BY CBHI CANADA

1ST EDITION

The Innovative Biotechnology

AIC FOR CALCIUM SIGNALING

Systemic Literature Review for the Effects of Anti-orbital Ionic Calcium Carbonate (AIC-$CaCO_3$)

AIC FORMULATION TECHNOLOGY

CERTIFIED
AIC FORMULATION
TECHNOLOGY

Q. What is the Solution for Age-Related Chronic Degenerative Diseases?

The calcium homeostasis is critical to maintaining our health as it affects our bone health which is associated with building muscles, blood vessels, nerves, and DNA replication.

Q. When Calcium Homeostasis is Disturbed:

P53 will become dysfunctional which causes cancers and chronic degenerative disease, such as astrocyte dysfunction, synaptic dysfunction, Alzheimer's disease, Parkinson's disease, Huntington's disease, diabetes, osteoporosis, arrhythmia, epilepsy, ADHD, autism, etc.

Q. What is the Solution? AIC!

"There are more than 60 trillion cells in the human body, and the cells are continuously replicated during the person's lifespan. However, the replication rate and the function gradually decrease as the human ages. Moreover, the calcium homeostasis in the body is also disturbed, which leads mitochondrial dysfunction. If the calcium level is altered, cells cannot function properly which ultimately results in cancer, osteoporosis and other chronic neurodegenerative diseases. As a result, balancing calcium level is crucial, and it can be the key to treating chronic degenerative diseases, including cancer."

Ph.D. in Chemistry,
Paul. K. Lee

BY CBHI CANADA

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Introduction of CBHI Canada

The Calcium and Bone Health Institute of Canada (CBHI Canada) is a scientific research-based corporation that was founded in 2001. The majority of our research is focused on the role of calcium in our bodies, and through this research, we have invented a new form of calcium, called Anti-Orbital Ionic Calcium Carbonate ($CaCO_3$). It is used as a physiologically active plasma ionic calcium in our bodies which enhances the calcium homeostasis and also, influx and efflux of intracellular calcium ion which as a result maintains our bodies in their optimal status. Moreover, as the appropriate influx and efflux of calcium ion modify mitochondrial function, it also improves protein synthesis which can be the key to treating chronic degenerative diseases, including cancer.

To develop our research, we also collaborate with many reputed researchers from many different disciplines. They are from SFU, UBC, UC

Davis, NSERC Canada and BC Government (BCBN). We also collaborate with many other research centers to combine our research with fundamental sciences such as mathematics, chemistry, physics and biology. Moreover, we work with many applied science faculties such as engineering and computing science to enhance the depth of the research.

The Calcium and Bone Health Institute of Canada (CBHI Canada) is one of the few Canadian research-based institutes that has been founded to study about calcium-related chronic diseases, such as cancer, osteoporosis and others. By conducting laboratory and clinical research, we are endeavouring to find effective prevention and treatment methods. Moreover, as we collaborate our study with many different medical and pharmaceutical companies, we are proving the efficacy of Anti-Orbital Ionic Calcium Carbonate ($CaCO_3$).

The mission of CBHI Canada is to provide better care for cancer, osteoporosis and arthritis patients by developing innovative and effective cancer, osteoporosis and arthritis treatments and to contribute to lower health care costs associated with cancer, osteoporosis and calcium-related disease.

Moreover, as osteoporosis is one of the high global burdens of diseases, especially for women who have experienced post-menopause, we are trying to find a way to decrease the cost due to subsequent fractures caused by osteoporosis. Since 2011, we have been providing comprehensive osteoporosis treatment using AIC products. As a result, more than 100,000 people worldwide have experienced the efficacy of AIC.

The CBHI is located in the Coquitlam, BC, Canada and we are committed to discovering the causes of and cure for calcium related diseases and believe this will be accomplished through research. By working together, we combine our innovative expertise to investigate questions related to all respects of bone and joint health.

"The CBHI Canada envisions a world without cancer, osteoporosis and any calcium-related diseases. Our mission is to achieve this vision through various profound researches and clinical trials."

Paul. K. Lee

1st Edition

Systematic Literature Review for the Effects of Anti-Orbital Ionic Calcium Carbonate (AIC)

CBHI Canada

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Preface

Tremendous medical advancements have marked the 21st century, and these advancements have extended the life expectancy of humans farther than ever before. However, progress in relation to bone-related and cancer-related disease seems to be at a standstill. In the majority of developed countries, the increase in aging population has resulted in higher risk of death caused by bone fracture. As a side effect, there is an increased social and economic burden on our society. Also, the substantial investment in the anti-cancer remedy has not helped in slowing down the rising death rate of cancer patients. This hopeless situation only frustrates doctors and adds greater frustration to the families of the cancer patients.

Therefore, at CBHI Canada, realizing the significant role of calcium in our health from the cellular level up, we continued to research and develop ways to pour the calcium directly into the cell. In March of 2009, CBHI was successful in creating AIC Calcium. In December of 2011, a clinical test on mice with osteoporosis proved successful in fully reversing the osteoporosis. CBHI then continued to treat osteoporosis patients across the spectrums of age, gender and race, proving the efficacy of this treatment. There are many existing osteoporosis treatments that have been were developed to treat the patients to date, including Bisphosphonates, SERMS, Teriparatide, and Calcitonin, but none of them have been successful in curing osteoporosis.

AIC Calcium proved that even in the absence of Vitamin D, it is able to permeate the cell membrane and is absorbed by diffusion effect. Additionally, the consumed small quantity of ionic calcium has the effect of physiological plasma calcium. Therefore, with AIC Calcium, CBHI was able to effectively treat diseases such as cancer, diabetes, and osteoporosis, which are caused by a lack of ionic calcium in the blood. AIC Calcium provided promising remedies for different types of cancers including multiple myeloma, brain tumor, colon cancer, breast cancer, and leukemia.

In November of 2013, news reached us from S. Korea that Ms. Molly Holt (Age 79, Female) was fighting multiple myelomas. Since all the anti-cancer remedies had proven ineffective for her, she was ready to call all her relatives from the United States to say her final words. With haste, CBHI sent AIC Calcium to Ms. Holt, and her condition significantly improved following treatment. Ms. Holt and her friends later visited CBHI and formally presented the plaque of appreciation, sincerely thanking me.

Both carcinoma and sarcoma have a close relation to calcium. According to many types of research, when the ionic calcium level decreases within the cell, the performance of the mitochondria, DNA, p53 protein, NK-kB protein, cytotoxic T-lymphocytes and all stem cells are decreased and could result in a DNA mutation, which then accumulates and turns into carcinoma or sarcoma.

Increasingly more researchers are actively writing about and proposing the importance of the role of calcium. For instance, the claim of The Neurology Association of the United States, "Calcium is a key to solve Alzheimer's Disease and Parkinson's Disease," tells us how crucial the role of the calcium ion is in our body. CBHI believes that if the healthy calcium level within all of our cells is well maintained, we will be free from hosts of debilitating diseases.

I hope AIC calcium brings renewed hope for many who are suffering and would like to thank all the members of the CBHI Canada research team who contributed to the publishing of this book.

Ph.D. in Chemistry,
Paul. K. Lee

March. 23rd, 2018

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I. Introduction

Through many academic pieces of literature, books, and media, people are well-informed that calcium is an essential mineral to maintain our bone health. As there are many calcium supplements available in the market, calcium is aggressively advertised for its positive effects on our bodies. As many pieces of research have shown, calcium plays a critical role in treating osteoporosis, which is one of the most prevalent aging-related chronic degenerative diseases. Moreover, as the demand for calcium is increasing, many medical scientists are focusing on the effects of intracellular calcium ion on our health. While cell health translates to our overall health, calcium is closely related to the mitochondrial function, which is the key to treating aging-related chronic degenerative diseases as well as cancers. Nevertheless, even though we had already established that balancing intracellular calcium ion is crucial for cell health and offers immense potency to treat the diseases that we have not yet eradicated, there were no particular calcium treatments that could directly affect the cellular calcium homeostasis.

Through many sources of published academic literature, it has been proven that calcium plays a crucial role in signaling mesenchymal and embryonic stem cells, replicating DNA, activating mitochondrial respiration, and balancing blood pH level. Whilst we already knew that restoring intracellular calcium ion is essential for the health of the mitochondria, there were no particular calcium supplements that directly affect the intracellular calcium ion level. However, remarkable progress has been made by the Calcium & Bone Health Institute of Canada (CBHI) with the invention of a new form of Calcium Carbonate ($CaCO_3$) that has a unique bonding structure with a significantly increased rate of calcium absorption. It directly affects extracellular and intracellular calcium ion levels, which modifies the mitochondrial function, offering effective treatment for chronic degenerative diseases. Existing as a physiologically active ionic form of calcium carbonate in water, this new calcium shows excellent efficacy in absorption rate, solubility, and bonding structure as compared to the regular calcium

carbonate currently on the market. We named it Anti-Orbital Ionic Calcium Carbonate (AIC).

While AIC provides physiologically active ionic calcium carbonate that is directly bio-available, the demand for calcium ion in our bodies is always high, especially for active lifestyles. AIC acts directly as plasma ionic calcium in the blood and cellular membrane as it is absorbed through the epithelial tissue of the stomach (Mucosa). Moreover, because AIC uses a passive transport system, it is incorporated into our cellular membrane without the use of any biomechanical energy. Regular calcium carbonate, however, uses an energy-draining active transport system, which is aided by Vitamin D to be absorbed through our cellular membrane. As a result, AIC is much more efficient and useful in balancing the calcium homeostasis of our bodies.

It is recommended to ingest 5 mL of AIC mixed with 500 mL of water because it uses osmotic pressure to be absorbed into the cell. Moreover, since AIC incorporates weak antiorbital bonding, it is easily broken down in the stomach with the osmotic pressure and low pH conditions. With the osmotic pressure and the chemical digestion reaction of the hydrochloric acid (HCl) in the stomach, AIC is easily broken down in the stomach and absorbed directly through the mucosa.

As AIC is directly ionized in the cellular membrane, it also has excellent solubility, with ionizing rate about 200 times greater than that of regular calcium carbonate. When a regular calcium supplement is taken, Vitamin D is often recommended, because of the role Vitamin D plays in helping calcium to bind to the peptide and be transported to the blood vessel. However, AIC bypasses all these processes by being directly absorbed through the cellular membrane in ionic form and is immediately bio-available. Regular calcium ends up as inactive protein-bound calcium instead.

Calcium exists in three different forms in serum plasma: 40-45% protein-bound calcium, 5% inorganic calcium, and 45-50% ionized calcium. The only calcium that is physiologically active and triggers osteoblast (bone forming

cell) and osteoclast (bone dissolving cell) activity is the ionized calcium. This physiologically active calcium signals the pituitary gland to secrete pituitary hormones, which in turn triggers the release of thyroid (TH) and parathyroid (PTH) hormones. These hormones stimulate the uptake of calcium in the bones by activating osteoblasts, which lead to the strengthening of bones. Moreover, it also triggers the activity of osteoclast to enhance the bone turnover rate to make the bone quality better. AIC, which elevates the serum concentration of ionic calcium, plays a critical role in activating these hormones to protect our bone health.

As AIC participates in the activity of intracellular calcium influx and efflux, it normalizes mitochondrial function, which in turn can treat cancers and other chronic neurodegenerative diseases such as Alzheimer's Disease, Epilepsy, Arrhythmia, Mitochondrial Dysfunction-Related Diseases, Osteoporosis and Type II Diabetes. Furthermore, by balancing the intracellular calcium influx and efflux, it can initiate the activity of mesenchymal and embryonic stem cells to regenerate cellular functions and provide the key to treating aging-related chronic degenerative diseases.

II. AIC (Anti-Orbital Ionic Calcium Carbonate)

"Anti-Orbital Ionic Calcium Carbonate (AIC): Physiological Ionic Calcium in Serum, Which Uses Passive Transport System when Absorbed in the Bloodstream."

i. Ionization, Solubility and the Bonding Structure

AIC has a unique chemical bonding structure as compared to other Calcium Carbonate ($CaCO_3$) that are sold in the market as dietary supplements. Although the molecule name of AIC is Anti-Orbital Ionic Calcium Carbonate, its distinct feature lies in its bonding strength. It is bonded with anti-orbital bonds, which are relatively weaker than regular ionic bonds. As it is weakly bonded, it is easily breakable when it is orally ingested with an adequate amount of water. With the osmotic pressure, it can be passively transported to the blood vessel system through the stomach lining. As other calcium supplements cannot be absorbed in the stomach due to its nature of ionic bonding structure, they have to be incorporated in the duodenum with the aid of Vitamin D and protein transporter into serum as a protein bound calcium.

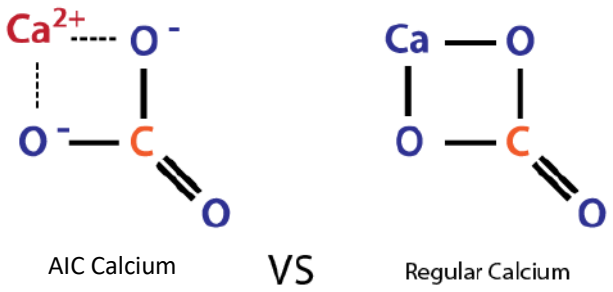


Figure 1. The Comparison of Bonding Structures of AIC and Regular Calcium Carbonate.

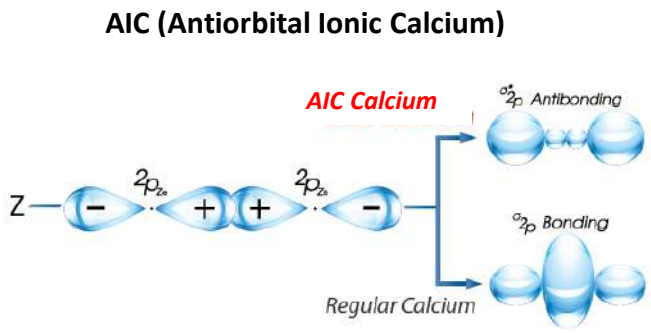


Figure 2. The Anti-Orbital of AIC.

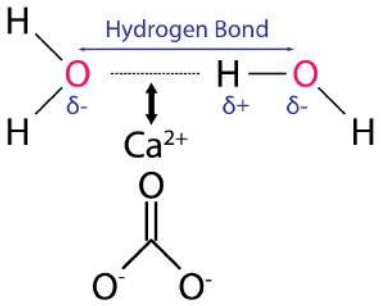


Figure 3. Calcium Carbonate Reacts with Water Molecule and with the Osmotic Pressure, Calcium Ion is Separated.

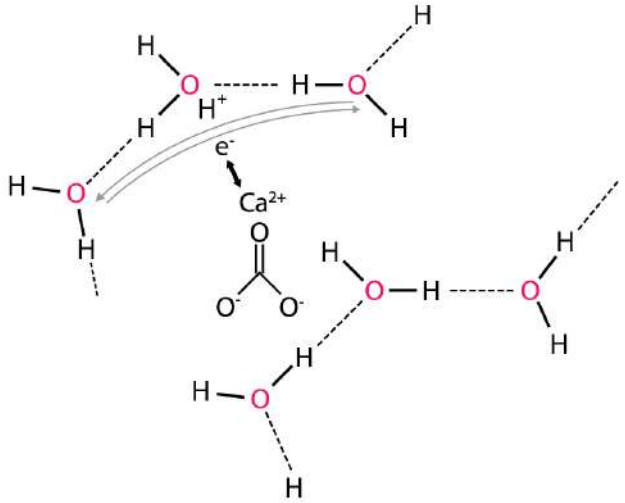


Figure 4. Water Hydrogen Bond 'Wires' Cooperativity by AIC.

Moreover, as AIC has much lower K_{sp} coefficient ($K_{sp} = 7.5\times10^{-7}$ to 8.7×10^{-7} at 25°C) than other regular calcium carbonates ($K_{sp} = 3.7\times10^{-9}$ to 8.7×10^{-9} at 25°C), it is more readily soluble in water which means that it can be more easily ionized in water. Whilst it is easily ionized in water, calcium ion can be physiologically used in our body; and it eventually activates the calcium signals in the intracellular membrane. As more calcium ion is refluxed into the intracellular membrane, calcium ion starts to oscillate, and enhances our body metabolism. Activating calcium signals is critically crucial because increased calcium ion initiates calcium signals that can modify protein synthesis by repairing mitochondrial activity; our bone health immunity can be strengthened. Moreover, as calcium balances lactic acid and ammonia in the cell, we can prevent neurodegenerative diseases. Also, as calcium normalizes mitochondrial function, it can maintain our body temperature, blood pH, and oxygen levels.

