rat was monitored until the platform is reached to be able to record the time it took to find the platform. If ninety seconds pass without the rat reaching the platform, it was gently guided to the platform as done in the training phase. Once it reaches the platform the rat was allowed to stay there for 15 seconds before conducting the second trial. After the two trials are done, animal was grabbed, dried off and put back to its cage. This process will be done for all rats.

Occasionally, the pool was cleaned and the water temperature was checked, also making sure that the platform is in its designated place making sure that there are no variables that may affect the behavior of rats. Each animal is dried gently with a towel after testing and placed back in its cage.

c. Maze Testing Phase – Long Memory Retention Latency:

This test was conducted on the 52nd day of the supplementation. The platform was hidden in the pool and each rat was allowed for one trial to find the platform, starting from a random starting position. If the rat fails to reach the platform in 90 seconds, it is gently guided to it. This test represents the long memory retention trial. Animals were dried gently with a towel after testing.

Parameters Measured in Acquisition and Testing Phases:

Retention Latency: The number of seconds it requires for the rat to escape to the platform is scored. If the animal fails to escape from the platform within 90 seconds it is given a score of 90.

Figure 7: Shot from the video recording during MWM acquisition phase

Figure 8: Rat locating the platform during the MWM testing phase

4.1.2 Blood Biomarker (ELISA TEST: Amyloid β-42):

ELISA The enzyme-linked immunosorbent assay is a laboratory method that is very known, common and prevailing for the detection of proteins by using the relevant antibodies (Xiao and Isaacs 2012). Enzyme-linked immunosorbent assay (ELISA) is a quantitative analytical test, where enzyme linked conjugate and enzyme substrate interact in an antigen-antibody reaction causing a color change. The intensity of the color change determines the presence and concentration of the target molecules to be detected. ELISA can detect molecules that are normally present in very low concentrations, such as hormones, vitamins, drugs and proteins, those molecules usually show high specificity towards the antigens/antibodies developed to react with. Since it is very uncommon that an antigen binds to a molecule that is not its own antibody, enzyme-linked immunosorbent essays are used to detect and measure substances that are present in very low concentrations. It is a two

ways relationship, having an antigen with known specificity to a certain substance, allows to identify the type and concentration of its antibody, and reversibly when the antibody is known, we can detect the presence and amount of the specific antigen. Enzyme immune-tests is the general term used to describe analytical methods using enzymes to detect antigen-antibodies reactions (Aydin 2015). The main worry of researchers is regarding any non-specific binding of reactants to wells in the microtiter plates, and as a solution, blocking agents like bovine serum albumin or non-fat dry milk are used. Those blocking agents also serve in stabilizing the molecules already fixed in the well and eliminating as much as possible any non-specific interaction (Xiao and Isaacs 2012).

The ELISA kit was purchased from Wuhan Fine Biotech Co., code ER0755. The kit is based on the sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates, and the biotin conjugated antibody was used as detection antibodies. Wash buffer was prepared by diluting 15 mL concentrated wash buffer in 375 mL distilled water. 1 mL sample dilution buffer was added to the lyophilized standard tube and labeled as zero tube. Serial dilution of the standard was done with 1/4 ratio by adding 0.3 mL of the zero tube to 0.3 mL sample dilution buffer in an Eppendorf tube, and then transferring 0.3 mL from the first Eppendorf tube to a second Eppendorf tube containing 0.3 mL sample dilution buffer. All test samples were diluted to the same ratio 1/4 following the same serial dilution step above. Sample dilution buffer was used for the blank.

Figure 9: ELISA kit and serum samples

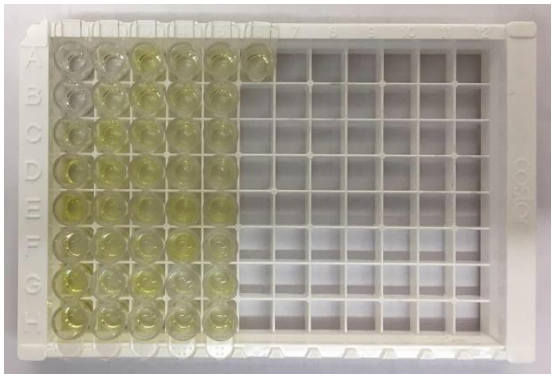


Figure 10: ELISA microplate after stop solution addition

Biotin-detection antibody was diluted with antibody dilution Buffer at 1:100 and mixed thoroughly. The HRP-Streptavidin conjugate (SABC) working solution was prepared by diluting

in SABC dilution buffer at 1:100 and mixed thoroughly. Plate was washed 2 times before adding standard, blank control and test samples. Aliquoted 100µl of the standard tube, sample dilution buffer (blank) and the samples into their respective wells. Their positions were recorded. Plate is sealed with cover and incubated at 37°C for 90 minutes. After that, cover is removed and plate content discarded, followed by washing the plate 2 times with wash buffer. 100µl Biotin-labeled antibody working solution were added into above wells (standard, test sample and blank wells) and plate was again covered and incubated at 37°C for 60 minutes. Another wash is applied by removing the cover and washing the plate 3 times with wash buffer by letting the wash buffer stay in the wells for 1-2 minutes each time. Next step was adding the HRP-Streptavidin Conjugate (SABC); 100µl of SABC working solution were aliquoted into each well, the plate was covered and incubated at 37°C for 30 minutes. Following the incubation, wash step applied for 5 times while the wash buffer allowed to stay in the wells for 1-2 minutes each time. Addition of 90µl TMB Substrate into each well, we then covered the plate and incubated at 37°C in dark for 20 minutes. Finally, reaction was stopped by adding 50µl stop solution into each well, following the same order of TMB substrate solution addition. The color turned yellow immediately. Optical density absorbance was read at 450 nm by using a microplate reader immediately after adding the stop solution.

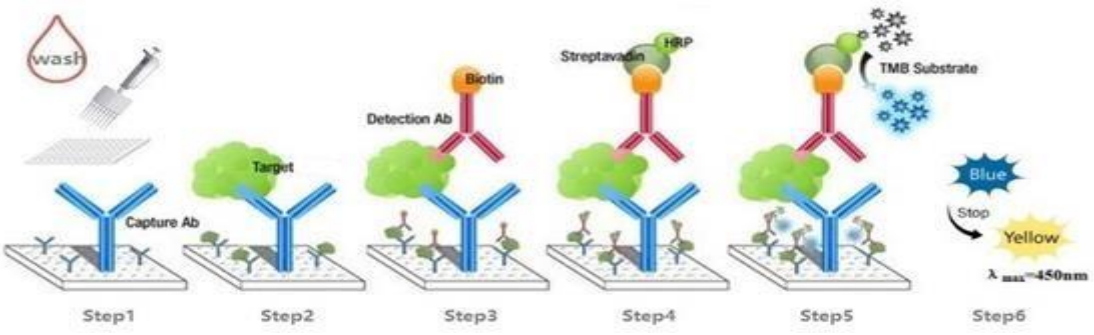


Figure 11: Sandwich enzyme-linked immune-sorbent assay procedure (ELISA)

4.1.3 Histopathological examination: Brain cross-section

In this study we applied two types of staining to examine the brain tissue of rats. The first one is the conventional hematoxylin and eosin stain, one of the main stains used in histology. The second stain is a more specialized type for the nervous tissue called Cresyl Violet Staining or mostly

known for Nissl Staining. We used the below protocols to stain the prepared brain cross section slides.

Hematoxylin and eosin staining protocol (H&E stain):

Sections were deparaffinized in xylene for 10 minutes. Hydration was performed in descending series of ethanol (100%-90%-80%-70%-60%-50%) followed by distilled water. Then sections were immersed into hematoxylin stain for 10 minutes, and then washed in tap water for 2 minutes, transferred to acid alcohol 1% (1 mL of conc. HCL (BDH, UK) in 100 mL of 70% ethyl alcohol) for acidification and removing the excess of hematoxylin stain. Slides were quickly washed under running tap water for 3 minutes for removing the excess of acid alcohol and establishing the hematoxylin stain, then sections were counter-stained in aqueous eosin stain for 3 minutes and followed by rinsing in distilled water. Dehydration was achieved using ascending series of ethyl alcohol, 5 minutes each. Clearing in xylene (2 changes, for 5 minutes each). Final step was mounting in DPX (distyrene, plasticizer and xylene) and slides were ready for the light microscope examination.

Cresyl Violet Staining (Nissl Staining):

Slides were washed briefly in tap water to remove any residual salts. They were immersed 2 times for 3 minutes in 100% ethanol. Defatting the issue by soaking 15 minutes in 100% xylene (2 changes), then 10 minutes in 100% ethanol. Rehydration done in 100% alcohol two times for 3 minutes each, followed by washing in tap water. Slides were stained in 0.1% Cresyl Violet for 15 minutes, quick rinse by tap water was applied to remove excess stain, followed by washing in 70% ethanol. Slides were dehydrated two times (3 minutes each) of absolute ethanol. Slides were also cleared in xylene then mounted in DPX, and finally allowed to dry in the fume hood. Slides are ready for histopathological examination by light microscopy.

4.2 Osteoporosis

In general, the techniques used in rodents for the assessment of bone mass, architecture and metabolism are more or less similar to those in humans. However, the need for more for deeper investigation in this field have been the main cause resulting in introducing new procedures and methods that are specifically catering for mice, that is why in a way these methods give more

accurate results in testing (Lelovas et al. 2008). There are two methods that will be further used in the testing the post consumption effect of micronutrients achieved after supplementation.

4.2.1 Biomarkers:

One easy and non-invasive method to diagnose osteoporosis is the measurement of serum bone turnover markers (BTMs), which include total osteocalcin hormone (OC), C and N-propeptide of type I collagen (PICP and PINP respectively), total calcium, ionized calcium and alkaline phosphatase ALP (Biver et al. 2012). Measuring calcium levels is an important step in osteoporotic risk assessment, and while total calcium (tCa) is the most common indicator, checking the ionized calcium (iCa) is more and more requested and considered as a gold standard (Guiducci et al. 2017). Different forms of calcium are present in blood, specifically 3 forms: ionized calcium is the most abundant form available and contributing to almost 50% of blood calcium, it is also referred to as free calcium. The second most present calcium form is the protein-bound calcium, mostly represented by albumin-bound calcium. The remaining calcium is bound to anions/minerals such as phosphate, citrate, lactate, bicarbonate and it represents around 10% of the total calcium. Ionized calcium is the most active form and is considered as the best indicator for calcium status. For instance, total concentration of calcium is affected by decreasing plasma proteins, but at the same time, this lowering is plasma proteins has weaker effects of the ionized calcium concentration. Therefore, measuring the ionized calcium is diagnostically more powerful than the total calcium and is considered as the golden standard (Guiducci et al. 2017). Increased blood calcium level is usually coming from leaching from bones and is an indication of bone resorption. Bone decalcification following menopause leads to increased serum calcium concentration. Therefore, measuring serum calcium (total and ionized) is a good diagnostic tool for osteoporosis (Biver et al. 2012). In post-menopausal osteoporosis, bone remodeling is happening, where there is more bone resorption than bone formation, and consequently calcium is leaching into the extracellular fluid (ECF), with an approximate rate of 20-40mg per day (or 8-16 g per year) (Houillier et al. 2005).

Alkaline phosphatase (ALP) is an enzyme present in the body but mostly found in bone and liver. It has phosphorylating properties and present in several isoenzyme forms, where the most abundant is tissue non-specific ALP. Two are isoforms are present under the tissue non-specific ALP, liver-specific ALP and bone-specific ALP (BALP), and both are available in serum

at equal levels (Biver et al. 2012). BALP is located in adherence to the osteoblastic cell membrane, with very low amounts released in serum. An increased bone remodeling, like osteoporosis, leads to increase BALP levels in serum (Biver et al. 2012). Consequently, bone mineral density loss in osteoporotic people is accompanied by increase ALP activity and higher serum calcium levels (Biver et al.).

Calcium (total and ionized) and alkaline phosphatase ALP were measured using automated devices at the Medical Experimental Research Center at Mansoura University (MERC).

- Total calcium (tCa) and alkaline phosphatase ALP were detected using Mindray semi-auto chemistry analyzer BA-88A.
- Ionized calcium (iCa) was detected using Biotecnica fully automated chemistry analyzer BT3500.