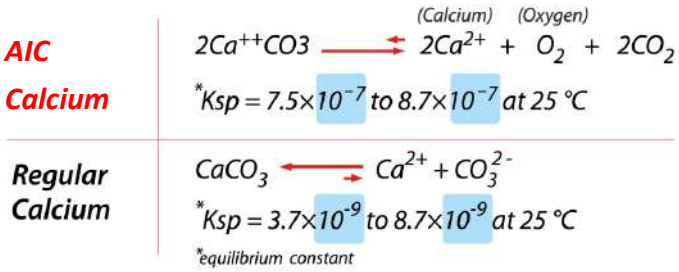


Figure 5. The Solubility and Ionization Comparison between AIC and Regular Calcium Carbonate.

ii. The Comparison of Chemical Reaction Rates of AIC with Regular Calcium Carbonate

Experimental value when reacting with acetic acid $CaCO_3 + 2CH_3COOH$?

$2\ (CH_3COO)Ca + CO_2 + H_2O$

The reaction rate (r) measures the amount of reduction of calcium ions per hour, and the chemical reaction formula is as follows.

$$-r_{Ca} = -\frac{d(Ca)}{dt} = \frac{K1(Ca)(ACT)^{1/2}}{k2+(ACT\ \ Ca)/(ACT)}$$

$$-\int_{Ca\ zero}^{Ca} \frac{d(Ca)}{d(Ca\ zero)} = k\int_{0}^{t} dt$$

ACT: Acetate

Figure 6. The Amount of Reduction of Calcium Ions Per Hour.

Time versus Concentration Run

Time (Sec)	Regular $CaCO_3$ [Ca] = [ACT] mole/liter	1/([Ca]	AIC $CaCO_3$ [Ca] = [ACT] mole/liter	1/([Ca]
0	0.225	4.444	0.225	4.444
15	0.221	4.524	0.1898	5.269
30	0.211	4.739	0.123	8.130
45	0.208	4.807	0.0967	10.341
60	0.19	5.076	0.0652	15.337

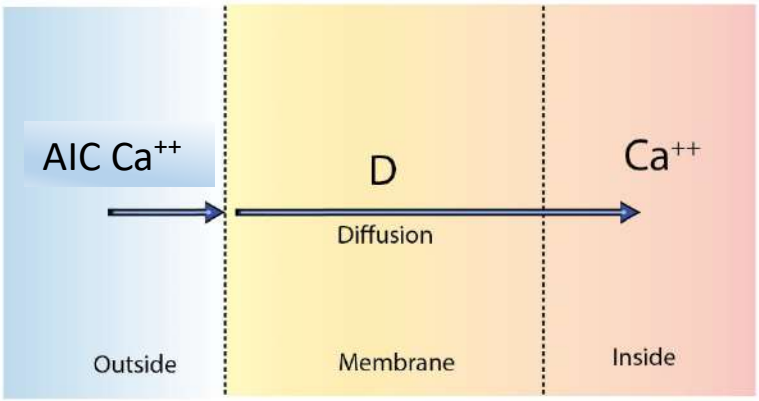
Table 1. The Comparison of Reaction Rate Between Regular Calcium Carbonate and AIC Calcium Carbonate.

Based on the data above, when comparing the reaction rate between regular calcium carbonate and AIC calcium carbonate, AIC calcium carbonate showed 3 times faster chemical reaction rate.

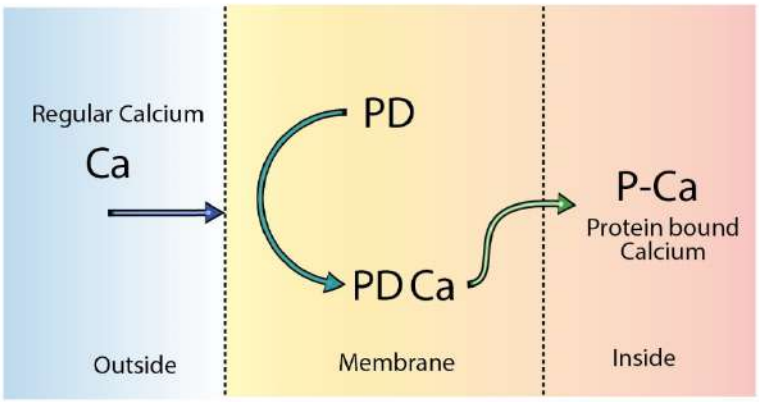
iii. AIC and the Absorption Route

Most of the calcium that we require is supplemented by foods we intake. However, due to lifestyle and dietary changes, "an estimated 126.6 million Americans were living with a musculoskeletal disorder [that is mainly caused by lack of calcium intake] in 2012." ("One in Two Americans Have a Musculoskeletal Condition Costing an Estimated $213 Billion Each Year in

Treatment and Lost Wages", 2016) To supplement the lack of calcium, people turn to nutritional supplements. Despite their effort, the regular calcium supplements in the market are not physiologically active ionic calcium. Although only a small amount of ionic calcium is needed for its physiological effects, daily recommended intake of calcium is much higher because of its absorption efficiency. The most prominent advantage of AIC over regular calcium supplements in the market is that AIC has 200 times more efficient absorption rate when compared to others.



<AIC Calcium Passive Diffusion Transport Mechanism>



<Calcium Active Transport Mechanism>

Figure 7. The Use of Protein Transports of AIC and Regular Calcium Carbonate.

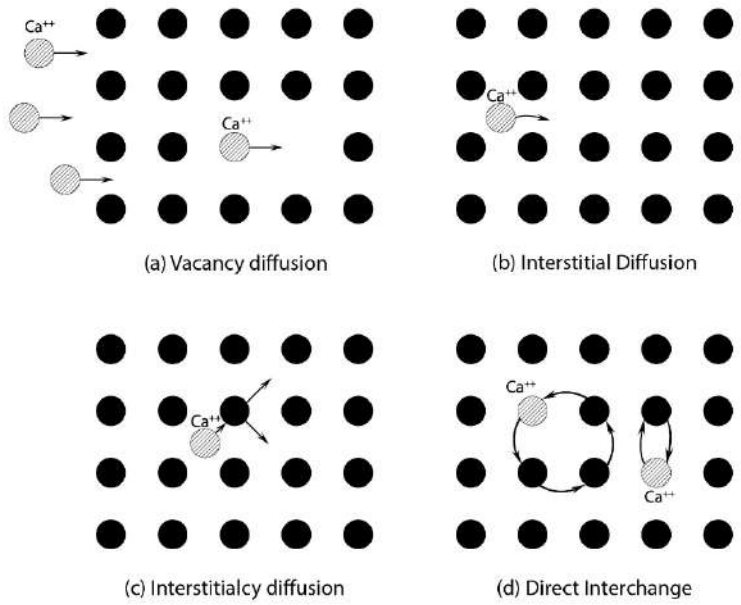


Figure 8. AIC Calcium Passive Transport Mechanism (Diffusion).

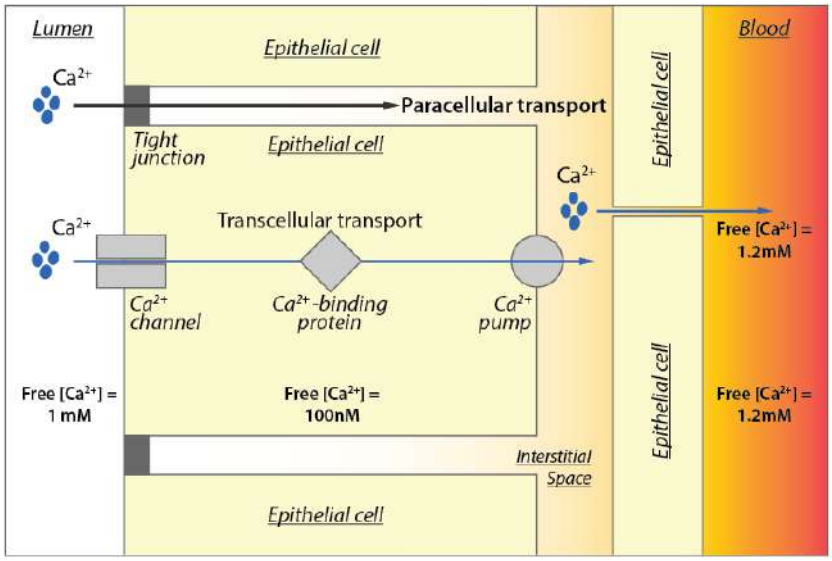


Figure 9. The Passive Transport Mechanism Through the Epithelial Cell to Blood Vessel.

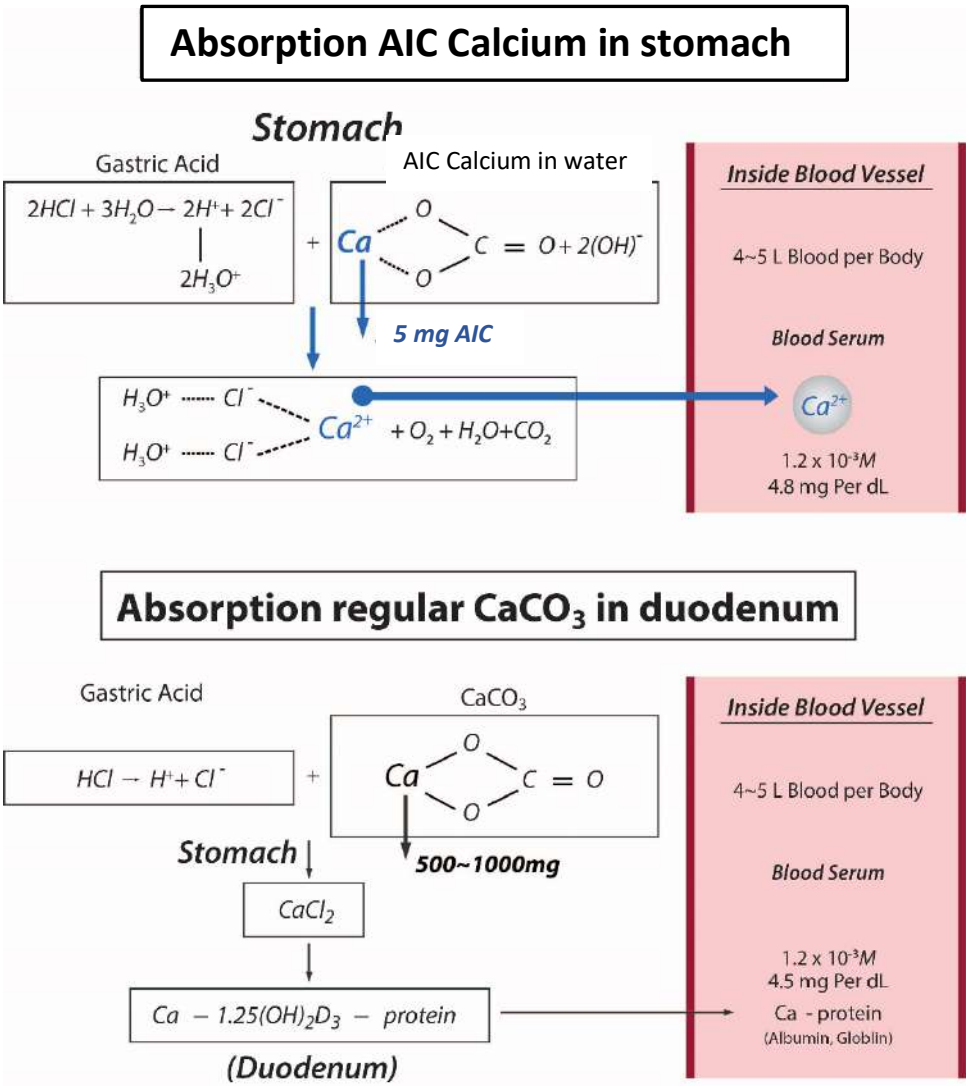


Figure 10. The absorption process of Passive Diffusion and Active Transport System of AIC and Regular Calcium.

It is prevalently known that calcium is absorbed in the epithelial tissue of jejunum, duodenum, and the small intestine with the aid of other biochemical energy. (Bronner, 2003) Since it is the protein-bound form of calcium, calcium requires Vitamin D to be absorbed into the epithelial tissue and needs protein porters to be transported into the blood vessel and cells. However, AIC uses a passive transport system that does not require any

biochemical energy to be absorbed. Moreover, since it is diffused into the blood vessels through epithelial tissue in the stomach (Mucosa), it can be directly used as ionic calcium to trigger the hormone regulation involved in calcium homeostasis.

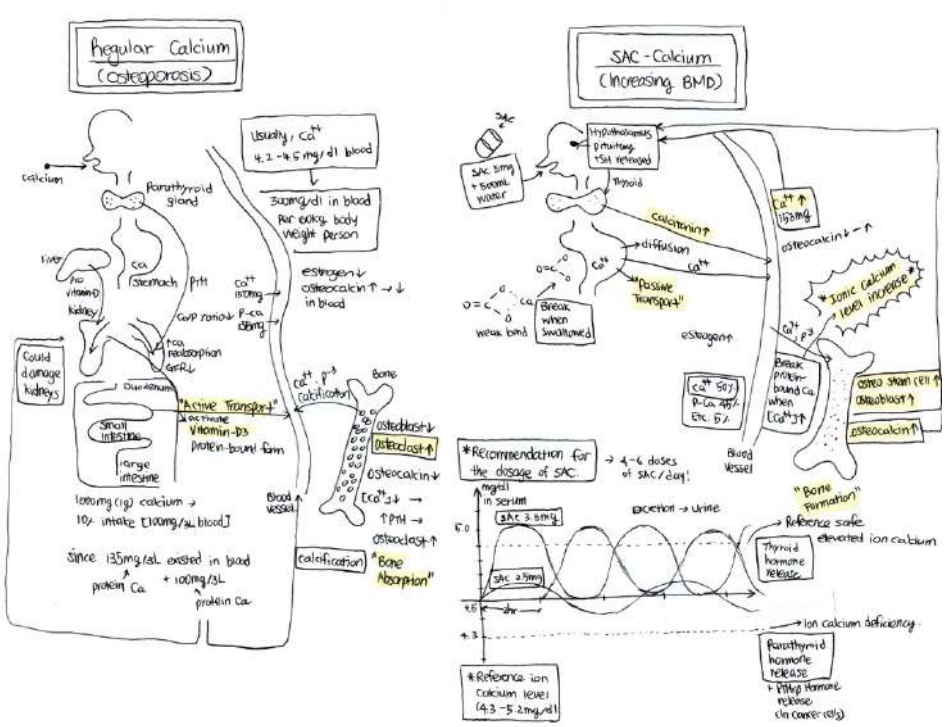


Figure 11. The Brief Depiction of Calcium Homeostasis for AIC.

iv. How Fast is AIC Absorbed into the Bloodstream?

Blood takes 25 seconds to circulate the whole body. Passively diffused AIC Calcium in the serum increases ion calcium level. The increased level of ion calcium is continued for three hours. People who are sensitive can sense the effect in 30-60 seconds.

v. Dosage Mechanism for AIC

"The Recommended Dosage of AIC: 5-10mg/ Day/ 60 kg of Body Weight; Even with the Small Amount of Dosage, A Lot of Changes Would have Occurred."

Compared to the recommended daily dosage of other supplements in the market, only a small amount of AIC Calcium is prescribed for consumption with adequate water. AIC acts as physiological ionic calcium in the blood to trigger the pituitary gland, which regulates thyroid hormone (TH) and parathyroid hormone (PTH). Moreover, as it also helps to break protein-bound calcium in our bodies and transform them to physiologically active ionized calcium as part of calcium homeostasis, the body calcium signals are finally activated and balanced.

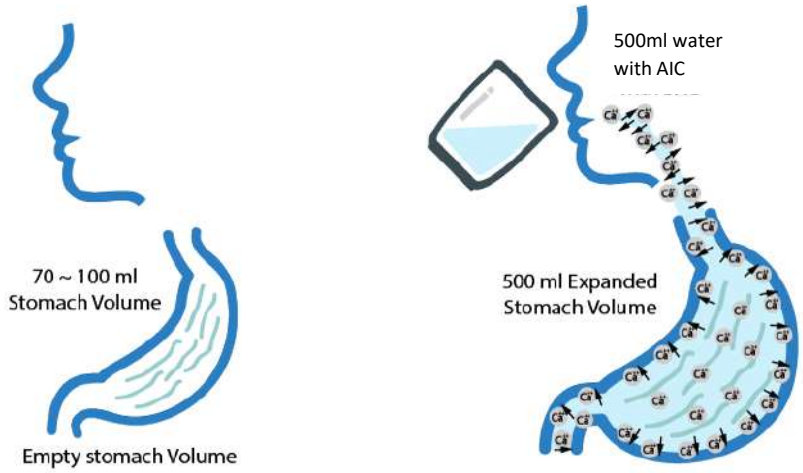


Figure 12. The Intake Method of AIC.

Calcium in serum exists in three forms: 40 to 45% of protein-bound calcium, 5% of inorganic calcium, and 45 to 50% of ionized calcium. In the case of a person who is 60 kg in body weight, the level of ionic calcium in serum exists at about 175 mg Ca^{2+}/3.5 L in blood. When the ionic calcium is

increased up to 177 to 178 mg after taking 5 mg of AIC calcium with 500 mL of water, thyroid-stimulating hormone (TSH) is released from the pituitary gland.

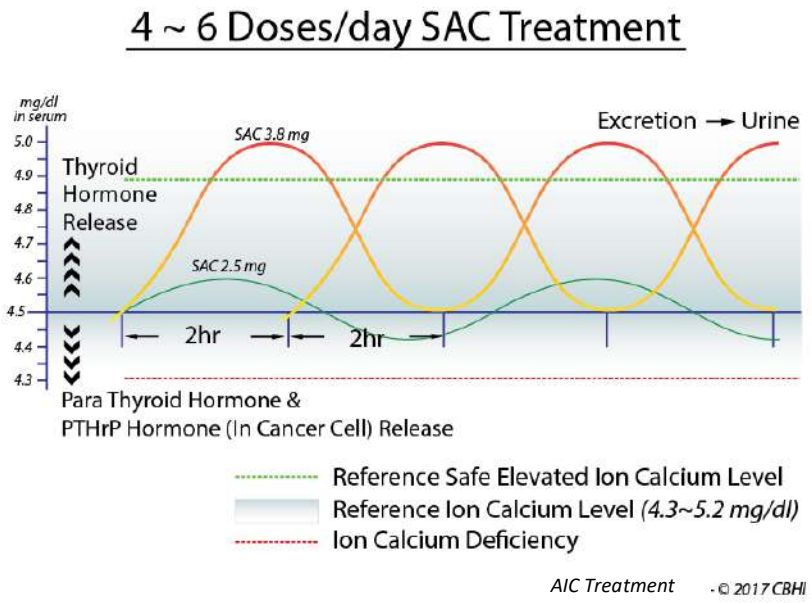


Figure 13. The Dosage Recommendation for AIC.

III. AIC and Osteoporosis

"Anti-Orbital Ionic Calcium Carbonate: The Innovative Approach to Treating One of the Top Ten Causes of Global Burden of Diseases, Osteoporosis"

i. Introduction

Osteoporosis is a skeletal system disorder associated with endangered bone strength, which is defined by low bone density and quality, consequently increasing the risk of fractures of the hip, spine, wrist, ribs, and shoulder. (Cooper, 1999) Thus it is characterized by low bone mass and deterioration of bone tissue, which leads to decreased bone strength, increased bone fragility, and risk of fracture. (Liberman *et al.,* 1995; Kanis, 2008; Baron & Hesse, 2012; Szulc *et al.*, 2010) Osteoporosis incites a critical public health issue, causing substantial morbidity and mortality.

Globally, more than 200 million women are suffering from osteoporosis, and most of these women are aged 45 years and older and have experienced postmenopause. (IOF, 2018) Moreover, nearly 8.9 million people are suffering fractures that are caused by osteoporosis. (IOF, 2018) In Europe, osteoporosis is a substantial economic burden because the disability caused by osteoporosis is higher than that produced by cancer (except lung cancer) and other chronic noncommunicable diseases such as asthma, rheumatoid arthritis, and cardiovascular diseases. (IOF, 2018)

WHO has defined osteoporosis as a level of Bone Mineral Density (BMD). (Kanis *et al.*, 1994) When patients are diagnosed with osteoporosis, they measure the Bone Mineral Density (BMD) which comes with T-score value. If the result is lower than -2.5, they are diagnosed with osteoporosis, and if the T-score value is in between -2.5 and -1.0, they are diagnosed with osteopenia. If the value is greater than -1.0, they are considered normal.

T-score	BMD (g/cm^2)	Relative Loss of Bone (%)
0	1.2	0% ↓
-1.0	1.08	10% ↓
-1.5	1.02	15% ↓
-2.0	0.96	20% ↓
-2.5	0.90	25% ↓
-3.0	0.84	30% ↓
-4.0	0.72	40% ↓

Table 2. T-Score and BMD Comparison Chart.

Although many scientists have researched to find a way to increase Bone Mineral Density (BMD), current treatment options conducted in the medical setting have not yet made any significant increase to BMD. Bisphosphonates, SERMS, Teriparatide, Calcitonin and Thiazide Diuretics are currently prescribed to treat osteoporosis. Moreover, some physicians recommend taking calcium supplements with Vitamin D, since an increase in calcium level in plasma would trigger the pituitary gland. By stimulating the thyroid gland, thyroid hormone (TH) is secreted, which initiates osteoblasts. However, it was painful to influx calcium ion in intracellular membrane because the regular calcium that is sold in the market is not the physiologically active form of calcium as it is bound to protein. Moreover, Paziana & Paziana (2015) have mentioned in their research that an excessive calcium intake could have adverse effects on the cardiovascular system because a sudden increase of calcium ion in the blood may cause calcification in blood vessels. Also, as Kim *et al.* (2017) discovered through their research, an excessive calcium intake can elevate the risk of metabolic syndrome for

men. Therefore, due to the adverse effects of calcium intake, calcium supplements could not be the primary source for treating osteoporosis.

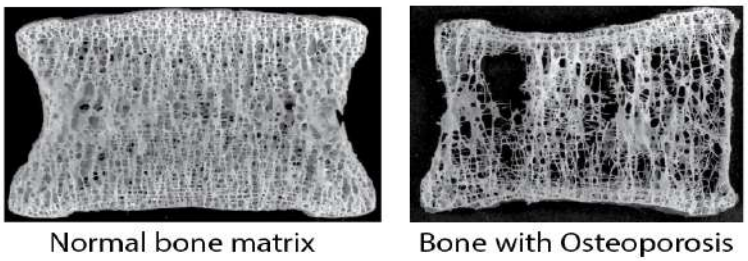


Figure 14. The Microscopic Images for Bone Matrix: Normal vs. Osteoporosis

ii. The Relationship between BMD and Osteoporosis

Despite the various treatment options for osteoporosis, the prevalence rate of osteoporosis is increasing because these treatments are yet to be proved for any significant efficacy. Bisphosphonate is the most frequently prescribed medication for patients with osteoporosis. Bisphosphonate mimics the structure of pyrophosphate and the activates the enzymes that are triggered by pyrophosphate. Therefore, it coordinates calcium and causes it to become accumulated in bones to increase BMD. However, it is still questionable if the BMD also improves the quality of bone. Even though bisphosphonates are the most prevalently used medication for osteoporosis, it is still unknown whether it is the best treatment option, since it only increases the density of bone and not the quality of bone. Furthermore, IOF (2018) has found out that bisphosphonate has many adverse side effects such as Hyperparathyroidism, Paget's Disease of Bone, Osteomalacia, Renal Failure, and Nephrolithiasis. For such reasons, it has been controversial whether patients with osteoporosis should continue to be prescribed bisphosphonates as their first line of medication.

AIC can be introduced as a new treatment option for osteoporosis because as shown by Choi *et al.* (2011), AIC proved its medical efficacy in their research. Through their study, they wanted to investigate if AIC increases

BMD and prevents bone turnover when ovariectomized rats orally ingested 0.0012% of AIC solution (83/μg/kg) for 12 weeks. As a result, the ovariectomized group treated with AIC had higher BMD (0.2276±0.012 mg/cm^2) rates as compared to the group that was not treated with AIC (0.1965±0.012 mg/cm^2). Moreover, the ovariectomized group treated with AIC had higher 17β-estradiol, lower osteocalcin, and Type I Collagen C-terminal Telopeptides (CTx), which result in an increase in bone turnover.

As AIC solution is registered as a dietary supplement in Canada, the United States, Republic of Korea, Indonesia and Japan, more than 100,000 patients with osteoporosis have been trialing it since 2012 and showing a significant improvement in laboratory BMD tests. In referring to one of the postmenopausal osteoporotic patient's data, after treating 5 to 15 mg of AIC orally every day for six months, her T-score increased to -0.8 from -3.4 which was profoundly osteoporotic. While there are no significant treatments available for patients with osteoporosis, the AIC solution can be seen as an innovative one, since it directly increases the serum calcium level as well as the intracellular calcium level, and decreases parathyroid hormone (PTH) secretion, which prevents unnecessary bone resorption. Moreover, as it has no adverse effect due to only a small amount of calcium being ingested to trigger calcium ion concentration in the intracellular membrane, it can be an active form of treatment for osteoporosis in the future.

Group	Estradiol (pg/ml)	Osteocalcin) (ng/mL)	C-terminal telopeptide
Sham	4.536±0.343 [a]	3.398±0.294 [a]	.244 [a]
OVX	3.920±0.277 [a]	4.047±0.501b [b]	.462 [ab]
OVX+AIC	4.217±0.247 [a]	3.558±0.154 [ac]	495 [a]

Table 3. 17ß-Estradiol concentration and bone metabolism markers of ovariectomized rats treated with AIC for 12 weeks. Adapted from "Effects of AIC on bone turnover and calcium balance in ovariectomized rats," by S.Y. Choi *et al*., 2011, *LAboratory Animal Research, 27(4)*, p. 305. Copyright 1985 by Korean Association for Laboratory Animal Science.

Group	Breaking force (kg)	Ash (mg/dL)	Ca (mg/dL)	P (mg/dL)
Sham	7.113±0.035 [a]	428.2±17.34	140.8±2.48 [a]	138.0±3.16 [a]
OVX	7.002±0.106 [b]	409.5±8.39	136.0±3.10 [b]	130.3±7.72 [ab]
OVX+AIC	7.1220±0.064 [a]	419.8±8.76	147.5±5.33 [c]	138.1±5.39 [a]

Table 4. Breaking force and values of ash, Ca and P of ovariectomized rats treated with AIC for 12 weeks. Adapted from "Effects of AIC on bone turnover and calcium balance in ovariectomized rats," by S.Y. Choi *et al*., 2011, *Laboratory Animal Research, 27(4)*, p. 305. Copyright 1985 by Korean Association for Laboratory Animal Science.

Organ (unit)	Group		
	Sham	OVX	OVX+AIC
Body weight (kg)	310.84±11.14	353.75±10.18	371.36±9.97
Uterus(g)	0.1889±0.05	0.0381±0.01	0.0444±0.02
Vagina(g)	0.1128±0.03	0.0501±0.02	0.0575±0.02
Liver (g)	2.3435±0.15	2.1675±0.10	2.1820±0.15
Kidney			
Right (g)	0.3236±0.02	0.2682±0.00	0.2626±0.02
Left (g)	0.3289±0.03	0.2712±0.01	0.2687±0.01
Spleen (g)	0.1840±0.02	0.1692±0.02	0.1856±0.02
Adrenal gland			
Right (g)	0.0127±0.0024	0.0093±0.0012	0.0094±0.0014
Left (g)	0.0128±0.0020	0.0092±0.0008	0.0094±0.0014
Brain	0.65550±0.03	0.6044±0.03	0.5526±0.02
Pituitary (mg)	0.0055±0.00005	0.0038±0.0002	0.0042±0.0010
Femur			
Right (g)	0.3296±0.02	0.2960±0.02	0.2902±0.02
Left (g)	0.3524±0.03	0.2930±0.01	0.2935±0.02

Table 5. The absolute organ weights of ovariectomized rats treated with AIC for 12 weeks. Adapted from "Effects of AIC on bone turnover and calcium balance in ovariectomized rats," by S.Y. Choi *et al.*, 2011, *Laboratory Animal Research, 27(4)*, p. 305. Copyright 1985 by Korean Association for Laboratory Animal Science.

iii. The Classification of Osteoporosis and the Cause of Disease Development

Osteoporosis is classified into two different types: Type I osteoporosis and Type II osteoporosis. Type I osteoporosis is mostly seen among women who have experienced postmenopause and Type II osteoporosis is mainly observed among men with hypogonadism. According to Funaro *et al.* (2013), hypogonadism is "one of the main risk factors for osteopenia and osteoporosis, which can be found in 8% of hypogonadal men younger than 50 years of age." (p. 19) As Chou *et al.*, (2016) investigated in their article, "older age (> 55 years), and low ERα mRNA levels in PBLs (≦ 250.39 copies/μg DNA) were associated with an approximately 9.188-, and 31.25-fold risk of osteoporosis." (p. 124) Whilst many scientists have assumed that osteoporosis is related to the alteration of sex steroids, the exact cause of the disease is still unknown. Moreover, a large number of scientists have aggressively researched the pharmaceutical medication for osteoporosis. However, none of the pharmaceutical options have shown significant efficacy for treating osteoporosis, and while the global population is getting older, the global economic burden of osteoporosis and osteoporotic fractures is increasing immensely.

iv. The Current Osteoporosis Treatment

a. Bisphosphonates

Currently, Bisphosphonate is the most frequently prescribed medication for osteoporosis. There are many different types of Bisphosphonate treatments in the market such as Alendronate, Risedronate, Ibandronate, Zoledronic Acid, etc. Bisphosphonate mimics pyrophosphate and acts as an antagonist, inhibiting activation of the enzyme that utilizes pyrophosphate. It also preferentially binds to calcium and promotes its storage in bones, strengthening bone health by increasing Bone Mineral Density (BMD). According to the National Osteoporosis Foundation (2018), some side effects have been observed in patients who were prescribed Bisphosphonates. Commonly, they complained of "joint or muscle pain, nausea, difficulty swallowing, heartburn, irritation of the esophagus and gastric ulcers." (NOF,

2018) Moreover, Bisphosphonates are not recommended for patients with low calcium levels, because some reports have indicated that low calcium levels may exacerbate symptoms of the disease. (NOF, 2018) Also, Bisphosphonates are not recommended for patients with severe kidney disease, because it can worsen the kidney calcification, which may cause nephrocalcinosis. (NOF, 2018)

Moreover, Green *et al.* (2010) have found out that "prescribing of [oral] Bisphosphonates over a period of about five years was associated with a doubling of the risk of esophageal cancer." (p. 545) Also, Shane (2010) claimed that long-term use of bisphosphonates can cause over-suppression of bone turnover, which can exacerbate the symptoms of osteoporosis. Moreover, Kennel & Drake (2009) have mentioned in their article, "this association was thought to be due to erosive esophagitis resulting from the suboptimal administration in patients who failed to maintain an upright posture for 30 to 60 minutes after ingesting medication with a full glass of water." (p. 633). As they (Kennel & Drake, 2009) have argued in their article, the dropout rates from the medication treatment is higher among patients with osteoporosis, especially for those who are prescribed bisphosphonates. Because of its complicated ingestion, the improper intake of the medication resulted in the decrease in the efficacy of the drug.

Through literature reviews for the efficacy of Bisphosphonates for patients with osteoporosis, it is confirmed that bisphosphonates have many adverse effects that can even exacerbate the symptoms of osteoporosis. Moreover, as the medication ingestion method is very complicated to follow, high dropout rates are observed. Therefore, we see an urgent need in finding alternative treatment for osteoporosis with much fewer side-effects and an easier consumption method. For this reason, AIC would be highly recommended, as its efficacy has been proven in increasing BMD and decreasing bone turnover rates. Furthermore, as AIC only contains the small number of calcium carbonates that can trigger calcium homeostasis in the body, there are much fewer side effects as compared to other calcium supplements in the market.