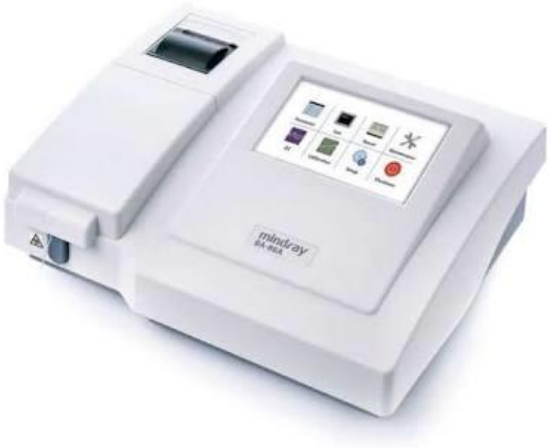


Figure 12: Mindray BA-88A used for tCa and ALP measurement

Figure 13: Biotecnica Bt3500 used for iCa measurement

4.2.2 Histopathological examination of the femur bone:

The second method is the invasive method using histomorphometry which offers a two-dimensional study with a very high resolution, portraying study of bone mass and architecture, it precisely assesses bone architecture and directories of bone fragility in an independent way from bone mass. The parameters measured include the number of osteocytes, osteoclasts, osteoblasts and active osteoblasts compared to bone perimeter, trabecular thickness number, and separation (Carbonare et al. 2005). The use of these methods in crucial in testing the validity of results since the models measuring BMD and histomorphometry can provide full insight on bone strength

(Dempster 2003). The femur bone was mounted longitudinally on glass slides and stained using the hematoxylin and eosin (H&E) staining protocol where sections were deparaffinized in xylene for 10 minutes. Hydration was performed in descending series of ethanol (100%-90%-80%-70%-60%-50%) followed by distilled water. Sections were immersed into hematoxylin stain for 10 minutes, and then washed in tap water for 2 minutes, transferred to acid alcohol 1% (1 mL of conc. HCL (BDH, UK) in 100 mL of 70% ethyl alcohol) for acidification and removing the excess of hematoxylin stain. Slides were quickly washed under running tap water for 3 minutes for removing the excess of acid alcohol and establishing the hematoxylin stain. Sections were counter-stained in aqueous eosin stain for 3 minutes and then rinsed in distilled water. Dehydration was achieved using ascending series of ethyl alcohol, 5 minutes each, followed by clearing in xylene (2 changes, for 5 minutes each), and finally mounting in DPX (distyrene, plasticizer and xylene). Slides were ready for histopathological examination.

Measurements of the thickness of the bone trabeculae were performed using BX45 Olympus optical microscope. AmScope MU1000 (USA) image processor with a calibration kit and Toupview software (version 3.7, 2013). Measurements were taken from different parts of the slide (at least 3) and expressed in micrometers.

5. Results

5.1 Dementia Arm:

5.1.1 Morris Water Maze Results:

Maze Acquisition (seconds) – Day 25				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (No $AlCl_3$)**	40.46	12.93	4.89
Group 2	**Positive Control ($AlCl_3$)**	46.39	18.68	7.06
Group 3	**Premix 1 + $AlCl_3$**	32.82	6.34	2.40
Group 4	**Premix 2 + $AlCl_3$**	32.43	2.82	1.07
Group 5	**Premix 3 + $AlCl_3$**	35.43	11.6	4.38

Table 3: Maze Acquisition Results

As shown in table 1, maze acquisition phase was performed on day 25 for all rats' groups, it was performed four times; each time from a different start point; north, south, east, west. Each group of rats reacted differently to the maze acquisition phase (the training phase), and an average per seconds was calculated for each group; the time each rat took to find the platform was calculated from each point and an average for the whole group was obtained. As noted before each rat had a time frame of 90 seconds to reach the platform, any rat failing to reach the platform within that range was gently pulled to the platform and remained there for 15 seconds before moving to the next start point. Results have shown that Group 4 (premix 2 + $AlCl_3$) had the best performance with an average of 32.43 seconds. Group 1; the negative control group not taking any premixes not even $AlCl_3$ with average of 40.46 seconds. Group 2; the positive control group, the group consuming $AlCl_3$ and not consuming any of the premixes, had the worst performance with an average of 46.39 seconds. Group 3; consuming premix 1 + $AlCl_3$ had an average of 32.82 seconds which was too close to group 4 with less than half a second behind. At last, group 5 consuming premix 3 + $AlCl_3$ had an average of 35.43 seconds.

Maze Test Short Term Retention Latency (seconds) – Day 26				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (No $AlCl_3$)**	21.93	9.02	3.41
Group 2	**Positive Control ($AlCl_3$)**	38.57	27.87	10.53
Group 3	**Premix 1 + $AlCl_3$**	10.14	3.70	1.40
Group 4	**Premix 2 + $AlCl_3$**	8.86	4.23	1.60
Group 5	**Premix 3 + $AlCl_3$**	23.79	20.00	7.56

Table 4: Short Memory Retention Results

On day 26th, second day after acquisition phase, two trials where done in this phase, the short-term retention phase. An average per seconds was calculated from two different random start points; trial 1 and trial 2, these two trials were performed for each rat from each group and an average per second was noted with a final average for the whole group. All groups showed an improved performance in comparison to the acquisition phase, however same process was done in retention phase a time frame of 90 seconds was set, failing to attain the platform, the rat was gently pulled towards it. Group 1: the negative control had an average of 21.93, while group 2: the positive control group had the worst performance again with an average of 38.57 seconds. Group 3 consuming premix 1 + $AlCl_3$ with an average of 10.14 seconds. However, group 4: consuming premix 2+ $AlCl_3$ had had the best performance in the retention phase with an average of 8.86 seconds and finally group 5 consuming premix 3 + $AlCl_3$ had an average of 23.79 seconds. The best performance was obtained by group 3 consuming premix 1 + $AlCl_3$.

Maze Test Long Term Retention Latency (seconds) – Day 52				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (No $AlCl_3$)**	19.17	4.31	1.63
Group 2	**Positive Control ($AlCl_3$)**	17.67	15.13	5.72
Group 3	**Premix 1 + $AlCl_3$**	10.50	3.39	1.28
Group 4	**Premix 2 + $AlCl_3$**	9.00	6.90	2.61
Group 5	**Premix 3 + $AlCl_3$**	16.00	20.56	7.77

Table 5: Long Memory Retention Results

All results in both dementia and osteoporosis testing were interpreted by statistical analysis, values are expressed as mean ± standard error of the mean; means of various groups were statistically compared by one-way ANOVA running on Sigma-Plot (version 14.0). As shown in table 3, that the number of rats has decreased by two rats one in group 4 consuming premix 2 + $AlCl_3$ and one in group 5 consuming premix 3 + $AlCl_3$. The rat from group 4 has a severe eye injury and was excluded from the long-term retention phase trial and the other rat was deceased. All other rats from different groups were nourished and consumed their specific dose of premix according to plan. The long-term retention phase was conducted on day 52 it included only one trial from a random starting point. Results have shown that again group 4 (premix 2 + $AlCl_3$) had the best performance with an average of 9 seconds, group 1; the negative control group had an average of 19.17 seconds, group 2; the positive control group had an average of 17.67 seconds, group 3 (premix 1 + $AlCl_3$) had an average of 10.50 seconds, and group 5 (premix 3 + $AlCl_3$) had an average of 16 seconds.

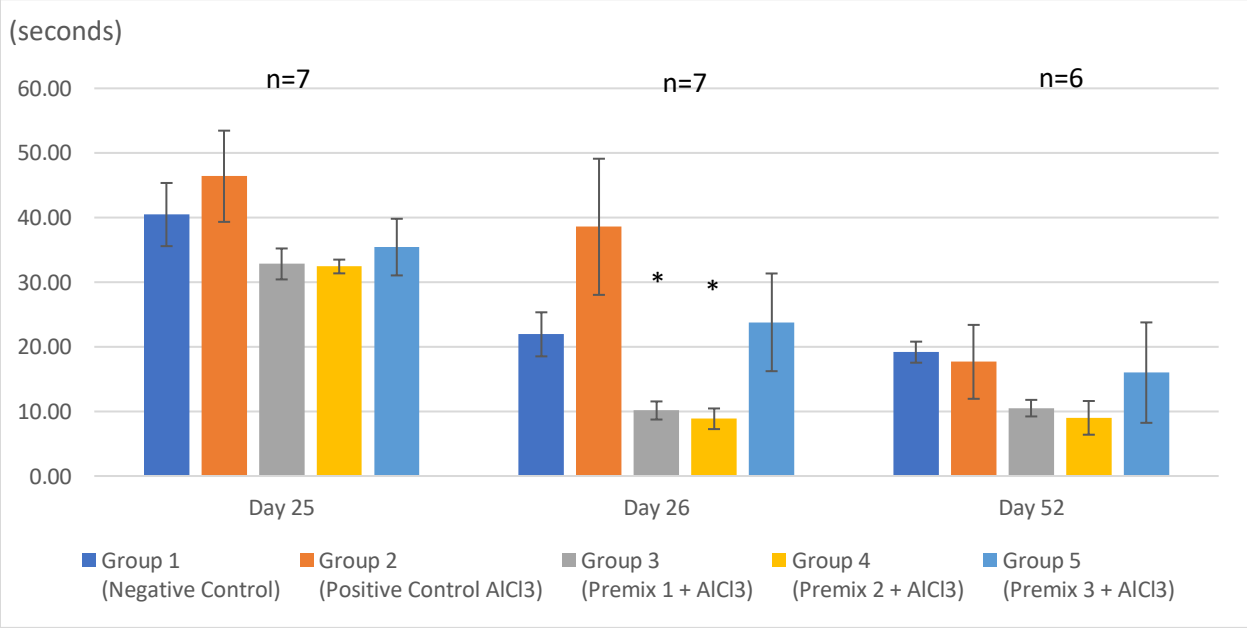


Figure 14: Comparison between the 5 groups within one day of MWM

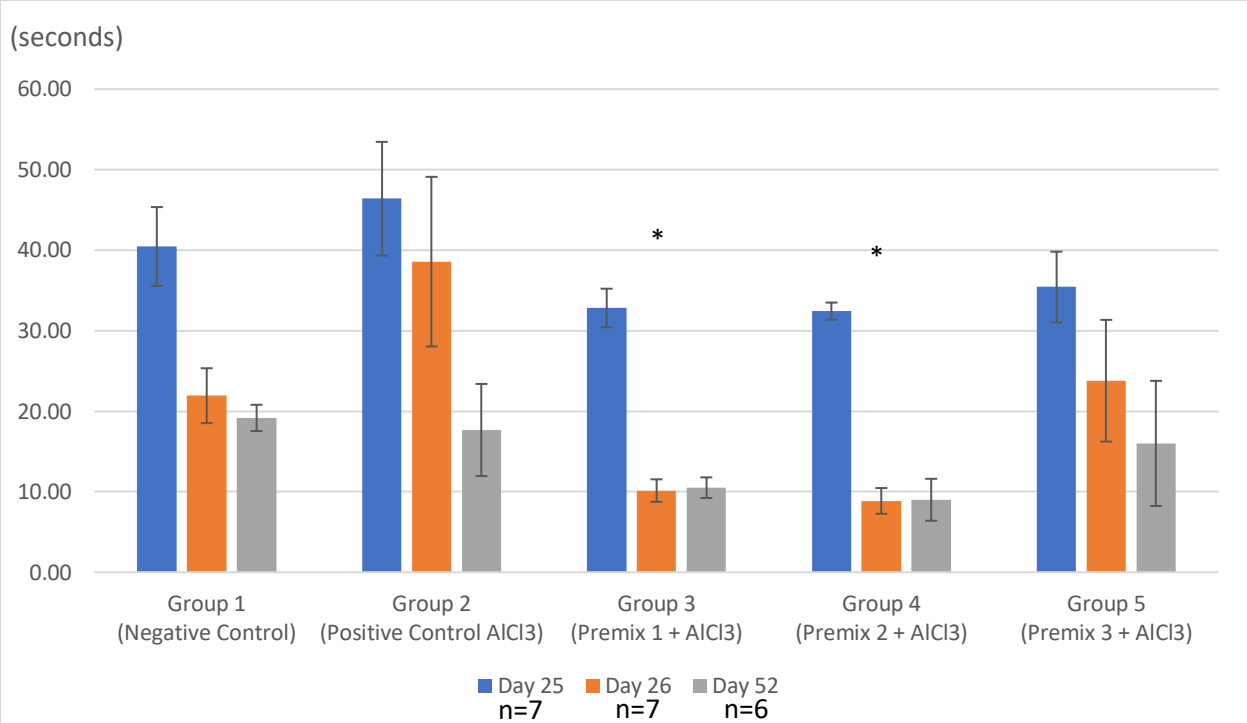


Figure 15: Evaluating the effect of premix 1 ($p < 0.05$), premix 2 ($p < 0.05$) and their combination (premix 3) on memory retention in rats through the Morris water maze test.