Figure 15. The Bisphosphonates Medication that are Currently Used.

b. SERMS (Estrogen Agonist/Antagonist)

Selective Estrogen Receptor Modulators (SERMS) are commonly prescribed for those who are suffering from postmenopausal osteoporosis, and it works by stimulating estrogenic action in bones. However, it is not recommended for women who were previously diagnosed with breast and uterine problems, because many researchers have shown the possible side effects of SERMS for those who have that medical history. There are many types of SERMS in the market. Raloxifene is the most prescribed medication for patients with postmenopausal osteoporosis. Bazedoxifene and Lasofoxifene are also commonly prescribed. While Raloxifene is specified for its efficacy to increase lowered serum estrogen level, its long-term use increases the risk of establishing endometrial cancer by two to three times. (Maximov, Lee & Jordan, 2013) Moreover, Bazedoxifene showed an increase in the risk of having venous thromboembolism. (Pickar & Komm, 2015) Since SERMS only targets repression of the serum estrogen level, it cannot control the entire homeostasis of calcium in the body. Therefore, it is necessary to find an alternative approach to reviving the underlying mechanism of the bone loss in postmenopausal women. For this reason, AIC can be a distinctive option to treat postmenopausal osteoporosis, while it triggers calcium homeostasis in the body. As it helps to balance the serum and intracellular calcium concentration, it can increase bone mineral density as well as the quality of bones.

Figure 16. The SERMS Medication that are Currently Used.

c. Teriparatide (Recombinant PTH)

Teriparatide is the recombinant form of parathyroid hormonal treatment. There are few different teriparatide medications, and one of the well-known parathyroid hormone (PTH) medications is called Abaloparatide. It acts as an anabolic agent to initiate bone growth, and it is identical to the form of PTH. It is used to activate more osteoblasts than osteoclasts and, increases bone formation. This type of medication also manifests adverse side effects such as such as limb pain, nausea, dizziness, and headache. (Rizzoli *et al.*, 2011) Moreover, as Bazaldua & Bruder (2004) have found in their research, "[PTH] causes osteosarcomas in rats, but the relevance to humans is unknown." (p. 1983) Even though it has not been extensively studied whether PTH may cause osteosarcoma in humans, the potential carcinogenicity of the drug may question the safety of PTH medication for some patients. Furthermore, patients who are suffering from Paget's disease of bone or open epiphysis or sudden elevation of serum alkaline phosphatase, as well as patients under radiation therapy involving bones, should not be prescribed Teriparatide since it can exacerbate the symptoms. (NOF, 2018) For this reason, it is crucial to investigate a treatment option that regulates both thyroid hormone (TH) and parathyroid hormone (PTH) to regulate bone absorption and resorption rates. Currently, PTH hormone only increases the bone absorption rate by forming osteoblasts; it cannot influence the bone turnover rate, which is

required for improving the quality of bone. Therefore, AIC is recommended for the new form of osteoporosis treatment, because while it triggers ionized calcium to be circulated in the body and regulates bone absorption and reabsorption rates, it can treat the fundamental cause of osteoporosis.

Figure 17. The Teriparatide Medication that are Currently Used.

d. Calcitonin

Primarily, calcitonin is produced by the thyroid gland, mainly by parafollicular cells that exist in the thyroid gland. Calcitonin reduces serum calcium, and it opposes the action of PTH. It is not commonly prescribed like any other osteoporosis medications. However, some physicians prescribe calcitonin to patients with Type II osteoporosis. It mainly acts to inhibit the activity of osteoclasts and for the minor effects, it inhibits tubular cells in the kidney and prohibits calcium and phosphate resorption by helping urine excretion. (Potts & Jüppner, 2008) However, as Carney (1997) has found, high calcitonin concentration in blood can increase urinary calcium and phosphate excretion, which causes hypocalcemia. Moreover, as the kidney becomes resistant to calcitonin, unregulated excretion of calcium would occur, especially to those patients who are suffering from thyroid tumors, which excrete excessive amounts of calcitonin. Also, Woo & Adachi (2001) have found that calcitonin showed "minimal changes in bone density." (p. 469)

Therefore, AIC could be an alternative option for osteoporosis treatment because as it increases both BMD and quality of bone by regulating bone turnover rate, it would be highly beneficial to treat patients who are suffering from the symptoms related to osteoporosis.

Figure 18. The Calcitonin Medications that are Currently Used.

v. AIC and Clinical Data

By using BeamMed™ MiniOmni™, we have conducted the Distal Radius Test Method, and to avoid experimental bias, we held the test three times. Moreover, we also used the Ultrasound BMD Tester to get more accurate results. Whilst women who are aged 55 years and older and have previously experienced post-menopause are at the highest risk for having Type I osteoporosis, we have investigated the BMD data for women who are aged 55 years and older after treating them with Anti-orbital Ionic Calcium Carbonate (AIC) for several months. The data below depict the difference in BMD after handling with AIC.

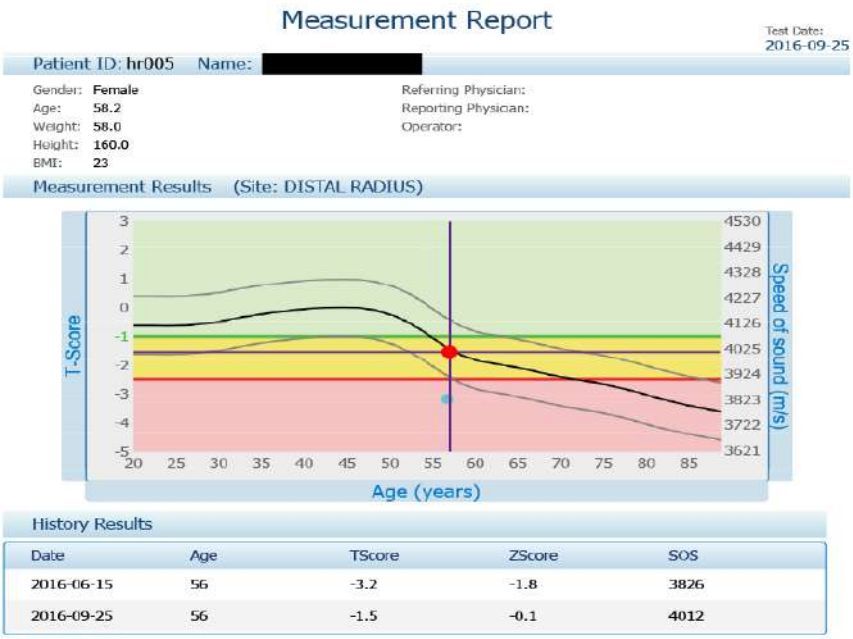


Figure 19. The BMD Change in an Asian Woman, Aged 56 Years Old After treatment with AIC for 3 Months.

Figure 20. The BMD Change in an Asian Woman, Aged 60 Years Old After treatment with AIC for 3 Months.

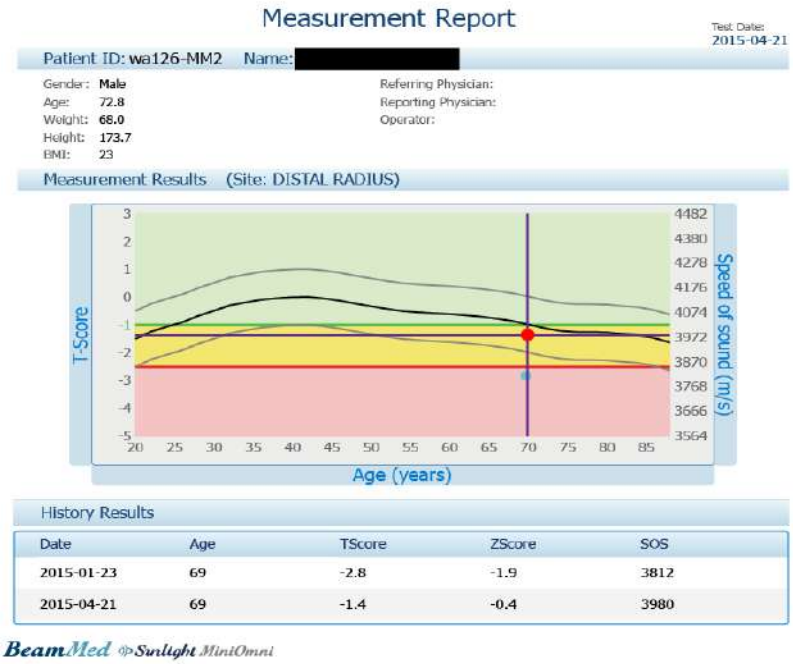


Figure 21. The BMD Change in an Asian Woman, Aged 69 Years Old After treatment with AIC for 3 Months.

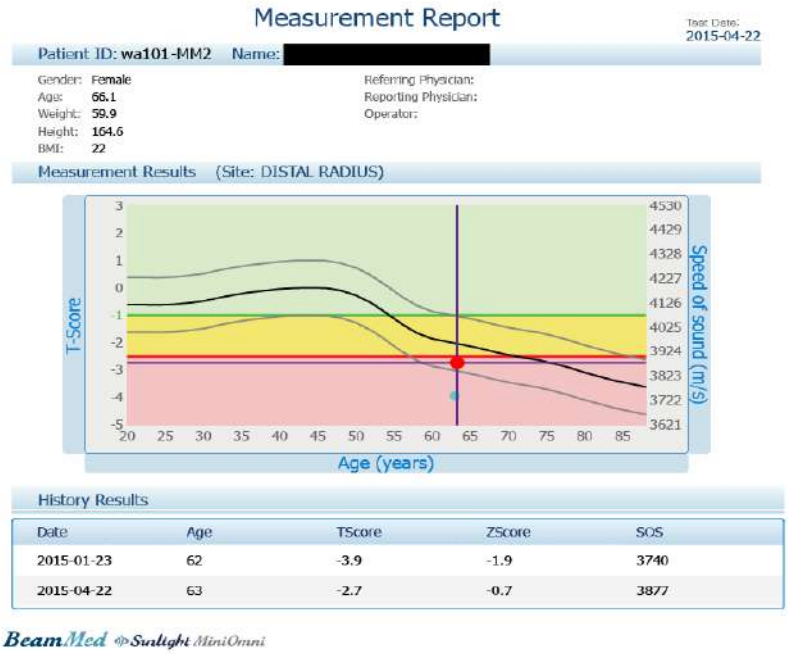


Figure 22. The BMD Change in an Asian Woman, Aged 62 Years Old After treatment with AIC for 3 Months.

IV. AIC and Dental Bone Health

"How to Increase BMD Without Bone Grafting? AIC Calcium Therapy Can Be an Option for Enhancing Dental Bone Health by Maintaining Periodontium and Pulpitis Healthily."

i. Introduction

With the advancement of medicines and dental technologies, the life expectancy of the world's population has considerably increased over the past few decades. As rising number of people live longer, and the quality of life of elders has gained much attention as with demands for improvements. With growing concerns about age-related diseases, oral health also accounts for a large part of the quality of life of elders.

Figure 23. Dental Bone Health Relates to Quality of Life.

Edentulism in the elderly population around the world is between 11% and 44%, inversely increasing to socioeconomic status. (Gaetti *et al.*, 2011) The use of implants is considered to be one of the most popular alternatives for its high predictability, durability, and functionality to rehabilitate patients

aesthetically and functionally. (Gaetti *et al.*, 2011) Moreover, loss of alveolar bone due to periodontitis is also a significant concern for all age groups, as it supports not only possible implants, but also natural teeth. (An *et al.*, 2012; Bosshardt, 2005)

ii. Type II Diabetes and Dental Bone Health

It is also very critical to figure that a growing number of patients with systemic disorders such as diabetes mellitus seek dental treatment. Such patients are associated with an increased risk of osteoporotic fractures, and depending on the type of diabetes, bone formation and osteoblast function are impaired. (Hamann *et al.*, 2012) Type II diabetes patients may have increased BMD. However, their bone quality is significantly reduced. (Hamann *et al.*, 2012) A study done by Abbassy, Watari & Soma (2010) suggests that uncontrolled diabetes mellitus decreases mandibular bone formation, reduces the rate of bone turnover in the alveolar wall, and affects the quality of bone. (Abbassy, Watari & Soma, 2010)

iii. Current Issues When Considering Dental Implants

When considering dental implants, there are two major types of treatment. For many years, subperiosteal implants were used successfully. However, this solution is no longer favored for its significant disadvantages such as its substantial surgical procedures, extensive fabrication requirement, and superstructure castings. (Von Fraunhofer, 1976) Even with endosseous dental implants being a significant part of prosthodontics and restorative dentistry and its advances in technology, the concern for potential clinical failure is still substantial for both dentists and patients. (Porter & Von Fraunhofer, 2005) In response to the interests of treatment implications, though, prosthetic design mainly governs the surgical placement of the endosseous implant, morphology, and quality of the alveolar bone are crucial to its success. (Wikesjo *et al.*, 2001) Also, the alveolar processes of the maxilla and mandible provide support for the teeth and the changes in these bones by osteoporosis affect tooth retention and the stability of dental implants as well as alveolar bone height. (Gaetti, 2011; Wactawski, 1996)

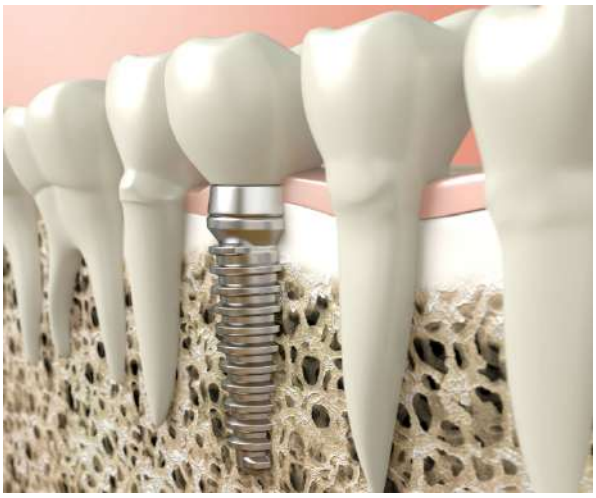


Figure 24. Dental Implants and Bone Health.

iv. The Relationship between Calcium and Periodontal Ligament Cell Facilitation

Furthermore, another crucial factor to consider in dental health is human periodontal ligament cells (hPDLCs). Human periodontal ligament cells reside within the periodontal ligaments and can differentiate into either cementoblasts to synthesize root cementum or osteoblasts to synthesize alveolar bone. (McCulloch, 1996) Koori *et al.* (2014) investigated the effects of extracellular calcium on cell proliferation and osteogenic differentiation of human periodontal ligament cells (hPDLCs). In the human PDLSC line they examined, calcium treatment increased the expression of genes of bone-related molecules such as BMP-2, OCN, OPN, and RUNX2, and also resulted in mineralization. (Koori *et al.*, 2014) These genes are found to facilitate the periodontal ligament cells (Ivanovski *et al.*, 2001) and are regulated by RUNX2. (Lee *et al.*, 2000) Also, increased concentrations of Ca^{2+} and P were observed to enhance cell proliferation, differentiation, and mineralization of hPDLCs. (An *et al.*, 2012)

v. AIC as a Treatment for Enhancing Dental Bone Health

In an effort to draw the effect of AIC on the restoration of the periodontium, we emphasize its outcome on the systemic regulation of

hormones associated with calcium homeostasis. It is widely known that significant regulators of the calcium homeostasis, and therefore of bone metabolism, are parathyroid hormone (PTH) and thyroid hormone (TH). (Liu *et al.*, 2016) As mentioned earlier, recombinant parathyroid hormone (PTH) is currently used as an alternative to anti-resorptive treatments for osteoporosis. In a rabbit model of osteoporosis, Bellido *et al.* (2010) show that administration of PTH almost wholly reversed the fall on bone mass density and calcium content in the peri-alveolar region, as a result reducing global mandibular bone loss. (Bellido *et al.*, 2010)

vi. AIC as a Trigger for Calcium Homeostasis: The Key for Improving Dental Bone Health

Moreover, the experimental study done by Li *et al.* (2017) suggests that PTH also promotes cementum formation by activating several cementogenesis- and differentiation-related biomarkers. Cementum formation and its repair process by cementoblasts (Bosshardt, 2005) are essential as it plays a crucial role in the development of functional periodontal ligaments. (Li *et al.*, 2017) It was found that intermittent low-dose PTH enhanced the mineralization capacity of cementoblasts and therefore promoted cementum regeneration. (Li *et al.*, 2017) However, it must be noted that dosage and duration of administration are essential details as PTH can apply both anabolic and catabolic effects on bone. (Liu *et al.*, 2016; Kasagi & Chen, 2013; Li *et al.*, 2017) AIC initiates systemic regulation of both thyroid hormones and parathyroid hormones involved in calcium homeostasis which, in turn, stabilizes the absorption and resorption of bones. Therefore, the systemic control of thyroid hormones by AIC can efficiently be used to restore the loss of bone in the peri-alveolar region as well as cementum formation in the periodontal area.

vii. AIC as an Initiator to Stimulate Mesenchymal Stem Cells for Growth of Dental Bone

There exist many pieces of research that reiterate the importance of calcium ion and its balance in maintaining and reviving bone health. In many

cases, synthetic bone replacement is used to fill and restore alveolar bone. (Caffesse, 2002) Targeting the underlying condition of calcium and hormone imbalance is essential. AIC as a physiological calcium ion triggers the systemic regulation of hormones and therefore calcium homeostasis and revives the core metabolism. Moreover, as Egusa *et al.* (2012) have found, "crosstalk between implanted donor cells and recipient immune cells plays a key role in determining clinical success that may involve the recently observed immunomodulatory properties of Mesenchymal Stem Cells (MSCs)." (p. 229) Also, they added, "CaP-based biomaterials have significant potential for bone regeneration." (p. 241) Therefore, is crucial to recommend additional calcium supplement intake for patients who are considering dental implants; as it improves dental bone regeneration.