Positive control group supplemented with $AlCl_3$ showed learning and memory deficit in the MWM compared to negative control group ($p = 0.146$) which is not reaching target level of significance $p < 0.05$, but taking into consideration the behavioral testing environment for the rats, the deviation from target significance should be considered minimal. Premix 1 ($p < 0.05$) and premix 2 ($p < 0.05$) offered treated groups significant protection against memory and learning deficit induced by $AlCl_3$. On the other hand, group 5 consuming premix 3, a combination of both premix 1 and premix 2, did not show significant statistical difference between the means compared to positive control, the results of this group are promising as they are relatively close to the negative control group not consuming $AlCl_3$.

5.1.2 ELISA Test (Amyloid β-42) Results:

ELISA Results Amyloid B-42 (Optical Density)				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (No $AlCl_3$)**	27.15	3.69	1.51

Group 2	**Positive Control ($AlCl_3$)**	111.09	11.18	4.56
Group 3	**Premix 1 + $AlCl_3$**	38.68	3.92	1.60
Group 4	**Premix 2 + $AlCl_3$**	23.64	1.94	0.79
Group 5	**Premix 3 + $AlCl_3$**	29.43	7.03	2.87

Table 6: Amyloid β-42 ELISA results

Elisa test results have shown that group 1 (negative control group) has a group average of 27.15 optical density O.D, while group 2 (positive control group; induced $AlCl_3$) has a group average of 111.09of O.D. Group 3, 4 and 5 had more optimistic findings; group 3 (consuming premix 1 + $AlCl_3$) has an average group of 38.68 O.D and group 4 (consuming premix 2 + $AlCl_3$) had a group average of 23.64 O.D. Finally group 5 (consuming the master mix premix 3 + $AlCl_3$) had a group average of 29.43 O.D. Group 4 again has the best results in all 5 groups even better than the negative control group.

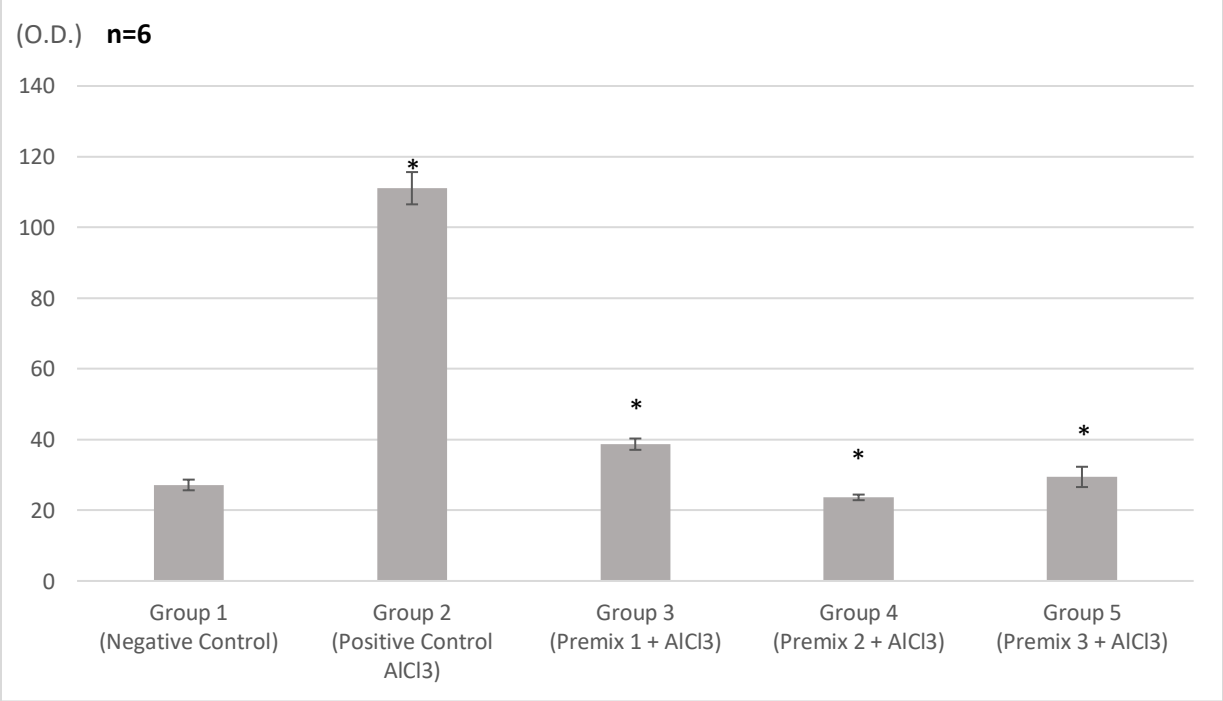


Figure 16: ELISA Amyloid β-42 results comparison between groups. Evaluating the effect of Premix 1 (p <0.001), Premix 2 (p <0.001) and their combination Premix 3 (p <0.001) on AD through the ELISA test detecting Amyloid β-42 in blood..

Positive control group treated only with $AlCl_3$ only showed significant presence of Amyloid β-42 compared to negative control group 1 (p <0.001). On the other hand, group 3, group 4 and group

5, supplemented consecutively with premix 1, premix 2 and premix 3 all showed significant protection against Aluminum Chloride effect to induce AD ($p < 0.001$) against positive control. Premix 2 was found to be more potent than premix 1 and premix 3.

5.1.3 Histopathology Examination of the Brain

5.1.3.1 Hematoxylin and Eosin stain (H&E)

When it comes to medical diagnosis the H&E is known as the most frequently used stain and is referred to as the gold standard. Results have shown that group 1 (Negative Control) demonstrated normal morphological features of different hippocampal layers with intact pyramidal neurons showing large vesicular nuclei with prominent nucleoli. Intact intercellular tissue was shown without abnormal alterations. Group 2 (Positive Control $AlCl_3$) showed many degenerated, shrunken neurons with dark pyknotic nuclei, perineuronal edemas as well as mild intercellular tissue edema of were observed in different layers. Groups 3, 4 and 5 (the 3 groups consuming micronutrients + $AlCl_3$): showed variable mixed records of apparent intact or degenerated neurons. However; more obvious perineuronal edema in pyramidal cells layer were observed, protection was observed among the three group with no evident difference between them.

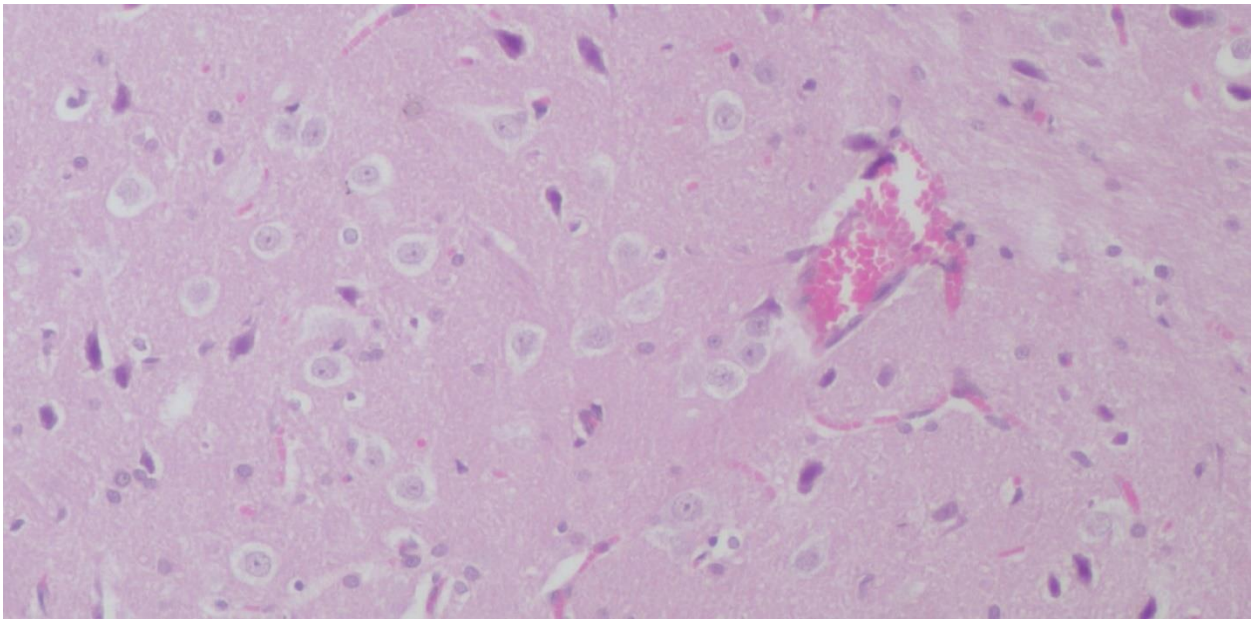


Figure 17: Group 1 (negative control) - Brain Cross Section using H&E stain demonstrating normal morphological features.

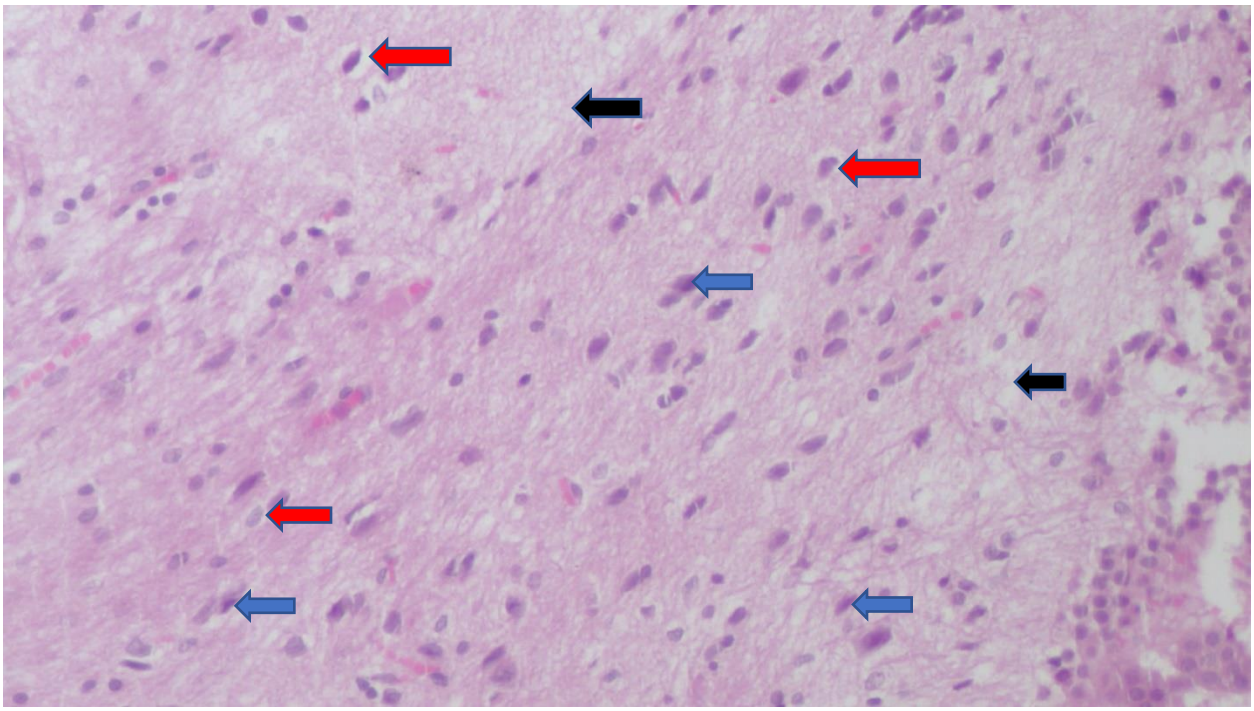


Figure 18: Group 2 (positive control $AlCl_3$ AD induced without premix) - Brain Cross Section using H&E stain. Showing many degenerated, shrunken neurons with dark pyknotic nuclei (blue arrows), perineuronal edemas (red arrows) and mild intercellular tissue edema in different layers (black arrows).