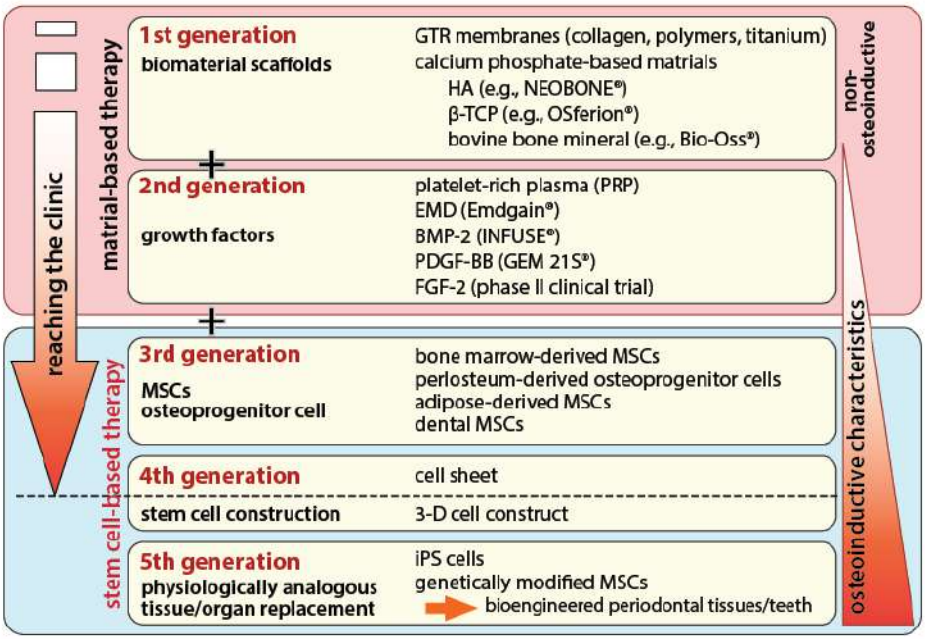


Figure 25. Progress in regenerative periodontal/bone therapies. Regenerative periodontal/bone therapies are broadly categorized as material-based therapies (first-generation biomaterial scaffold-based approach and second-generation growth-factor-based approach) and stem-cell-based therapies (third-generation MSC/osteoprogenitor cell-based approach, fourth-generation stem-cell construction-based approach, and fifth-generation physiologically analogous tissue/organ-replacement approach). Adapted from "Stem cells in dentistry – Part II: Clinical applications," by H. Egusa *et al.*, 2012, *Journal of Prosthodontic Research, 56 (4)*, p. 231. Copyright 2012 by the Elsevier Ireland.

viii. Case Study for AIC Treatment in Dental Bone Health

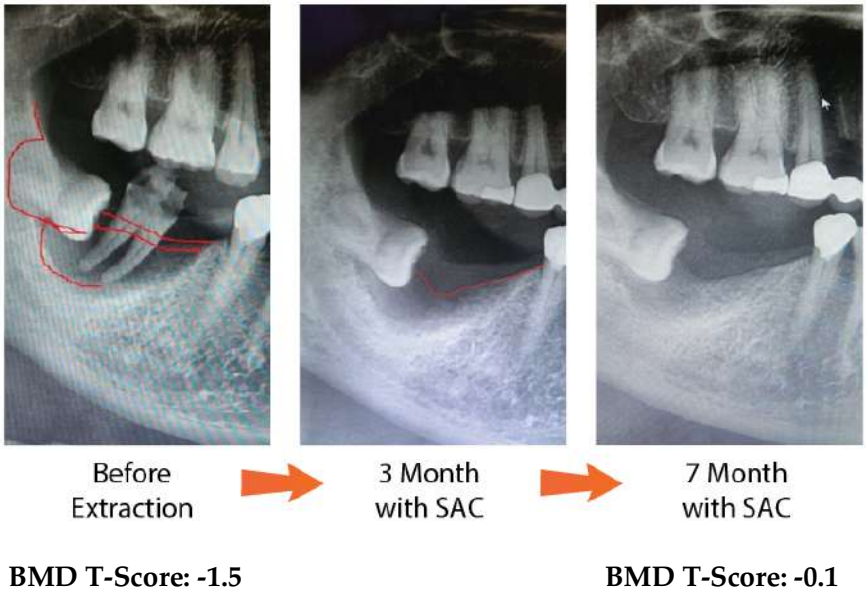


Figure 26. The X-Ray Image for an Asian Male Who is 58 Years Old, Conducted Molar Tooth Extraction; The Dental Bone Growth After Treating AIC for 7 Months without any Cotreatment.

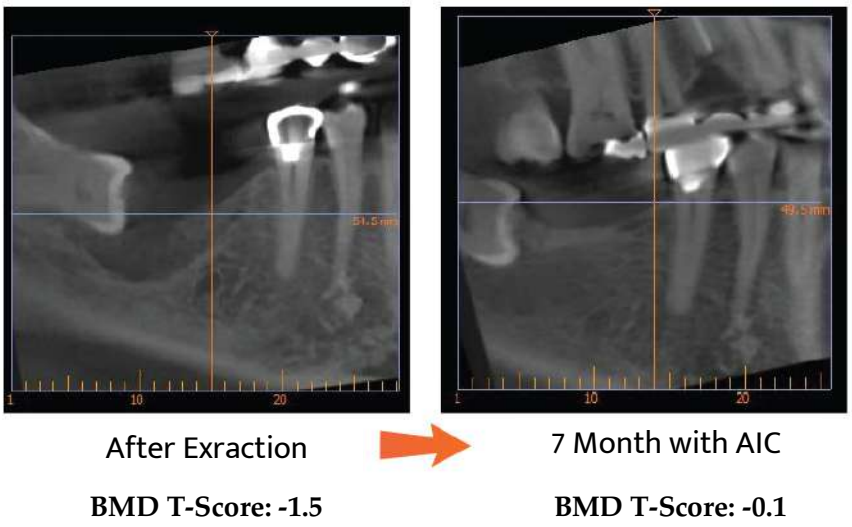
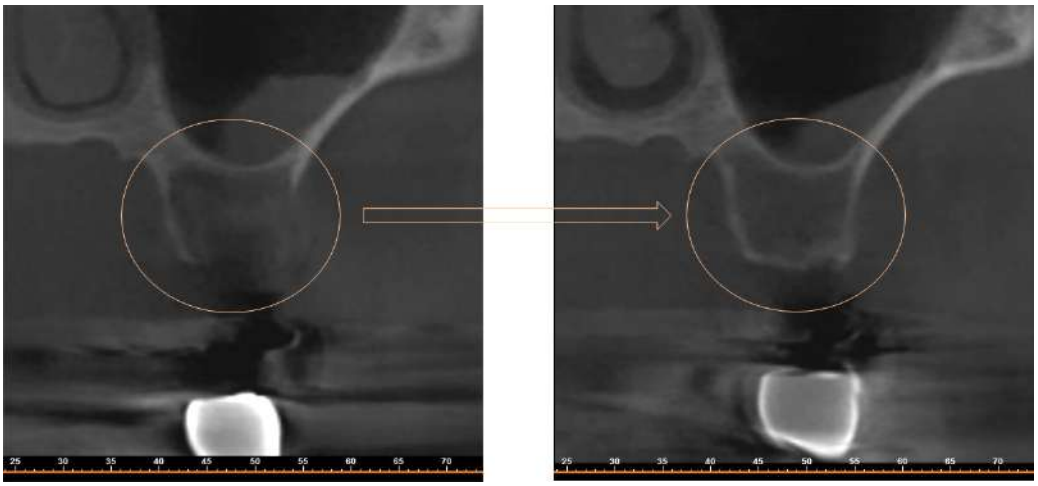


Figure 27. The X-Ray Image for An Asian Male Who is 59 Years Old Showed Dental Bone Growth After Treating AIC for 7 Months Without Any Treatment. (The T-Score for the Dental Bone is Significantly Improved)

Before (06.21.2017) **After (03.20.2018)**



BMD T-Score: -1.5 **BMD T-Score: -0.3**

Figure 28. The X-Ray Image for an Asian Male Who is 58 Years Old Showed Dental Bone Growth After treating AIC for 10 Months without any Cotreatment. (The T-Score for the Dental Bone is Significantly Improved)

ix. AIC and Gingivitis and Periodontal Disease (Gum Disease)

"Lack of Calcium Level Results in Severe Gum Disease. So, by treating with AIC, Dental Bone Health Can be Improved."

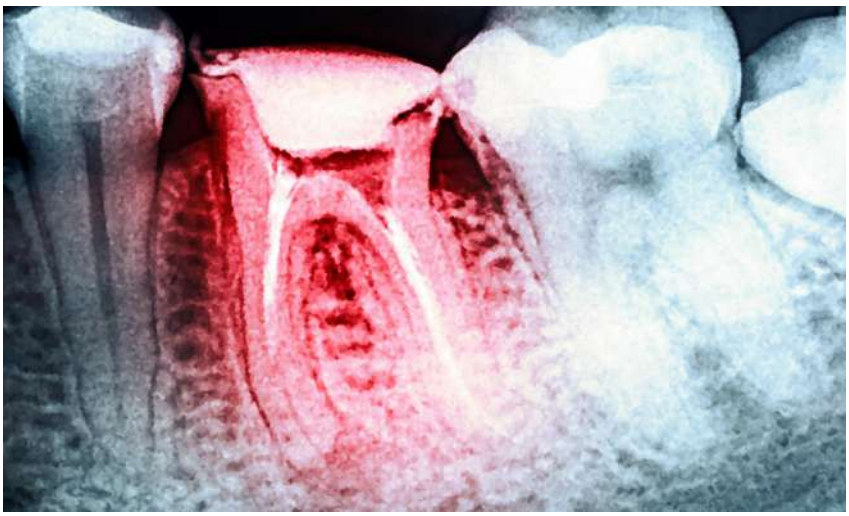


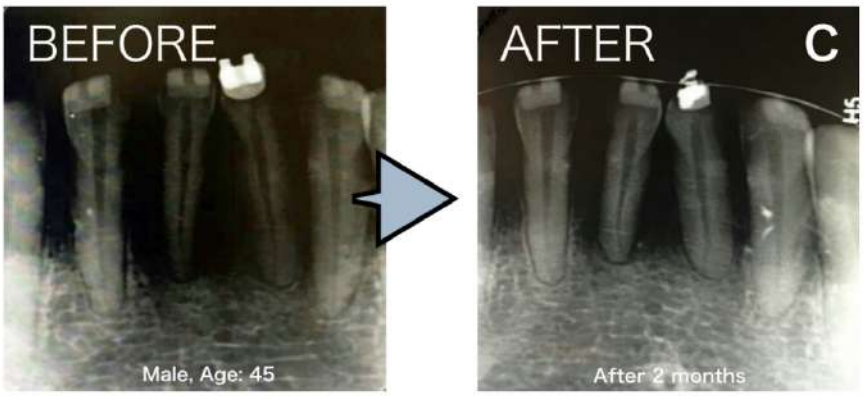
Figure 29. The Gum Disease Image.

It is well-known that one-third of the population in developed countries are suffering from gum and periodontal disease. (ADHA, 2008) Both oral health and general health are affected by the food you choose to eat and how often you consume it. Bacteria cause gingivitis or periodontal dental disease, which can destroy the tissue that surrounds the teeth. The difference between gingivitis and periodontitis is that periodontitis is usually precedent from the gingivitis; but not all gingivitis progresses to periodontitis. In the early phase of gingivitis, the bacteria in the plaque causes inflammation on the gums to bleed even while tooth brushing easily. (Abbas *et al.*, 1986) The teeth are still firmly planted in their socket even though the gums may be irritated. In this phase, there are no evident signs of irreversible bone or other tissue damage. Gingivitis advances to periodontitis when it is not treated. It is prominent in the periodontitis patients that the inner layer of the gum and bone have pulled away from the teeth and formed pockets. The formed pockets between

teeth and gums collect debris and can cause infection. The immune system fights with the bacteria which grows beneath the gum line.

a. The Cause of Gum Disease and AIC Treatment

Plaque is the primary cause of gum disease. However, changes in hormone during pregnancy, purity, menopause, and menstrual period can cause gums to be sensitive, which can lead to periodontal disease. (Rajesh *et al*., 2016) Moreover, diseases like HIV and cancer can interrupt the immune system. Type II Diabetes patients are especially vulnerable to gum disease because the disease is affected by the amount of sugar uptake. (Mansour & Abd-Al-Sada, 2005) Therefore, Type II Diabetes patients are at higher risk of developing infections including periodontal disease and cavities. Medications can interfere with oral health, because some reduce the flow of saliva, which has a protective effect on teeth and gums. Drugs like anticonvulsant medication, "Dilantin and Adalat can cause abnormal growth of gum tissue." (Darley, n.d.) Moreover, smoking can prevent gum tissue from repaired itself. Without the constant effort to brush and floss, gingivitis disease can quickly develop. (Drocolinrichman, 2014) Family history of dental disease can be related to the development of gingivitis. (Medical News Today, 2018) Until now, there have been many gingivitis and periodontal disease treatments in the market but these did not show remarkable improvement in the condition of the patient. Calcium deficiency or unbalanced level of ionic calcium can be contributing factors to gum and periodontal diseases. Therefore, AIC calcium helps balance the calcium level to prevent growth of bacteria in the gum and improves the gingivitis and periodontal health.



T-Score: -2.4 **T-Score: -1.5**

Figure 30. The X-Ray Image for an Asian Male Who is 45 Years Old Showed Dental Bone Growth After treating with AIC for 2 Months with splint only (The T-Score for the Dental Bone and his bad gum health is Significantly Improved.)

V. AIC in Mitochondria and Stem Cell

"Anti-Orbital Ionic Calcium Carbonate: Helps to Regenerate Mitochondrial Function and Initiate Mesenchymal Stem Cell Activity Which is the Key to Treat Chronic Neurodegenerative Diseases, Including Cancer."

i. Introduction

Within the past ten years, many significant pieces of research have been conducted to investigate the relationship between mitochondrial dysfunction and the development of chronic neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's Disease (PD) and Alzheimer's Disease (AD). Recently, there were also notable findings that increased influx of calcium ion causes excitotoxicity of mitochondria, which results in chronic neurodegenerative diseases. Therefore, it is critical to balance up the ionized calcium concentration in the cytosol and mitochondrial matrix to prevent over-influx of calcium concentration in the mitochondrial matrix, which causes the mitochondrial dysfunction. Moreover, mitochondrial dysfunction increases the production of Reactive Oxygen Species (ROS), which affects alteration of RNA synthesis, which mutates mitochondrial proteins such as SOD1, FUS, and TDP-43. The misfolding of the proteins would alter the permeability for ionized calcium. Therefore, as the calcium buffer system is collapsed, calcium excitotoxicity would occur, which is the primary cause of the development of chronic neurodegenerative diseases. Thus, it is crucial to regulate the ionized calcium concentration in the cytosol and mitochondrial matrix.