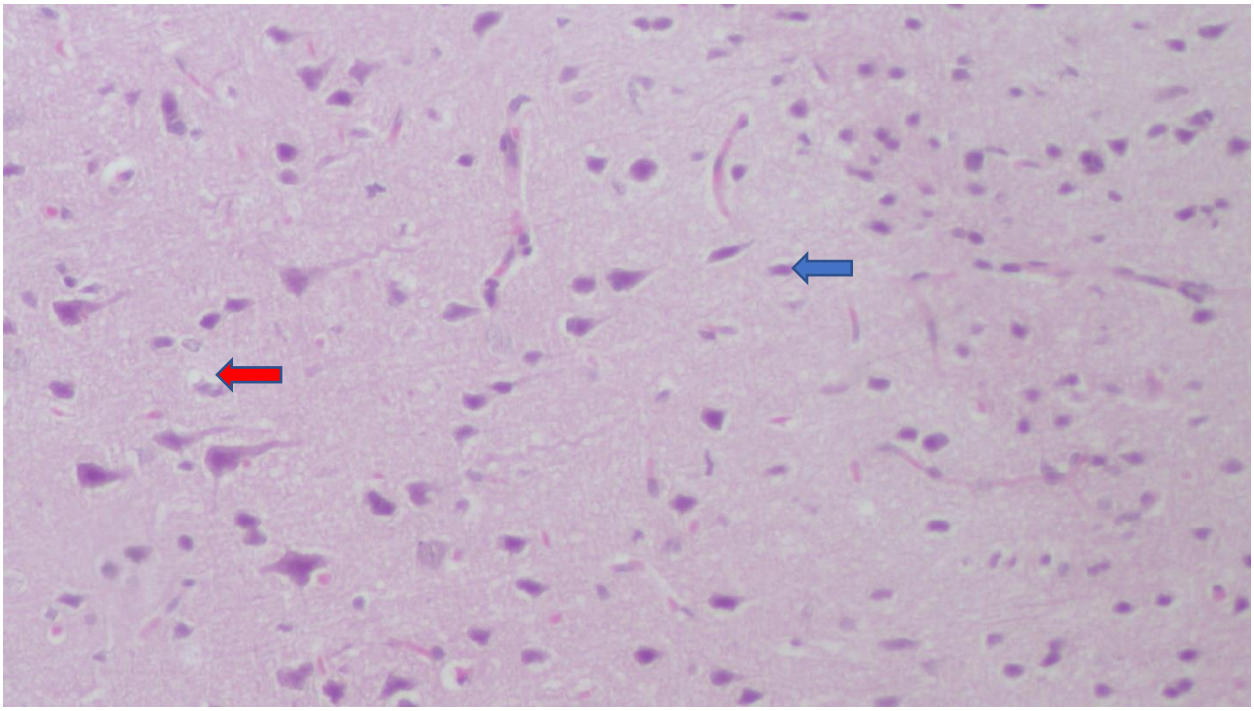


Figure 19: Group 3 ($AlCl_3$ AD induced taking premix-1) - Brain Cross Section using H&E stain showing protection against $AlCl_3$ effect with limited degenerated neurons.

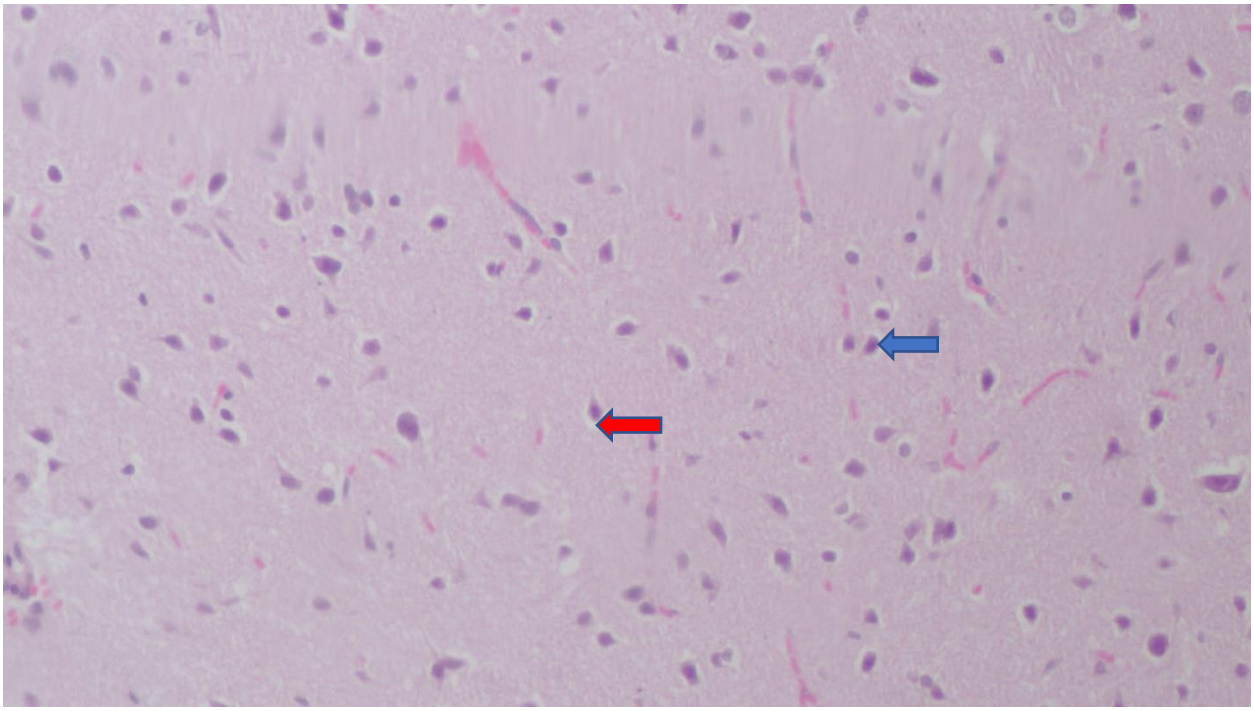


Figure 20: Group 4 ($AlCl_3$ AD induced taking premix-2) - Brain Cross Section using H&E stain showing protection against AlCl3 effect with limited degenerated neurons.

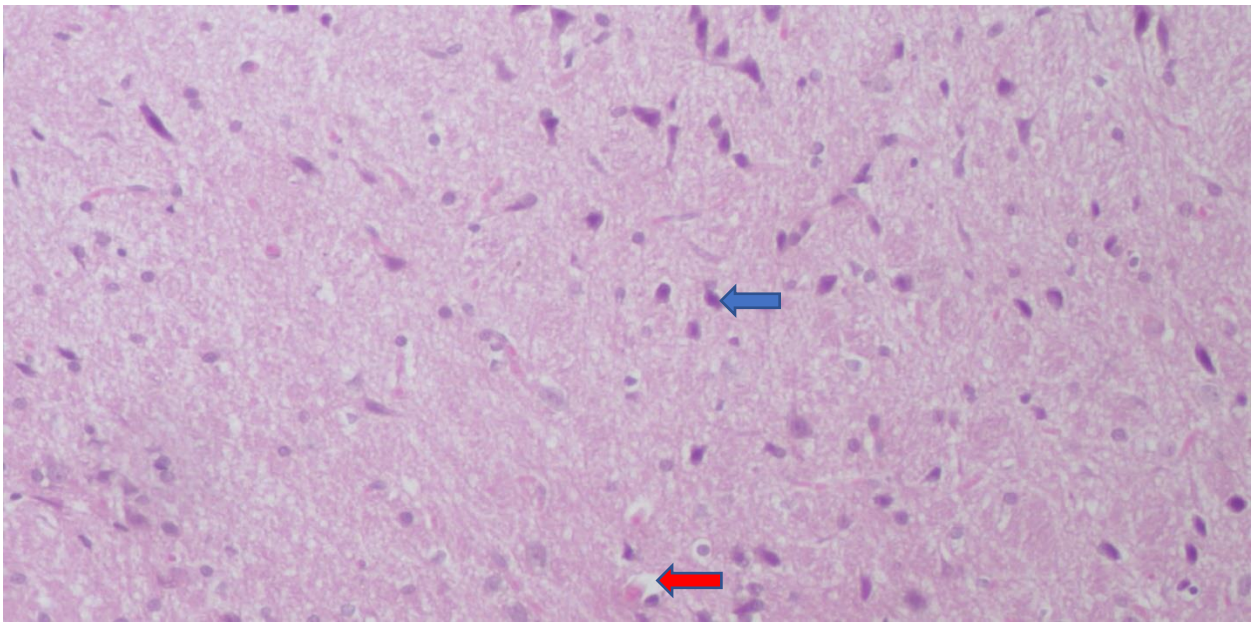


Figure 21: Group 5 ($AlCl_3$ AD induced taking premix-3) - Brain Cross Section using H&E stain showing protection against AlCl3 effect with limited degenerated neurons.

5.1.3.2 Nissl stain (Cresyl Violet Staining)

Nissl is a type of stain that is conventionally used when it comes to nervous tissues, results have shown that group 1 (Negative Control) Nissl staining of the CA1 zone showing intact neuron with normal density of neuronal sheets, Group 2 (Positive Control + $AlCl_3$) showed damaged neurons as well as decrease in density of neuronal sheets in the relevant zone of hippocampus. Groups 3, 4 and 5 showed number of intact neurons, with more preserved density of neuronal sheets compared to positive controls, however, no differences were shown between the three groups.

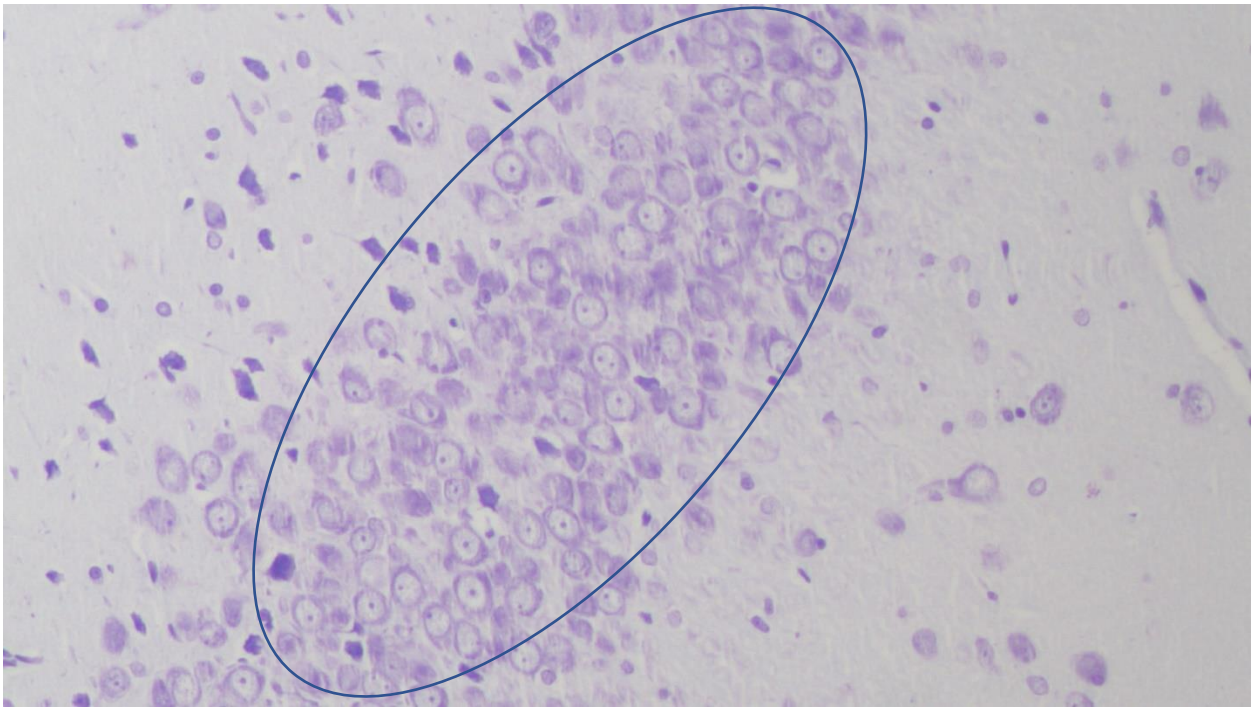


Figure 22: Group 1 (negative control) - Brain Cross Section using NISSL stain showing normal density of neurons sheets in the hippocampus zone.