To control the calcium homeostasis, regulating thyroid hormone (TH) and parathyroid hormone (PTH) is also essential to solving imbalance of calcium homeostasis. While mitochondrial dysfunction results in chronic

neurodegenerative diseases due to the alteration of RNA, DNA and protein synthesis, it is also crucial to regenerate mitochondrial function by triggering depolarization of the mitochondrial intracellular membrane. As Duchen (2000) has insisted in their article, calcium uptake depends on mitochondrial membrane potential, which is followed by transient mitochondrial depolarization. Therefore, calcium signaling should be switched on by exposing the small amount of calcium in cytosol that activates the action potential in the mitochondrial matrix. Impaired mitochondria can be recovered through the regeneration of calcium depolarization by increasing the permeability of calcium in the mitochondrial matrix. As mitochondrial function is regenerated, more ATP would be produced which may initiate the Mesenchymal Stem Cells (MSC); and this can be the key to treat chronic neurodegenerative diseases. For instance, as ALS occurred due to the dysfunction of mitochondria in muscle cells, if the mitochondria are regenerated and able to produce sufficient amount of ATP, it can activate MSC, which are located in muscle tissue. Therefore, as the muscle cells are regenerated, it can treat the symptoms of ALS. Since the problem is how to regulate calcium homeostasis in the body, we can assert that AIC can act as a trigger to initiate calcium homeostasis. As Choi *et al.* (2011) have found out, AIC has shown its efficacy to regulate calcium homeostasis by proving the change in Bone Mineral Density (BMD) in mice; which is resulted by the secretion of TH and PTH hormones.

ii. Mitochondrial Dysfunction and the Development of Chronic Neurodegenerative Diseases

Damiano *et al.* (2006) have found in their research that poor calcium uptake mutates SOD1, which ultimately causes defection in spinal cord and brain tissue. Moreover, Nguyen *et al.* (2009) investigated that mitochondrial inner membrane depolarization depends on "calcium influx into motor terminals." (p. 2009) By analyzing their experimental results, it seems clear that mitochondrial defect can mutate protein uniporter. Mutated protein uniporter such as SOD1 can cause the alteration of calcium uptake into the mitochondrial matrix. Elevated intracellular calcium concentration contributes to excitotoxicity in mitochondrial membrane (Kawamata &

Manfredi, 2010) and it ultimately leads a mitochondrial dysfunction. Moreover, mitochondrial dysfunction elevates ROS production (Wei *et al.*, 1998), which can cause damage to lipids, DNA, and proteins; this ultimately causes aging-related chronic degenerative diseases. (Lin & Beal, 2006) Therefore, it is crucial to regenerate mitochondrial dysfunction.

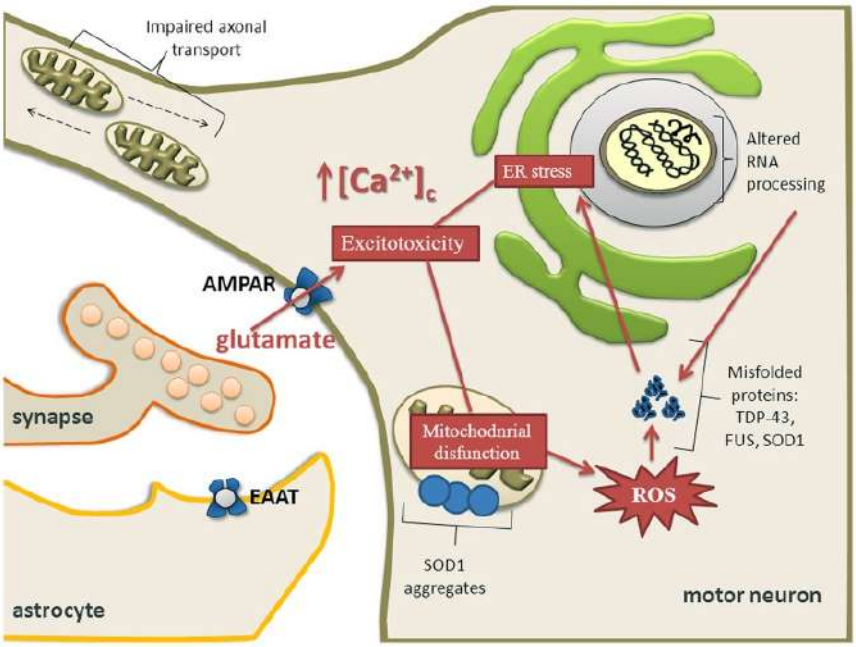


Figure 31. Calcium dysregulation, ER stress and mitochondrial impairment are major components of excitotoxicity in motor neurons. Mitochondrial dysfunction causes activation of proteolytic and ROS generating enzyme systems. Mutant SOD1 forms insoluble aggregates in mitochondria at the surface of the outer membrane. Motor neurons might also undergo transcriptional dysregulation and abnormal RNA processing which together with depleted ER Ca2+ levels and overproduction of ROS contribute to aberrant protein folding. Aberrant proteins form aggregates leading to ER stress and ultimately activating apoptotic pathways, especially when the unfolded protein response is exhausted. Impaired axonal transport may also contribute to an energy deficit in the distal axon and the dying back axonopathy that is observed in ALS. Adapted from "The ER mitochondria calcium cycle and ER stress response as therapeutic targets in amyotrophic lateral sclerosis," by V. Tadic *et al.*, 2014, *Frontiers in Cellular Neuroscience, 8 (147)*, p. 10. Copyright 2014 by PMC.

Amyotrophic Lateral Sclerosis (ALS) is a well-known chronic neurodegenerative disease that is caused by calcium excitotoxicity in the intracellular mitochondrial matrix. Kawamata & Manfredi (2010) mentioned

that the relationship between calcium dysregulation, mitochondria dysfunction, and neuronal cell death is the key to understanding ALS. They also stated that mutation of SOD1 is the key to explaining the occurrence of ALS. Initially, SOD1 works as the antioxidant to remove superoxide, which is produced in the respiratory system. Mutation of SOD1 results in mitochondrial dysfunction, which is impaired calcium handling. (Kawamata & Manfredi, 2010) It leads to disturbance of homeostasis of calcium ion. Imbalance of calcium ion levels results in neuronal cell death which occurs in ALS.

Moreover, Lüdtemann & Abramov (2018) have mentioned that calcium dysregulation has a significant relationship with Parkinson's disease. This research shows that mitochondrial calcium ion overload and dysfunction is related to PD-risk genes. One of the PD- risk genes is a-synuclein. Activation of Alpha-synuclein induced calcium ion dysregulation and oxidative stress. Another one is PTEN-induced putative kinase 1 (PINK-1). "Ca^{2+} extrusion in PINK-1 deficient neurons were severely inhibited, resulting in mitochondrial Ca^{2+} overload, increased reactive oxygen level species production, PTP opening, and ultimately neuronal cell death." (Lüdtemann & Abramov, 2018, p. 88) The mechanism of PD-risk genes hinders efflux of calcium ion and ultimately leads to cell death, which occurs in Parkinson's disease.

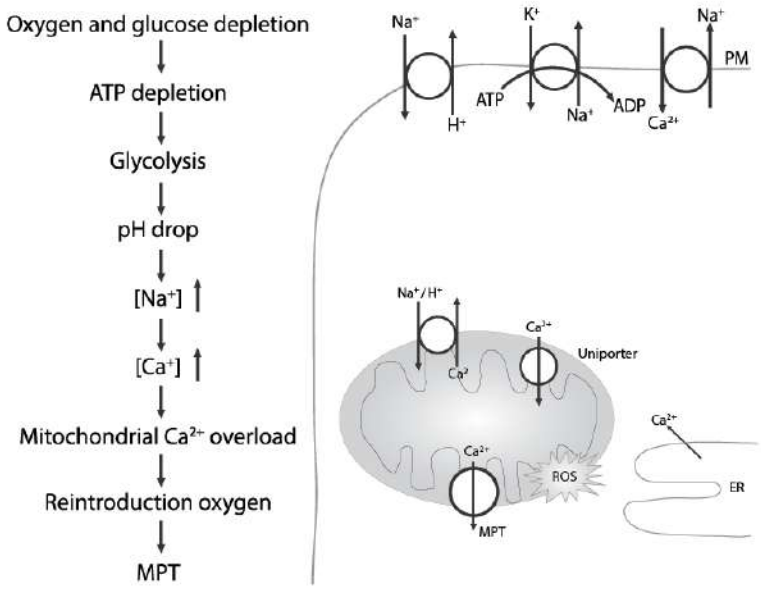


Figure 32. Mechanisms of mitochondrial calcium overload and subsequent mitochondrial permeability transition. Depletion of oxygen and glucose during ischemia induces the shutdown of oxidative phosphorylation and results in ATP depletion. The consequent increase in anaerobic glycolysis lowers pH. To counteract this pH drop, the cell activates the Na^+/H^+-antiporter, causing an increase in Na^+. The excess Na^+ cannot be pumped out via the Na^+/K^+-ATPase because of reduced ATP availability. So, the cell becomes loaded with Ca^{2+} due to reversal of the Na^+/Ca^{2+}-antiporter. In addition, cytosolic Ca^{2+} can also be elevated by Ca^{2+} influx from the ER. Calcium entering the mitochondria via the Ca^{2+}-uniporter cannot be pumped out by the mitochondrial Na^+/Ca^{2+}-antiporter due to saturation, and the result is mitochondrial calcium overload. Reperfusion re-introduces oxygen, the damaged mitochondria produce a burst of ROS, and intracellular pH is restored. These changes, in combination with the calcium overload and other factors, induce MPTP. Adapted from "Molecular Mechanisms and Pathophysiology of Necrotic Cell Death," by N. Vanlangenakker *et al.*, 2008, Current Molecular Medicine. *8(3), p. 209.* Copyright 2008 by Bentham Science Publishers.

iii. AIC and Calcium Homeostasis

AIC plays a critical role when ingested into the blood system, because it acts as a hormone trigger, which initiates calcium homeostasis. Calcium homeostasis is essential to trigger calcium influx in the intracellular mitochondrial matrix. As Kawamata & Manfredi (2010) have insisted, calcium homeostasis maintenance is required to make calcium influx dynamic. In other words, to initiate mitochondrial function and regulate

calcium influx and efflux in the mitochondrial matrix, controlling the secretion of thyroid hormone (TH) and parathyroid hormone (PTH) is essential. Moreover, Loskutova *et al.* (2009), have found that bone mineral density (BMD) is linked to the "bone loss in early Alzheimer's disease." (p. 1) According to their research, in the early stage of Alzheimer's disease, patients have shown significant bone loss. (Loskutova *et al.*, 2009) Moreover, brain atrophy and memory loss have frequently been observed among patients who experienced early symptoms of Alzheimer's disease. (Loskutova *et al.*, 2009) Through their research, we could assume that if AIC could increase BMD rates, it could prove that AIC acts as a trigger to initiate calcium homeostasis. Also, if calcium homeostasis is controlled, we can also regulate the intracellular calcium influx and efflux; which means that we can eventually regenerate mitochondria. While mitochondrial modification is essential regarding stem cell initiation and restoring motor neurons, AIC can be the key to treating chronic neurodegenerative diseases.

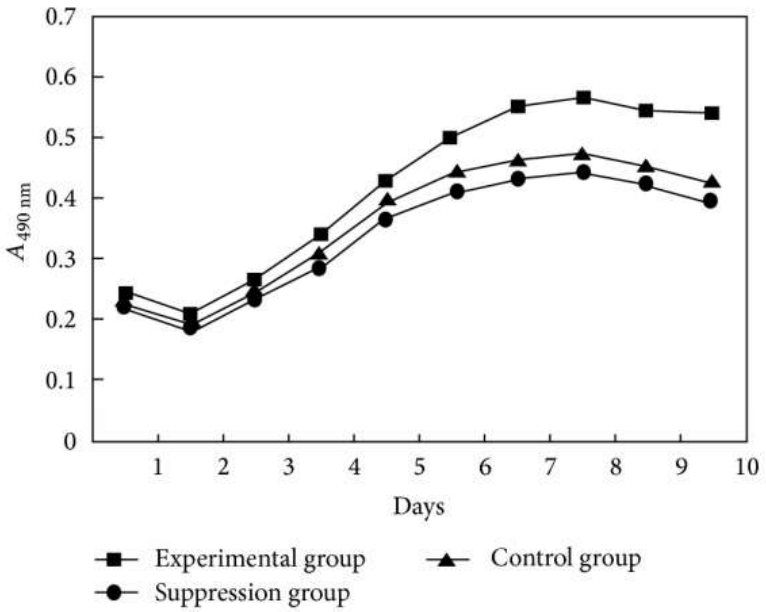


Figure 33. The growth curve of BMSCs in the experimental, suppression, and control groups ($\Delta P <$ 0.001 between experimental group versus suppression group and control group; $*P > 0.001$ between suppression group and control group). Adapted from "Effects of Parathyroid Hormone on Calcium Ions in Rat Bone Marrow Mesenchymal Stem Cells," by Y. Chen *et al.*, 2014, BioMed Research International. p. 3, Copyright 2014 by Hindawi.

Group	n	Calcium ion fluorescence intensity	
		7 d	14 d
Experimental group	6	7.91 ± 2.34*	5.15 ± 1.58**
Control group	6	5.37 ± 2.55**	2.71 ± 0.89*

**$P < 0.001$ between two sampling times, or between groups.
*$P > 0.001$ between experimental group on the 14th day and control group on the 7th day.

Figure 34. Calcium ion fluorescence intensity of BMSCs in the experimental and control group. Adapted from "Effects of Parathyroid Hormone on Calcium Ions in Rat Bone Marrow Mesenchymal Stem Cells," by Y. Chen *et al.*, 2014, BioMed Research International. p. 4, Copyright 2014 by Hindawi.

As Choi *et al.* (2011) have found in their research, AIC plays a crucial role in elevating BMD, as well as enhancing bone quality. They have orally ingested 0.0012% of AIC solution to ovariectomized rats for 12 weeks. They have also examined the change in osteocalcin, type I collagen C-terminal telopeptides (CTx), 17β-estradiol, breaking force, BMD, bone ash, and calcium and phosphorus in femurs. They compared the control (sham) with two different groups: ovariectomized group (OVX) and AIC-treatment group (OVX+AIC). The experimental results have shown that AIC has significant efficacy in elevating 17β-estradiol and osteocalcin, as well as decreasing the CTx rate. Moreover, it has been demonstrated that AIC has considerable effectiveness in increasing breaking force, BMD, bone ash, as well as calcium and phosphorous in femurs. As renal excretion also occurs when calcium homeostasis is regulated, they have compared the mass of kidneys of the rats. The results showed a significant decrease in renal mass. Therefore, we could confirm that AIC acts as a hormone trigger in the body to initiate calcium homeostasis.

iv. AIC and Calcium Signaling for Mitochondrial Regeneration and Mesenchymal Stem Cell Initiation

Calcium signaling effects profoundly on initiating Mesenchymal Stem Cells (MSC) because as calcium ions influx and efflux through the Ca^{2+}

channel, the action potential is activated, which gives inaugurating signal to MSC. According to Zhang & Morad (2016), "the contribution of mitochondrial Ca^{2+} signaling to spontaneous beating activity of human-induced Pluripotent Stem Cell-derived Cardiomyocytes (hiPSC-CM) has been recently examined." (p. 102) As they inspected, mitochondrial calcium signaling is essential to induce MSC; which means that the imbalance of intracellular and extracellular calcium ion can cause mitochondria-related chronic degenerative diseases. Therefore, it is essential to maintain calcium homeostasis to provide an adequate amount of ionized calcium into the mitochondrial intracellular matrix. Moreover, while mitochondrial calcium signaling can initiate the activity of MSC, it can help to restore the cells that were deactivated due to mitochondrial dysfunction.

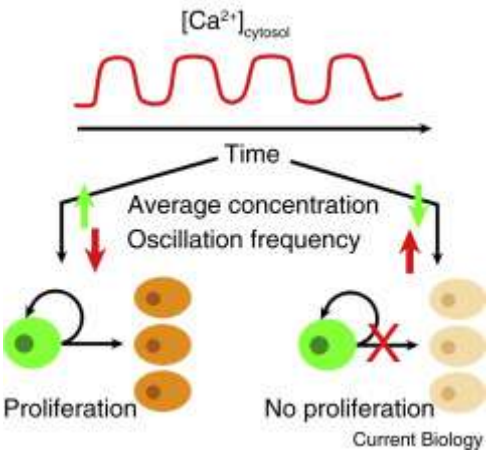


Figure 35. Ca^{2+} oscillation frequencies and stem cell proliferation. Decreased Ca^{2+} oscillation frequency and increased average Ca^{2+} concentration in the cytoplasm of Drosophila intestinal stem cells (green) is associated with increased rate of stem cell proliferation. A reduction in Ca^{2+} concentration and an increase in Ca^{2+} oscillation frequency is observed in quiescent stem cells. Adapted from "Intestinal Stem Cell: Got Calcium?," by M. Mászai & J. B. Cordero, 2016, Current Biology, 26, p. 118, Copyright 2016 by Elsevier.

Furthermore, Mahajan *et al.* (2011) have insisted, "calcium deficiency also reduced ($p<0.05$) the in vivo proliferation of MSC by ~50%." (p. 373) The insufficient calcium intake reduces the MSC proliferation. In counter approach, it can be thought that if an sufficient amount of calcium is taken,

the number of MSC that is generated would be increased; thus, it would be useful to make the MSCs specialized into the specific forms of cells, which can be helpful to treat chronic degenerative diseases such as osteoporosis. Also, it has been noted that "neonatal Ca nutrition is crucial for bone integrity and suggest that early-life Ca restriction may have long-term effects on bone integrity via programming of MSCs." (Mahajan *et al.*, 2011, p. 373) As they suggested, dietary calcium supplements are critically needed for those who are pregnant, because as early-life calcium supplementation is crucial to building bone integrity that is programmed by MSCs. For this reason, AIC can play as a trigger to regulate adequate calcium homeostasis in our bodies, which can be helpful to initiate human-induced MSCs, the key for treating chronic degenerative diseases.

VI. AIC and Cancer

"Anti-Orbital Ionic Calcium Carbonate: By Regenerating Mitochondrial Function and Regulating Lactic Acid and Ammonia Secretion, It Can Efficiently Modify p53 Function Which is the Key to Treating Cancer."

i. Introduction

According to WHO (2018), cancer is one of the top ten leading causes of death in high-income countries. As dietary habits and lifestyles have changed, the number of developing chronic degenerative diseases are increasing. Therefore, it is crucial to pay careful attention to prevent developing cancers at an individual level. It has been reported that 8.8 million people died due to tumors in 2015 worldwide (WHO, 2018) and the global burden of cancer exceeds USD 1.16 trillion. (EI, 2010) Also, approximately 14.1 million new cases of the disease occurred in 2012 globally (EI, 2010). For Canada alone, about 206, 200 new cases of cancer are diagnosed, and 80,800 fatalities caused by the disease were reported in 2017 worldwide. (EI, 2010)

Current cancer treatments include surgical operation, chemotherapy, radiation therapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant and precision medicine. Even though there are a lot of cancer treatment options, none of them has shown significant effects for conquering cancer. According to Pignon *et al.* (2000), Only four percent of tumor cancer patients survived through chemotherapy. Also, Juan *et al.* (2009) mentioned that lung tumor patients remain poor, despite the use of radiational therapy. The reason is because the current cancer treatment options are only focusing on decreasing the number of pre-existing tumor cells. Caley & Jones (2012) mentioned that chemotherapy concentrates on the number of tumor cells by interfering with tumor cell division. However, to treat the tumor, we must

understand the underlying mechanism of the proliferation of cancer cells in the human body to move closer to the actual cure.

Mutated cells will keep duplicating if the overall body metabolism and functions of the cell are not normalized. It is crucial to develop new cancer treatment technology that restores the whole-body mechanism and regenerates the cellular function, which eventually prevents the subsequent proliferation of tumor cells. Therefore, AIC can be utilized to regenerate malfunctioned mitochondria and endoplasmic reticulum (ER), which ultimately prevent possible protein mutation such as tp53. By stimulating hormone secretion that plays a critical role in calcium homeostasis and fluctuating influx and efflux of calcium ions in the mitochondrial intracellular membrane, we can finally trigger the apoptosis of cancer cells by efficiently releasing apoptosomes such as cytochrome c.

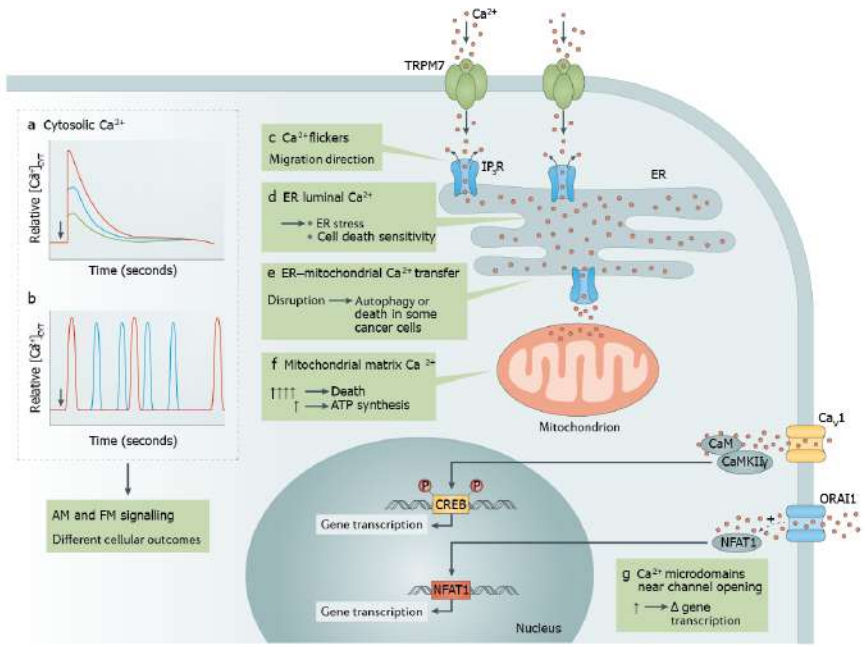


Figure 36. Ca^{2+} signal diversity. This figure illustrates the diversity in Ca^{2+} signalling and how this may contribute to processes that are relevant in the context of cancer progression. This diversity and its consequences include, but are not limited to, the examples shown. a | The degree of increase in cytosolic free Ca^{2+} ($[Ca^{2+}]$ CYT) (amplitude modulation (AM)), which may control cell fate (for example, proliferation or cell death); the different coloured lines on the graph represent cytosolic Ca^{2+} increases of different amplitude. b | The frequency of $[Ca^{2+}]$ CYT oscillations (frequency modulation (FM)), which may control the nature of gene transcription and hence different cellular outcomes and expression remodelling; the different coloured lines on the graph represent cytosolic Ca^{2+} increases of varying oscillation frequency. c | Highly localized and transient increases in Ca^{2+} at the edge of some cells (Ca^{2+} flickers), which control directional migration. d | Free Ca^{2+} levels within the endoplasmic reticulum (ER) that may influence cell stress and the sensitivity to apoptotic stimuli, which act in part through the release of Ca^{2+} from internal stores. e | The confined transfer of Ca^{2+} from the ER to the mitochondria that when reduced may induce autophagy or even death in some cancer cells. f | Levels of Ca^{2+} within the mitochondrial matrix, where modest changes may promote ATP synthesis but sustained high levels may promote cell death. g | Localized very high levels of Ca^{2+} (known as Ca^{2+} microdomains) achieved upon the opening of plasmalemmal Ca^{2+} channels can specifically regulate gene transcription pathways. In some cells, Ca^{2+}-bound calmodulin (CaM) produced near the opening of CaV1 L-type voltage-gated Ca^{2+} channels can be delivered by Ca^{2+}/calmodulin-dependent protein kinase II subunit γ (CaMKIIγ) to the nucleus to promote the phosphorylation of cAMP responsive element-binding protein (CREB) and gene transcription; in other cellular systems, localized Ca^{2+} increases produced upon the opening of calcium release-activated calcium channel protein 1 (ORAI1) channels is sufficient via a series of signalling pathways (indicated by the small dashed arrow and the plus symbol) to produce the translocation of nuclear factor of activated T cells 1 (NFAT1) to the nucleus. In contrast ORAI1-activated gene transcription via NFAT4 also requires increases in nuclear Ca^{2+} (not shown on this figure). Δ denotes changes in a pathway or levels, ↑ denotes an increase in levels or activity. IP3R, inositol 1,4,5-trisphosphate receptor; TRP, transient receptor potential. Adapted from "The calcium–cancer signalling nexus," by G. R. Monteith, N. Prevarskaya & S. J. Roberts-Thomson, 2017, Nature Reviews Cancer, 17, p. 370, Copyright 2017 by Macmillan.

ii. The Lactic Acid Production in Cancer Cells

Healthy cells use both cellular respiration and anaerobic glycolysis using mitochondria. Through oxidative phosphorylation, they produce 36 ATP per one mole of glucose and also produce 2 ATP per one mole of glucose when they have an insufficient amount of oxygen for breathing. However, tumor cells produce lactic acid via aerobic glycolysis that is unique metabolism which does not use oxygen during catabolism. (Heiden, Cantley & Thompson, 2009) The reason why tumor cells utilize aerobic glycolysis is to build physiological constructing elements for cancer cell proliferation. To explain the mechanism in detail, tumor cells require increased glucose uptake for rapid spread. Therefore, they use inefficient cellular respiration. Moreover, by using aerobic glycolysis, they can produce more acidic products (lactic acid), which makes a favorable environment for cancer cells. Furthermore, they go through the biosynthesis pathway for producing palmitate. (Carta *et al.*, 2017)

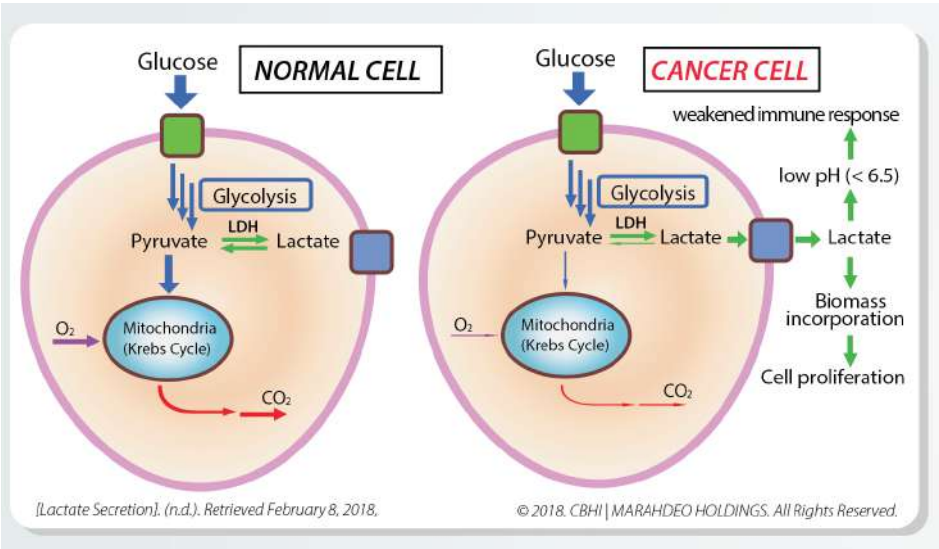


Figure 37. The Lactic Acid Production from Cancer Cells.

Han *et al.* (2013) have elucidated that the excessive amount of lactic acid production via cancer cells would activate the secretion of proteases; which causes extracellular degradation, leading to metastasis of cancer cells.

Moreover, the enormous secretion of lactic acid would increase the synthesis of HIF-1 proteins, which are actively involved in the proliferation of cancer cells by invading and metastasizing the healthy cells. (Han *et al.*, 2013) Therefore, it is crucial to neutralize the acidic environment in the cell, and for the method, we suggest AIC solution because as calcium ion in the AIC solution would be reacted with the lactic acid in the cell, it produces Calcium Lactate, which is the neutral form of salt. As cancer cells favor an acidic environment as it interferes the activity of natural killer cells such as T-lymphocytes and NF-kB cells, by forming Calcium Lactate, the viability of cancer cells would be decreased.

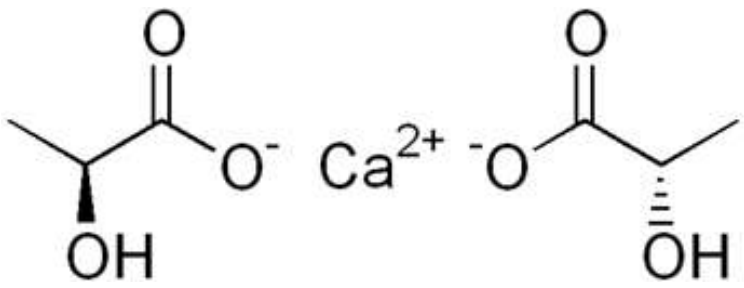


Figure 38. Calcium Lactate bonding structure.

According to Cooper *et al.* (1992), the patients suffering from lactic acidosis showed lack of ionized calcium concentration in the intracellular membrane. Moreover, Gupta (2016) argued that cancer cells use aerobic glycolysis which "[increases] degradation of glutamine leads to metabolic acidosis with pH of the solid cancers as low as pH 6.0 - 6.5." (p. 1) In other words, lack of calcium ion causes an increase the presence of lactic acid in the cellular membrane, and therefore, supplementing the calcium concentration at an intracellular level is critical to stabilizing the acidic pH level. Strictly speaking, adequate calcium intake is highly recommended for those who are suffering from cancer.

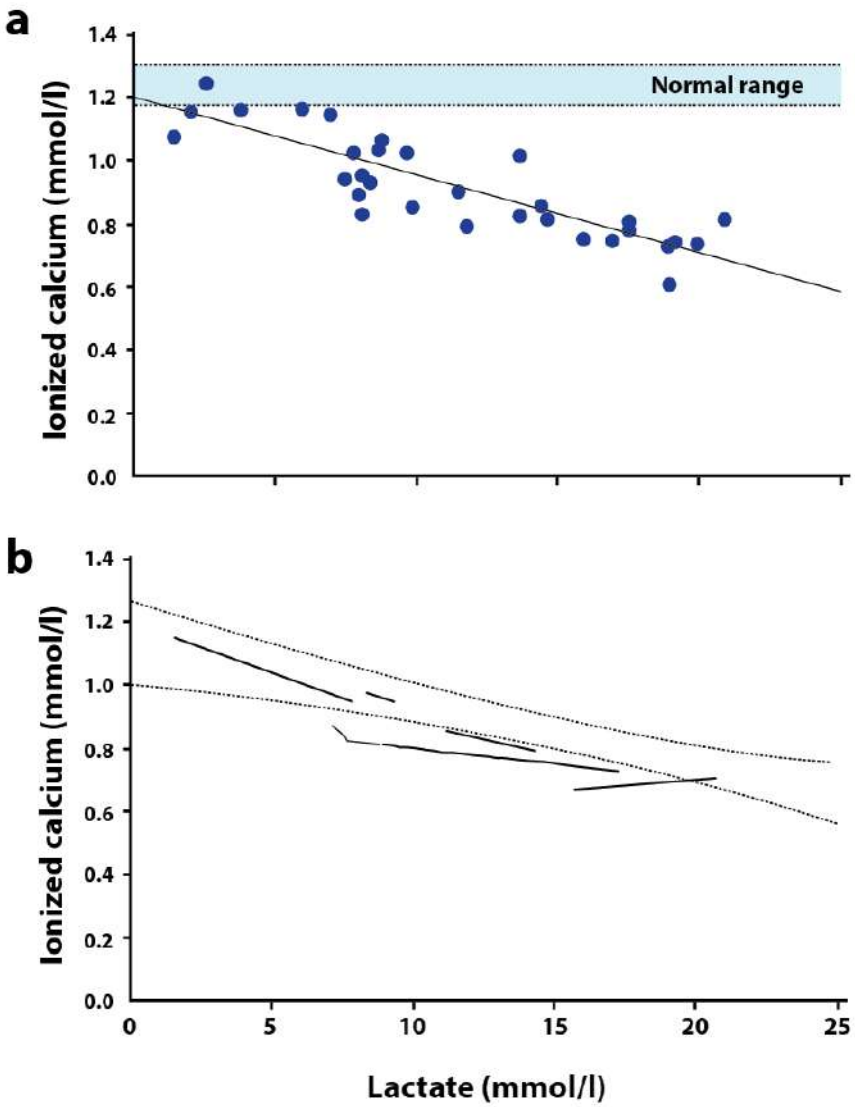


Figure 39. Relationship between [Ca^{++}] and plasma lactate concentrations in nine critically ill patients who had metabolic acidosis. [Ca^{++}] decreased as lactate increased. (a) Linear regression analysis for the group (r^2=0.78, p<0.001) (b) Random effects regression and 95% CI for the 6 patients who had 3 or more data points [slope -0.018 (SE 0.005); intercept 1.11 (SE 0.08)]. Adapted from "Plasma ionized calcium and blood lactate concentrations are inversely associated in human lactic acidosis*," by D. J. Cooper *et al.*, 1992, *Intensive Care Medicine, 18*, p. 287. Copyright 1992 by the Springer-Verlag.

As different from the regular calcium supplements that are sold in the market, AIC does not contain severe adverse effects such as renal failure.