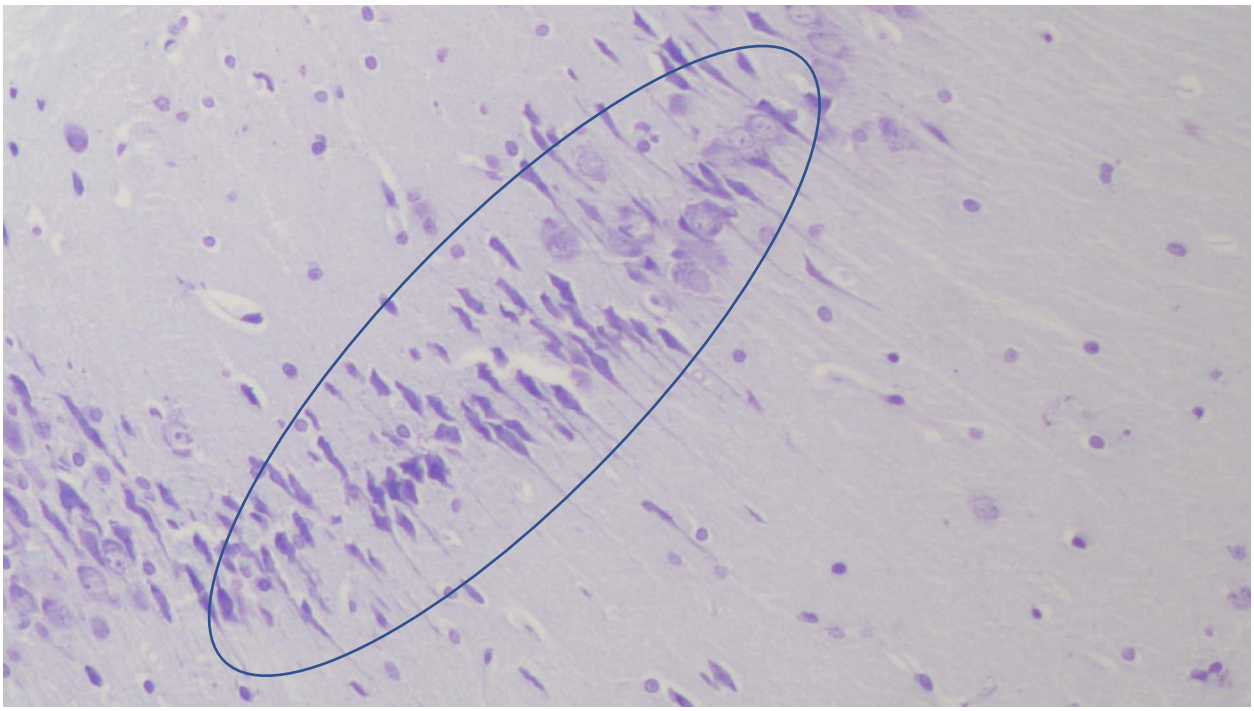


Figure 23: Group 2 (positive control $AlCl_3$ AD induced without premix) - Brain Cross Section using NISSL stain showing decreased density in neuronal sheets in the hippocampus.

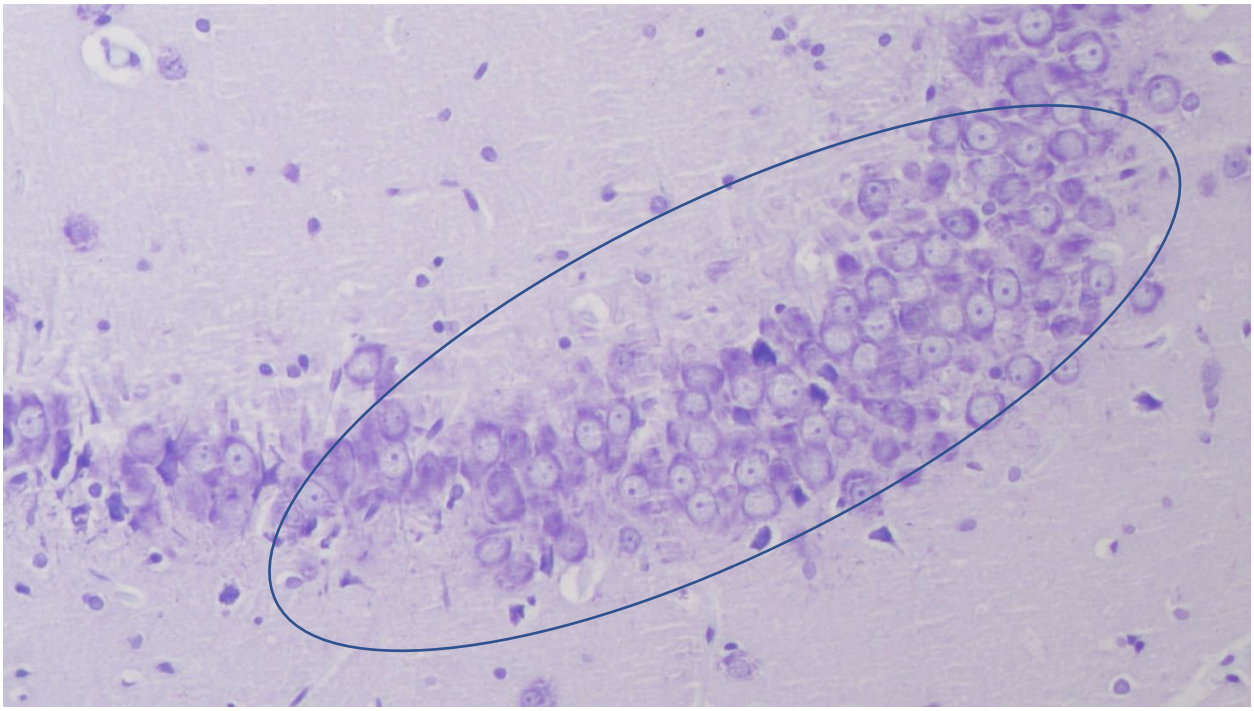


Figure24: Group 3 ($AlCl_3$ AD induced taking premix-1) - Brain Cross Section using NISSL stain showing preserved density of neuronal sheets.

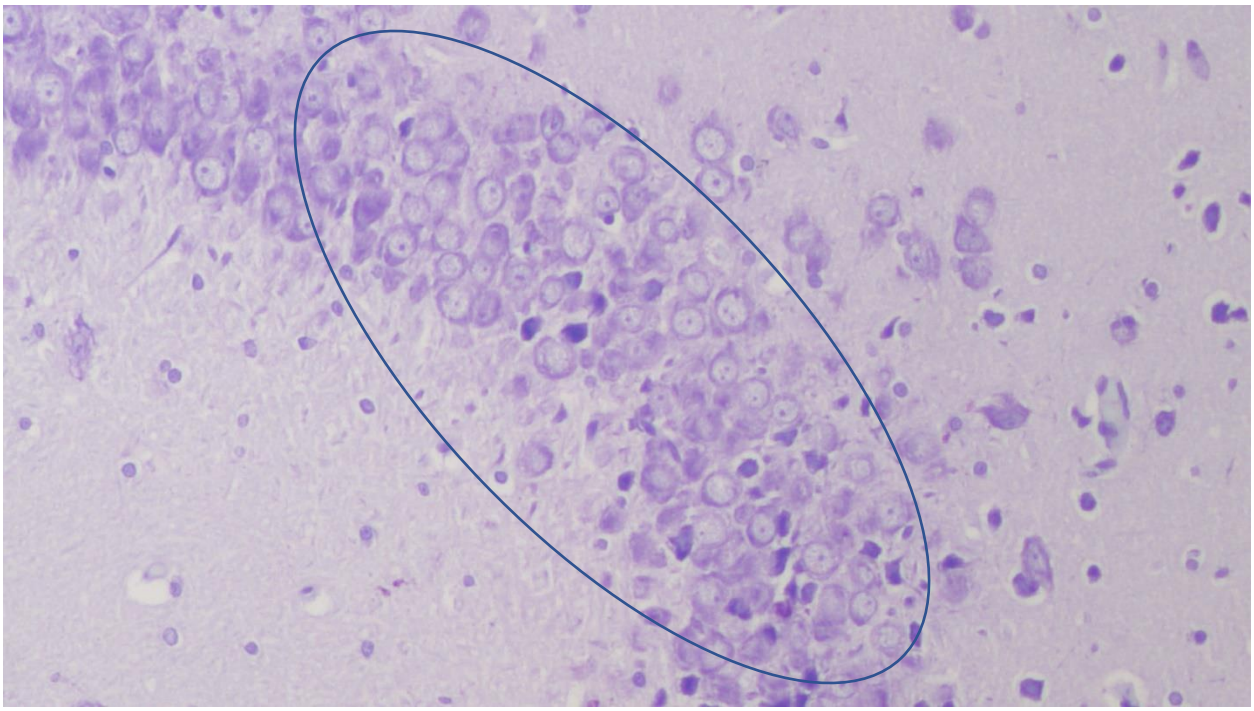


Figure 25: Group 4 ($AlCl_3$ AD induced taking premix-2) - Brain Cross Section using NISSL stain showing preserved density of neuronal sheets.

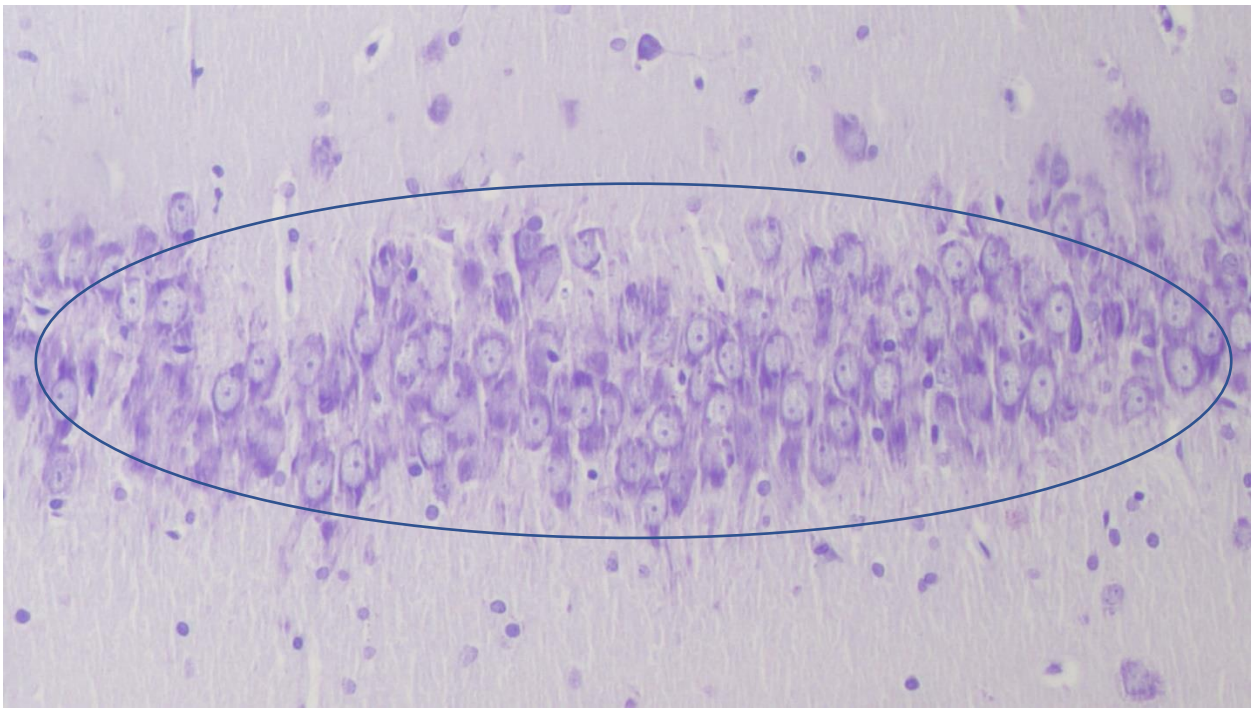


Figure 26: Group 5 ($AlCl_3$ AD induced taking premix-3) - Brain Cross Section using NISSL stain showing preserved density of neuronal sheets.

5.2 Osteoporosis Arm

5.2.1 Blood Biomarkers:

5.2.1.1 Blood Calcium (Total and ionized):

Total Blood Calcium - (mg/dl)				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (SHAM)**	7	3.14	1.41
Group 2	**Positive Control (OVX)**	6.9	0.34	0.15
Group 3	**OVX + Premix 4**	6.92	0.46	0.20
Group 4	**OVX + Premix 5**	6.8	0.37	0.16
Blood Calcium Ionized - mmol/l				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (SHAM)**	0.932	0.08	0.03
Group 2	**Positive Control (OVX)**	0.966	0.07	0.03
Group 3	**OVX + Premix 4**	0.946	0.06	0.02
Group 4	**OVX + Premix 5**	0.938	0.07	0.03

Table 7: Blood Calcium (Total/Ionized) Results

For the osteoporosis biomarkers result of calcium detection (total & ionized), results have been controversial. Results have shown that for group 1, the negative control group (who was not ovariectomized but only sham operated, total calcium average of 7 mg/dl and a total Calcium ionized of 0.932 mmol/l. Group 2 the positive control group which was ovariectomized without taking any supplementations has a total calcium average of 6.9 mg/dl and a total Calcium ionized of 0.966 mmol/l. Group 3 and group 4 these two groups were ovariectomized and respectively consuming authorized and content premix. Group 3 had total calcium average of 6.92 mg/dl and a total Calcium ionized of 0.946 mmol/l while group 4 had total calcium average of 6.8 mg/dl and a total Calcium ionized of 0.938 mmol/l.

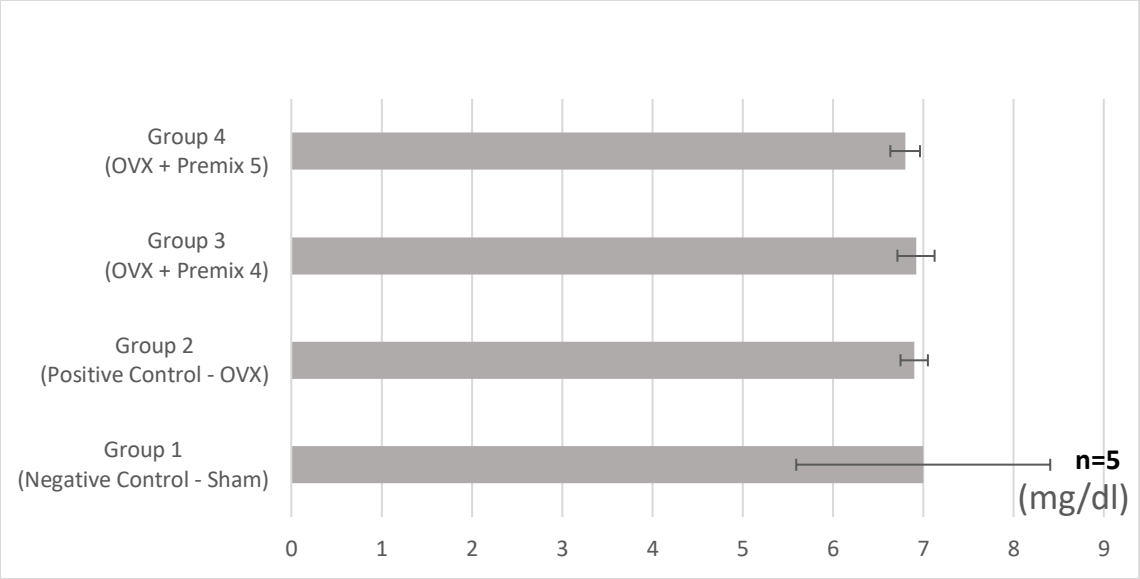


Figure 27: Total Calcium results

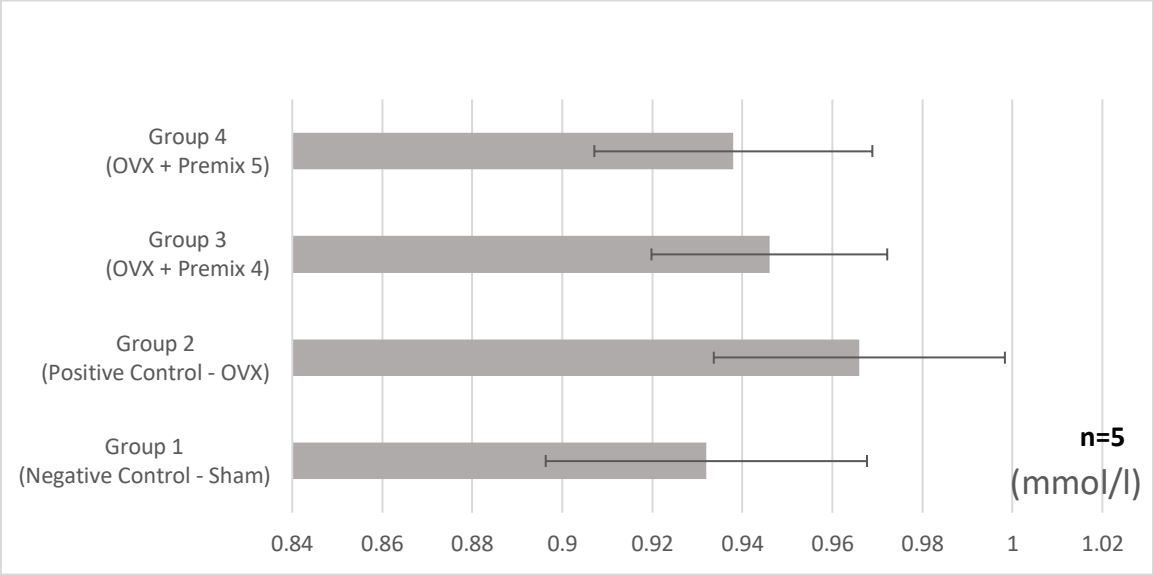


Figure 28: Ionized Calcium results

By running one-way ANOVA on Sigma-Plot (version 14.0), the differences in the mean values among the treatment groups and compared to the control groups were not significant enough to show a visible relation between the control groups and groups undergoing micronutrients supplementation. Total blood calcium and ionized blood calcium results were insignificant to conclude any interpretation of this study arm.

5.2.1.2 Alkaline Phosphatase (ALP):

Alkaline Phosphatase Results (U/l)				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (SHAM)**	159.77	49.47	22.12
Group 2	**Positive Control (OVX)**	174.50	51.41	22.99
Group 3	**OVX + Premix 4**	172.87	50.65	22.65
Group 4	**OVX + Premix 5**	118.13	29.85	13.35

Table 8: Alkaline Phosphatase (ALP) Results

Results of blood biomarkers of osteoporosis concerning alkaline phosphate (ALP), findings have shown that group 1 the negative control group is showing an average group total of 159.77 u/l. Group 2 the positive control group is showing a total group average of 174.50u/l which is the highest finding in the four groups and confirms the presence of osteoporosis. Group 3 and group 4 have lower findings; group 3 with a total average ALP of approximately 172.13 u/l and group 4 of an average group total of ALP of 118.13 u/l.

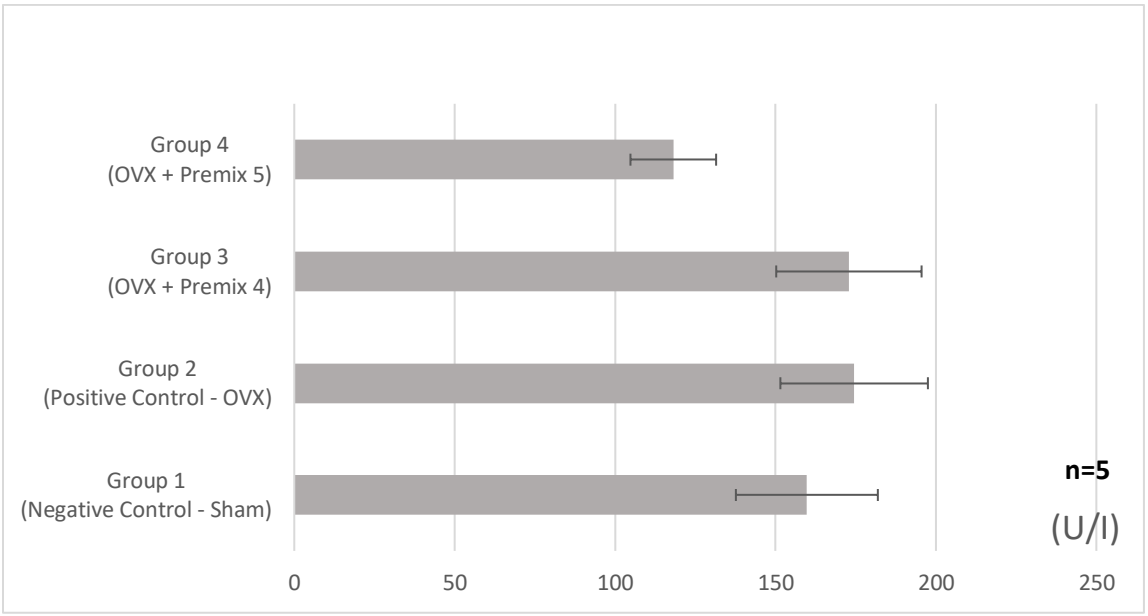


Figure 29: Alkaline Phosphatase results

After analyzing the results using one-way ANOVA on Sigma-Plot (version 14.0), no statistical significance were measured, however, by relying on the group average results, group 4 showed a measurable trend compared to the positive control group 2. Alkaline tests results did not show indicative significance to have a clear interpretation.

5.2.2 Histopathology Examination of femur Bone:

Mice Femur Trabeculae Thickness (µm)				
		Group Average	**Standard Deviation**	**SEM**
Group 1	**Negative Control (SHAM)**	277.134	58.73	26.26
Group 2	**Positive Control (OVX)**	120.26	7.74	3.46
Group 3	**OVX + Premix 4**	251.62	42.92	19.20
Group 4	**OVX + Premix 5**	152.28	51.62	23.08

Table 9: Femur Trabeculae Thickness Results

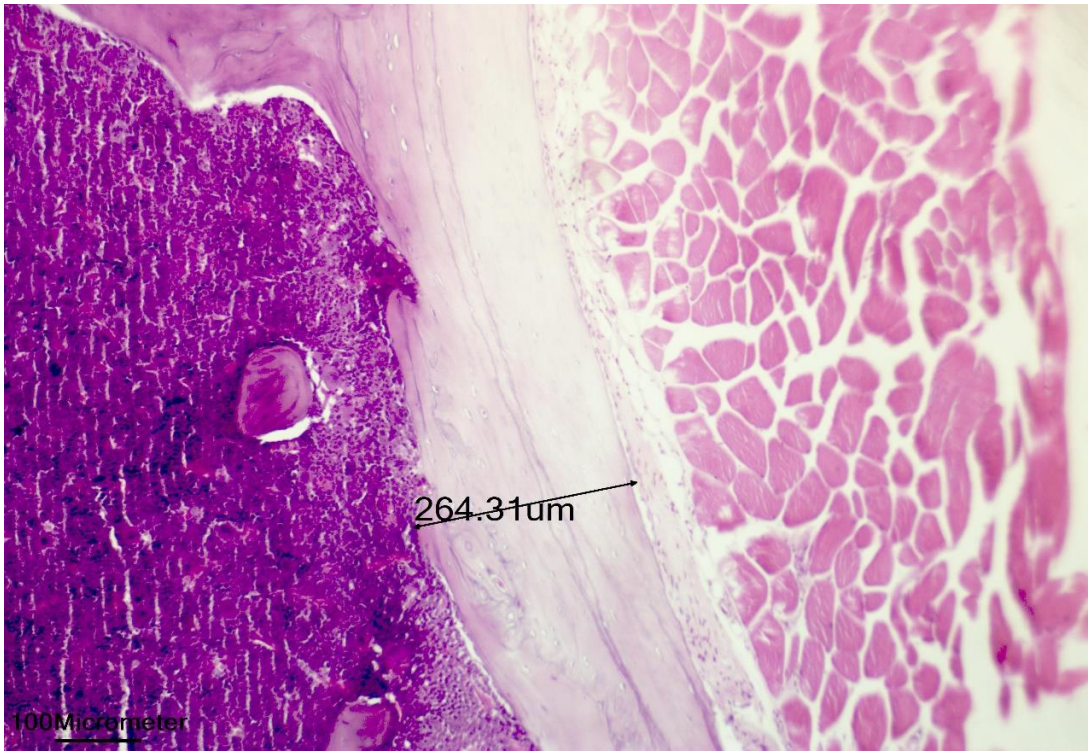


Figure 30: Group 1 (negative control – sham operation)

Femur Longitudinal Section using H&E stain showing the trabeculae thickness

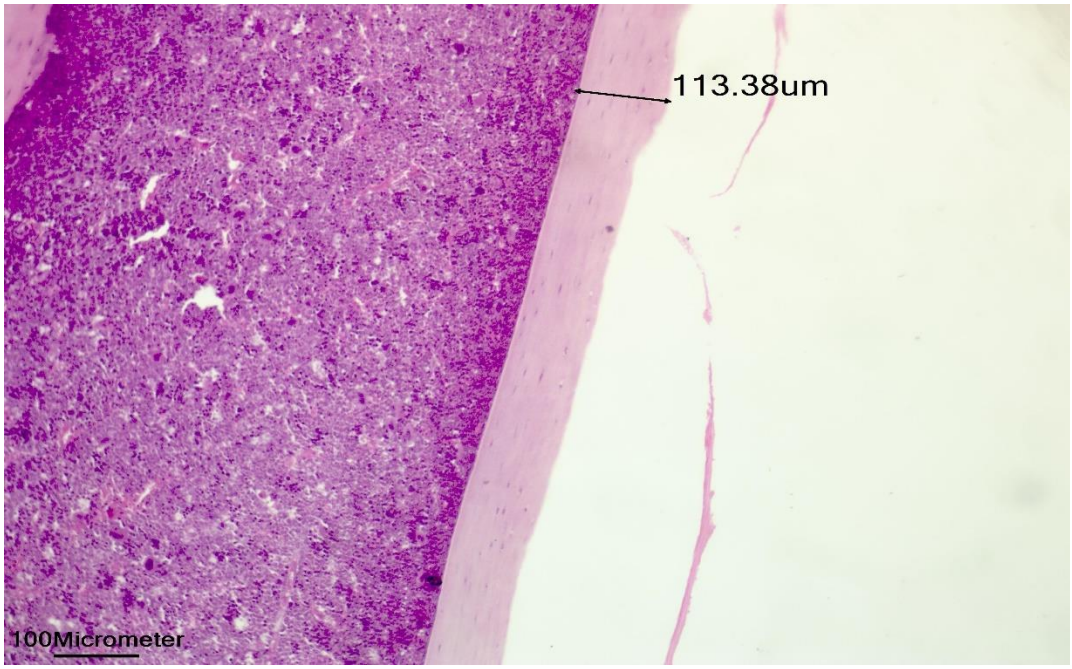


Figure 31: Group 2 (positive control – ovariectomized mice)

Femur Longitudinal Section using H&E stain showing the trabeculae thickness

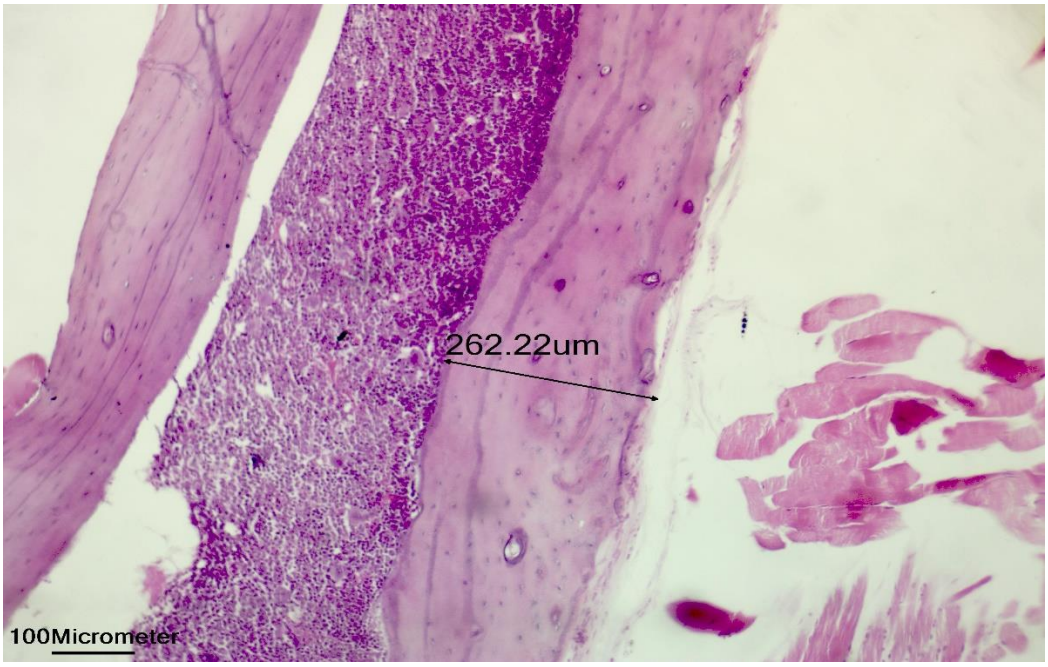


Figure 32: Group 3 (ovariectomized mice taking premix 4)

Femur Longitudinal Section using H&E stain showing the trabeculae thickness

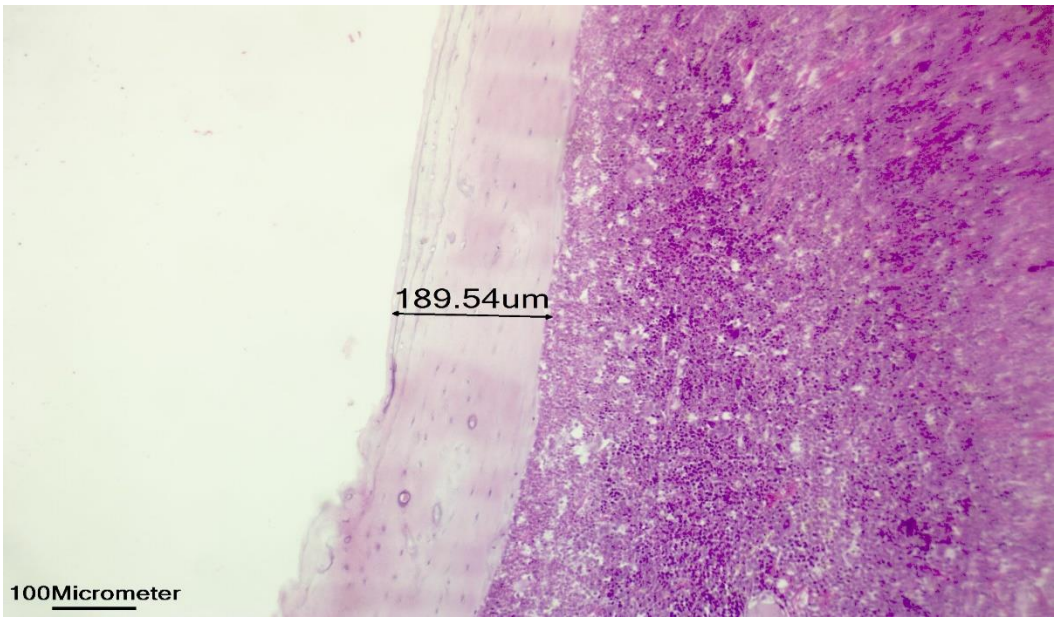


Figure 33: Group 4 (ovariectomized mice taking premix 5)

Femur Longitudinal Section using H&E stain showing the trabeculae thickness

In the results of the histopathological examination of the longitudinal section of mice femur bone stained by hematoxylin and eosin, we measured the thickness of the trabecular bone. The thickness of bone trabeculae is one of the main features of osteoporosis found at the microscopic level of pathological examination. The normal thickness was calculated in negative control group then compared with positive control groups and the treated groups. The thickness of the bone trabeculae in the treated groups is used to measure the effect of the drugs on the rates of osteoporosis subjected to the ovariectomy.

Group 1 (negative control) showed the highest group average with a 277.134 µm thickness, while group 2 (positive control) had the lowest average with 120.26 µm trabecular bone thickness. Group 3 and group 4 showed an average thickness of 251.62 µm and 152.28 µm consecutively, lying on between the measurements of the positive and negative control.

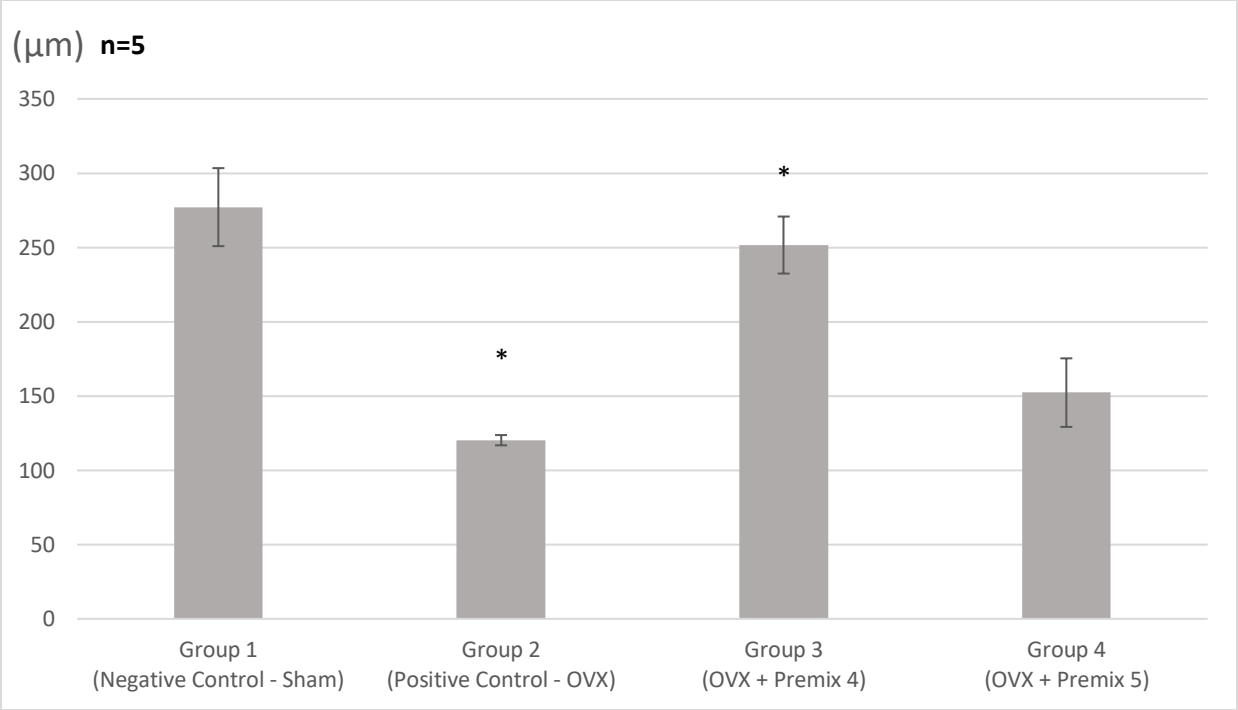


Figure 34: Trabecular bone thickness. Evaluating the effect of Premix 4 (p <0.001) and Premix 5 vs. control groups on osteoporosis by measuring the thickness of the femur trabecular bone.

Positive control group of ovariectomized mice showed significant difference of femur thickness compared to negative control group 1 of mice with SHAM operation ($p < 0.001$). On the other hand, group 3 and group 4, supplemented consecutively with premix 4 and premix 5 gave protection against osteoporosis when compared to the positive control, where group 3 ($p < 0.001$) showed strong differentiation while group 4 didn't reach statistical significance but relying on the group average results it definitely showed moderate premix 5 potency.

6. Discussion

Dementia has aggressive symptoms, affecting memory and impairing the ability of conduction simple problem solving, as results have shown that group 1 the negative control who is not consuming $AlCl_3$ thus not experiencing Alzheimer disease symptoms, was improving over time and training like any normal animal acquiring a new skill and remembering by time the place of the platform with an improvement in rapidity. On the other hand, group 2 the positive control consuming $AlCl_3$ thus experiencing dementia was slightly improving through time and training but still few rats were reaching the 90 seconds average time and were gently pulled to the platform since they were experiencing Alzheimer disease symptoms and could hardly find the platform.

The three other groups consuming premix 1, 2 and 3 were showing better performance, quicker ability to find the platform in the three phases training, short-term retentions and long-term retention. Specially group 3 and group 4 had slightly different performances in the short-term and in the long-term with group 4 having the better performance. One can say that both premixes 3 and 4 are performing with more or less similar efficiency while group 4 is excelling in results. This means that consuming vitamin E and phosphorus has nearly the same effect of consuming Vitamin B1, B2, B3, B5, B6, B7, B9, B12, vitamin C, vitamin E, magnesium, zinc, Iron, Iodine, calcium, phosphorus when it comes to memory improvement. Vitamin E and phosphorus have made a powerful statement through this whole study by improving memory.

The ELISA test results, have shown that all three groups consuming premixes are performing better than the positive control group, the group that is not consuming any premixes with induced AD which has the highest average of optical density O.D. All results from the ELISA test have given an average group number for O.D, the higher this number the higher the presence of amyloid-β 42 which is present when AD is present. This been told the negative control group that has been only fed with no induction of $AlCl_3$ (= no AD) has a low level of optical density, which is a rational result however group 4 consuming vitamin E and phosphorus is performing even better than the control group which is an outstanding result, giving hope in preventing AD while consuming these micronutrients together. Group 5 consuming the master premix is also having good findings a bit higher than group 4 but still low optical density.

The histopathology results whether it was the H&E stain or the Nissl stain showed more or less the same results that group 1 the one not having induced AD showed normal and intact neurons, however group 2 with induced AD not consuming any of the premixes (vitamins and minerals) showed degenerated or damaged neurons which is natural outcome of AD presence in such group. The three other groups consuming three different premixes, all with induced AD, whether it was the authorized premix, the content premix or the master mix which is a combination of both authorized and content premix showed more or less same results with no main differences between any of these groups. They all showed a mix of degenerated and intact neurons showing that the presence of AD thus dementia is there but still consuming these premixes is helping the brain protect its neurons. These three groups showed better neurons than the group not consuming any of the premixes.

Exploring osteoporosis results, there have been many controversial findings. Starting with blood biomarkers by detecting calcium total and ionized, high values of total calcium group average and total ionized calcium group average indicate the presence of osteoporosis. So, the higher these numbers are the higher the presence of the osteoporosis. What comes to attention that group 2 which is the positive control group the one where all the mice were ovariectomized, the one that should have had the highest total and ionized calcium is having lower or approximately same value of supplementation groups. Also negative control group 1 which was not ovariectomized shows minimal differences than the ovariectomized groups. Group 5 that was supposed to consume the master mix (authorized and content premixes) was eliminated from the study due to death which even weakened the study scope. The remaining two group; group 3 and 4 who were expected to portray low average group of ionized and total calcium are not showing those results.

On the other hand, exploring the findings of the blood biomarkers of osteoporosis when it comes to alkaline phosphatase (ALP), results are explainable. Showing group 2 (the ovariectomized group without taking any micronutrients) leading the results with the highest figures, which confirms the presence of osteoporosis without any kinds of prevention. Group 1 not consuming any micronutrients and not having ovariectomized mice also had acceptable moderate presence of ALP. Group 4 is having better findings than group 2 which is promising, meaning that premix 5 consumption is managing to prevent osteoporosis. Group 4 (consuming content premix)

had the best results showing the lowest alkaline phosphatase which proves that it had the best performance within the four groups. However, one concerning point that in group 3 consuming authorized claim premix 4 is not significantly different that the positive control value.

The histopathological examination of the longitudinal section of mice femur stained by the hematoxylin and eosin stain showed promising results. We measured the thickness of the trabecular area in micrometers, where the thinner the bone is, the stronger the impact of osteoporosis, indicating bone loss and bones are becoming more and more porous. The first group which is the negative control group with sham operation had the thicker trabecular bone with a group average of 277.134 µm, while the second positive control group of ovariectomized mice showed the thinner trabecular bone results with a group average of 120.26 µm. Those results prove that the ovariectomy actually induced osteoporosis and that the mice bones were highly impacted with the positive control group having less than half the thickness of the negative control group. Group 3 and 4 of ovariectomized mice consuming premixes both showed results that fall in between the negative and positive control groups, with group 3 having more significant results this time with an average thickness of 251.62 µm, while group 4 having an average of 152.28µm. Those results demonstrate that consumption of premix 4 consumed by group 3 with authorized claim micronutrients significantly diminished the impact of osteoporosis, where the trabecular bone thickness only decreased 9.2% from the negative control group. Group 4 that consumed premix 5 with content claim micronutrients showed moderate results, where the trabecular bone is thinner than the negative control with 45.1%, while the positive control group is thinner by 56.6% compared to the negative control group.

Overall, the vitamins included in premix 5 in the osteoporosis study, vitamin E, vitamin B9 and vitamin B12 showed promising results that show the positive impact those micronutrients have on preventing osteoporosis.

7. Research Limitations

- For the rats in group 4 of the dementia arm consuming the content claim premix, vitamin A and vitamin D were excluded of the premixes given to rats due to study's size and capacity, it was impossible to be able to weight these two vitamins separately in order to assess the individual consumption for each rat. Calculations have shown that individual daily consumption should be approximately 0,000003 mg for vitamin A and 0.00000004 mg for vitamin D, which made it not possible to weight and include in the premix.
- Results may have been compromised by the moderate size of the study's sample, and the low doses of some micronutrients used in comparison to other vitamins and nutrients.
- For the osteoporosis study; lots of mice could not endure the ovariectomy process which resulted in many deaths in mice, specifically fifteen mice died during the ovariectomy, eliminating an entire group sample (group 5) and 2 to 3 mice from the other groups. The group who was supposedly consuming the master mix premix, which includes authorized claim premix of group 3 and content claim premix of group 4, was completely eliminated from the osteoporosis study due to death. Some of the death occurred during the ovariectomy process while other took up to two weeks, weakened each day until they died.
- This study have fallen under time and cost constraints, since the premixes ordered waited too long in Egypt's customs for approval and by then the rodents available for the study became older which would have jeopardized results of the study due to the aging. We had to start the study all over again with new younger rats and mice.
- This study is examining the effect of 3 different premixes, containing different substances, on Alzheimer's dementia, and 3 different premixes with different micronutrients and their results on osteoporosis. Results of a certain premix will not provide detailed examination of each of the vitamins and minerals used in such premix on AD. For example, if the content claim premix has proved negative relationship with osteoporosis, it cannot be generalized that vitamin E has a negative relationship with osteoporosis since the effect of each component is not examined but instead the premix as a whole. One or few of the vitamins used in any of the premixes may be of no value or low effect on osteoporosis and still results can show positive effect because of the other micronutrients.

8. Future Recommendations

- In the dementia study; it is crucial to explore independently the effect of each micronutrient consumed by group 4 since this group results were promising in terms of the prevention of dementia. In other words, exploring the effect of vitamin E in a separate study and also the effect of phosphorus in another study. This will give a better understanding of the weighted importance of each micronutrient to dementia prevention. It will also eliminate any doubts that one micronutrient can be more beneficial than the other when it comes to dementia symptoms.
- Future studies can be implemented on humans since these micronutrients are not new or have any side effects on the human body. Voluntarily human sample will give a better understanding of the effect of these micronutrients on the human body.
- Future studies can take place in undeveloped or developing countries like Egypt were most of the population are not following a healthy diet or consuming additional supplements. Results of such study will be interesting and enlightening.
- Experiment the treatment model instead of the prevention model used in this study. Which means we can use rodents that are already osteoporotic or suffering from AD and apply same premixes used. In this study, we induced osteoporosis and AD at the same start point of supplementation.
- For the osteoporosis study, since results were controversial in the blood markers testing, future studies could explore the effect of these specific micronutrients on rats' sample instead of mice sample. The reason behind the sample change is that there were a lot of death in the mice sample and maybe rats could have had better results to help us understand the effect of these premixes on osteoporosis prevention.
- Further studies should be implemented on single ingredients of premix 5 used in osteoporosis arm. Vitamin E, B9 and B12 showed promising results in reducing osteoporosis impact and should be further explored.

9. Conclusion

To sum up, aging is associated with many neurological diseases and deteriorations such as dementia which includes mainly but not limited to memory impairment, the main cause of dementia is Alzheimer Disease AD. Also, while aging the body's storage of vitamins and nutrients is diminishing leaving the bones very weak and fragile more prone to fractures with no severe traumas and it is more common in women but also inevitable for men only a bit later on, mainly represented in osteoporosis. These two diseases are grabbing the attention of scientists and researchers since they are affecting a large portion of the population leaving them not socially and economically active and also bearing cost on the government by their medical insurance. The number of people affected by those two diseases are expected to also dramatically increase in the coming years, unless there is a solid scientific proof or preventing or controlling these diseases. In this research two separate studies were conducted, one exploring dementia and ways to prevent it or ameliorating its negative effects and the second was targeting osteoporosis for the same aim to prevent it or ameliorate its negative effects. For the dementia as well as the osteoporosis the sample was divided into five groups.

For the dementia it was a sample of rats while for the osteoporosis it was a sample of mice. For the dementia study, group 1 was the negative control group; this group was only fed and nourished without having $AlCl_3$ induction, thus without having AD and dementia symptoms. Group 2 was the positive control group having $AlCl_3$ induction thus having AD since $AlCl_3$ in proven to have neurological damages similar to these of AD. The remaining three groups were all also $AlCl_3$ induced but also consuming certain premixes of vitamins and minerals. Group 3 was consuming premix that had authorized claims, which means that they have approved proof by the European Food Safety Authority (EFSA) to have a positive relationship with dementia. Group 4 consumed a premix that obtained micronutrients with a content claim with means that there are some proofs to have a direct relationship with dementia but still these proofs are not approved by EFSA. Finally Group 5 was consuming a master mix which is a premix including micronutrients consumed by group 3 and group 4.

To test the results and be able to discuss them, three mediums were used; Morris Water Maze, ELISA test and histopathological examination of the rats' brain (H&E stain and Nissl stain). Results of the three groups taking micronutrients supplementation were promising by

all these mediums; MWM showed results from the training phase and then results from the acquired phase. In agreement, the ELISA test and histopathology all showed more or less the same outstanding results that all three groups consuming premixes showed, improvement in their results whether it's in memorizing the platform when it comes to MWM or the low presence of Amyloid β-42 peptides in ELISA test or protected and/or intact neurons in histopathological examination. These were all signs that compared to group 2 with induced AD, showed that these three latter groups where performing really well with group 4 in particular leading such success. Consuming vitamin E and phosphorus should be further researched on their own for more specific results and maybe even tested on humans.

For the osteoporosis study mice were divided into similar five groups, group 1 negative control; sham operated, only fed and nourished, group 2 the positive control group; mice were ovariectomized to induce osteoporosis in these mice without consuming any premixes. The three remaining groups were all ovariectomized as well but also consuming specific premixes of micronutrients. Group 3 was consuming authorized premix; premix that is proved and approved by EFSA to have a positive relationship with osteoporosis, group 4 consuming content premix; premix that claim to have a positive relationship to osteoporosis but still not approved by EFSA and group 5 the master mix, mice are consuming both the authorized and content premix ingredients of group 3 and 4. To test osteoporosis study results and be able to discuss them two mediums were used; blood biomarkers through detection of blood calcium (total & ionized) and alkaline phosphatase ALP and histopathological examination of mice femur bone. Results of blood biomarkers were divided into two findings the detection of blood calcium (total and ionized) has debatable findings with showing no improvement at all when consuming micronutrients not only that but the group not consuming any premixes was even having better results than other groups. The second finding concerning ALP was showing again group 4 the content group having the most promising results of all. On the other hand, the histopathological examination showed promising results where group 3 and 4 consuming premix 4 and 5 consecutively had much thicker trabecular bone compared to the osteoporotic positive control not consuming any micronutrients. The effect of consuming vitamin E, vitamin B9 and vitamin B12 that do not have an authorized claim for mobility enhancement should be further studied.

10. Acknowledgments

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  - Dr. Aya El Sayed
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- **DSM Nutritional Products**

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