Moreover, with its unique structure and efficient absorption method, it can efficiently trigger calcium homeostasis in the body. Also, as it effectively triggers the calcium signaling with the tiny amount of calcium carbonate solution, it can be highly useful to neutralize the cellular pH level as it is ingested. To investigate the efficacy of AIC in offsetting cellular pH level, Korean National Sports University held an experiment in 2014. They examined whether AIC would decrease the amount of lactic acid and glucose production in the cell for 20 Korean professional swimmers after they receive hard physical training. The result was noticeable because the average lactic acid level for the swimmers was notably decreased after orally ingesting 5 mL of AIC twice a day for two weeks. As it shows significant efficacy to reduce the amount of lactic acid, we can use it for treating cancer as acidosis issue is serious among many cancer patients. Moreover, as their personal performance record improved after taking AIC, we can assume that AIC regenerates mitochondrial function, which results in higher ATP production.

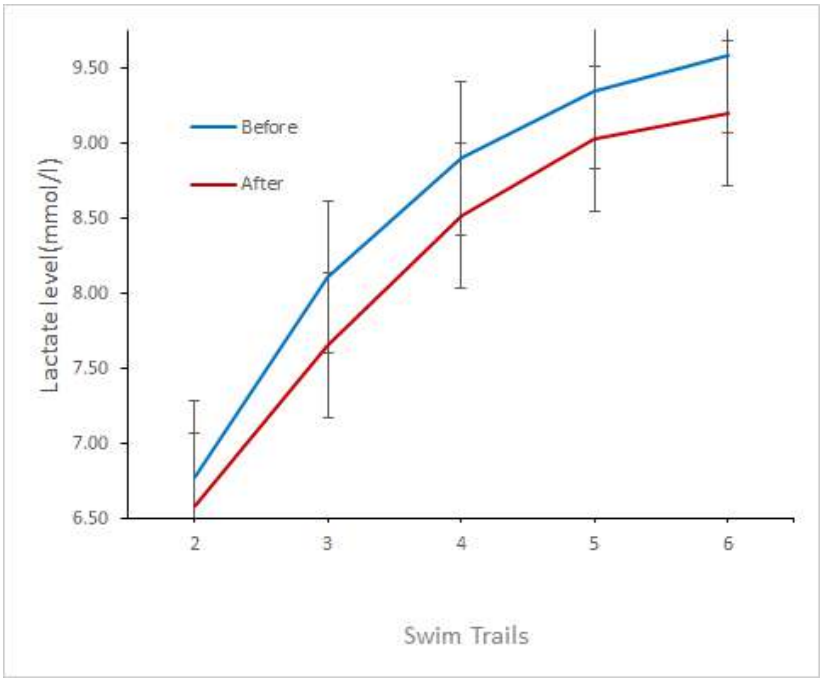


Figure 40. The Decrease in Lactic Acid Production After Treating with AIC.

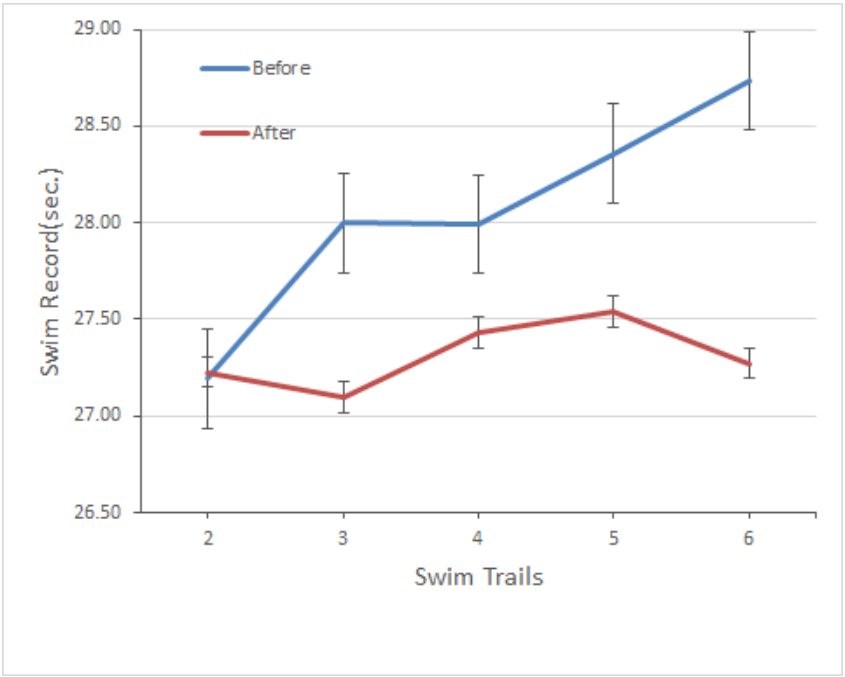


Figure 41. The Improvement in Swimming Record After Treating with AIC.

Figure 42. Pictures Taken When the Research was Conducted.

iii. The Ammonia Production in Cancer Cells

While cancer cells are generated, they require a lot of nutrients to form their building blocks. Also, they uptake a massive amount of glucose; as glucose is the primary source of energy that activates their cellular activity. However, other metabolic waste is produced during the catabolism, called

Ammonia (NH_4). Ammonia is produced through tumor cell metabolism and referring to the research conducted by Spinelli *et al.* (2017), ammonia produces glutamine when they need to obtain energy to metastasize. As Eagle (1955) found, glutamine is the most utilized nutrient for tumor cells, so it is the key to repressing the glutamine production to inhibit cancer cell proliferation.

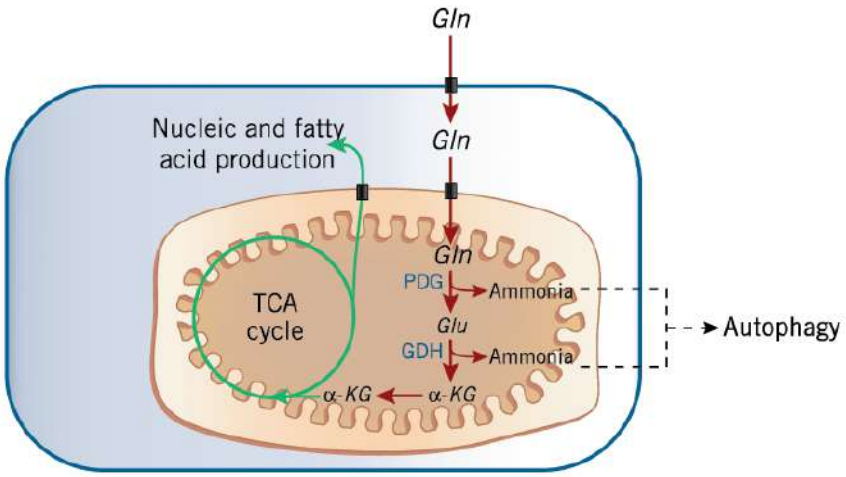


Figure 43. Ammonia as a stress signal in cancer. Ammonia has long been seen as a toxic by-product of glutamine (Gln) metabolism in mitochondria. Adapted from "Sending ammonia signals," by S. Edelson, 2010, *SciBX, 3(19)*, p. 1. Copyright 2010 by Nature Publishing Group.

Furthermore, when there is too much ammonia concentration in the body, the urea cycle is activated in the liver to convert toxic ammonia into uric acid. (Ali, Gurusamy & Ramachandr, 2014) However, as cancer cells produce an excessive amount of Ammonia, the excretory system does not work correctly, which ultimately causes the body to become highly basic. (Spinelli *et al.*, 2017) Therefore, as cancer cells are generated, a significant concentration of ammonia remains in the cardiovascular system and extracellular mitochondrial matrix (Tarantino *et al.*, 2009); consequently, this can cause the dysfunction of mitochondria and alteration in protein synthesis. (Niknahad *et al.*, 2017)

According to Spinelli *et al.* (2017), tumor cells use glutamine to protect themselves from the attack of natural killer cells such as T-lymphocytes and

NF-kB Cells. As they repress the expression of TXNIP protein, cancer cells can invade healthy cells without interruption. (Morrison *et al.*, 2014) According to Morrison *et al.* (2014), when they held an experiment on a mouse with thyroid cancer, they found out that TXNIP expression can weaken the growth of the cancer cells. Moreover, as Kaadige *et al.* (2009) investigated, TXNIP expression is remarkably activated in the presence of glucose. However, it significantly decreased in the presence of both glucose and glutamine. As they concluded that glutamine deactivates the expression of TXNIP (Kaadige *et al.*, 2009), it is critical to repress the production of ammonia, which leads the mass production of glutamine.

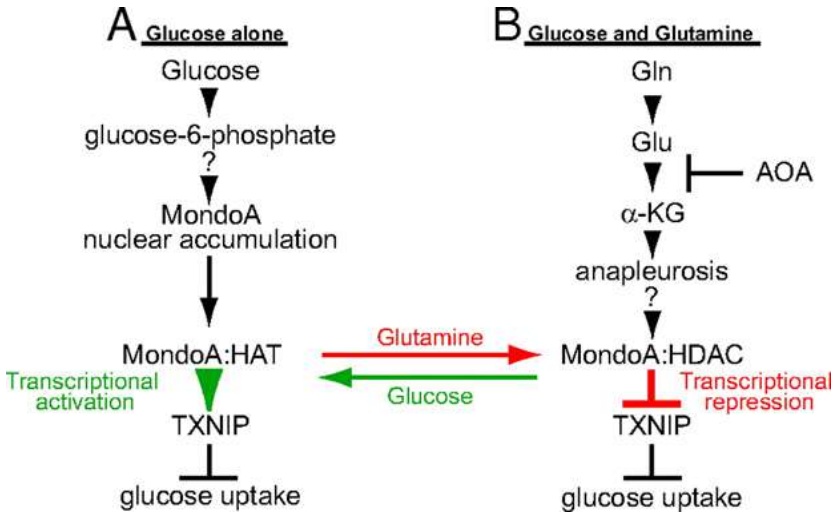


Figure 44. MondoA is a nutrient-dependent transcription factor. See text for details. As indicated, the mechanisms controlling how MondoA accumulates in the nucleus in response to glucose and how glutamine-dependent mitochondrial anapleurosis converts MondoA to a transcriptional repressor are not known. HAT, histone acetyl-transferase; HDAC, histone deacetylase; -KG, -ketoglutarate; AOA, aminooxyacetate; Gln, glutamine; Glu, glutamate. Adapted from "Glutamine-dependent anapleurosis dictates glucose uptake and cell growth by regulating MondoA transcriptional activity," by M. R. Kaadige *et al.*, 2009, *PNAS, 106(35)*, p. 287. Copyright 2009 by the National Academy of Sciences.

As Dickman, Senozan & Hunt (1970) mentioned, calcium ammoniate is naturally formed as the following reaction occurs; $Ca(NH_3)_6(s) \leftrightharpoons Ca(s)+6NH_3(g)$. They demonstrated that serum ionic calcium could be reacted with 6 moles of ammonia by forming the ionic salt, called

calcium ammoniate ($Ca(NH_3)_6$). Therefore, we assume that AIC can be a treatment for cancer by referring to this equation. As AIC significantly increases the serum ionic calcium level, it might also be able to repress the mass ammonia concentration efficiently in the cell. As ammonia produces glutamine, which interferes with the activity of natural killer cells, it is essential to suppress the production of glutamine by causing ammonia to react with AIC.

$$NH_3 + H_2O \rightarrow NH_4(OH);\ pH\ 9\text{-}10$$
$$Ca^{2+} + 6NH_3 \rightarrow Ca(NH_3)_6^{2+}$$
$$Ca^{2+} + NH_4(OH) + H_2O \rightarrow Ca(OH)_2$$
$$4NH_3 + 2(H_2O) \rightarrow$$
$$[Ca(NH_3)_4(H_2O)_2]^{2+} + 2OH^- \rightarrow$$
$$[Ca(NH_3)_4(H_2O)_2](OH)_2$$

Figure 45. Ammonia Inhibition with Calcium in Cancer Cells.

iv. AIC Triggers p53 Modification and Apoptosis

As it is well-known, p53 is a tumor suppressor that can inhibit cancer cell proliferation and regulate the cell cycle by arresting cellular growth. However, when the calcium level is imbalanced in the body, mitochondria do not work correctly, especially when there is excitotoxicity due to the massive influx of calcium ion into the mitochondrial matrix. This will destroy the mitochondria, which ultimately causes the mutation in protein synthesis. Then, p53 protein is mutated and converted into the tp53 form of protein, which helps the rapid proliferation of cancer cells. As p53 is mutated, apoptosis does not occur correctly; therefore, it is critical to maintain calcium homeostasis to prevent mitochondrial dysfunction.

According to Stanika *et al.* (2009), calcium excitotoxicity causes mitochondrial dysfunction. Moreover, Murphy (2013) stated that the ROS production due to the malfunction of mitochondria causes misfolding of p53 proteins. Also, Hughes, Adam & Gottschling (2012) investigated that the imbalance of pH leads the mitochondrial dysfunction. As mitochondrial dysfunction causes the p53 mutation, it is critical to regulate mitochondrial function. While calcium signaling is activated through calcium homeostasis, ionized calcium is influx and efflux through the calcium ion channel; then it initiates the mitochondrial function.

As Giorgi, Bonora & Pinton (2015) investigated, p53 plays a role in stimulating calcium signaling in order to activate mitochondrial activity to control oncogenes. However, as p53 is mutated, calcium signal will not work correctly anymore, which ultimately causes the mass production of ROS, and in turn affects the development of cancer cells. (Macip *et al.*, 2003) Moreover, Kaur & Sanyal (2011) insisted that calcium signaling should regularly oscillate since the imbalance of calcium influx and efflux in the mitochondrial matrix can cause tumor formation. They also added that when the calcium homeostasis is adequately regulated, apoptosomes, such as Apaf-1, are produced. (Kaur & Sanyal, 2011)

For this reason, we suggest AIC as a new form of cancer treatment, as it regulates calcium homeostasis in the body; which initiates mitochondrial activity. Moreover, AIC has proven its efficacy in controlling intracellular calcium ion by regulating pituitary hormones. Therefore, we can assert that AIC can be a treatment option to cure cancer as it neutralizes serum pH level and regulates mitochondrial activity, which prevents p53 mutation and helps to produce apoptosomes.

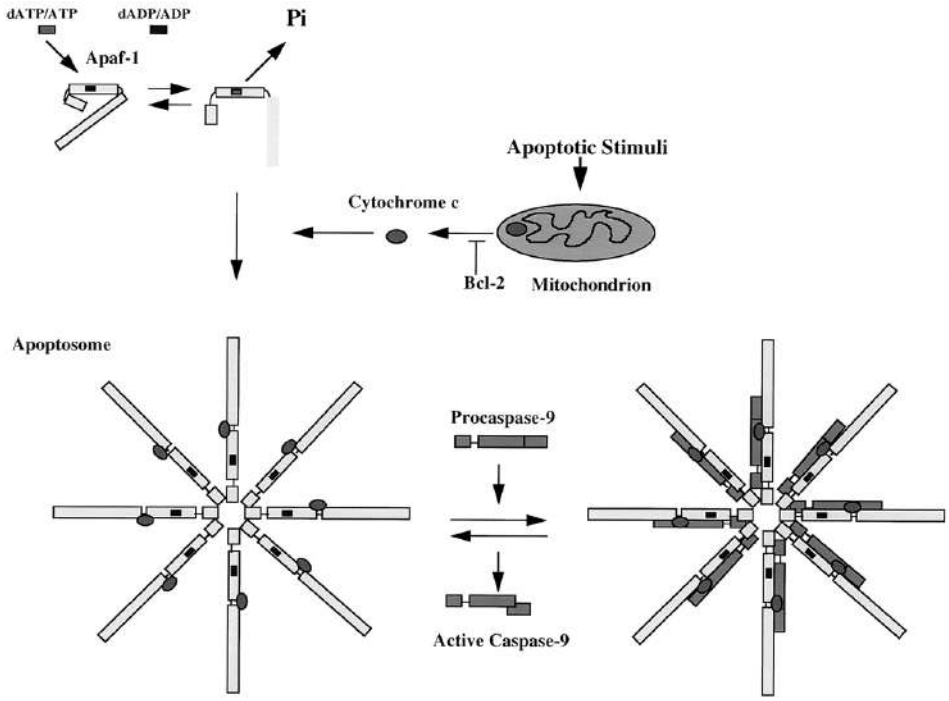


Figure 46. Model of caspase-9 activation. See the text for details. APAF-1, cytochrome c, caspase-9, dATP, and dADP are indicated. Adapted from "An APAF-1z Cytochrome c Multimeric Complex Is a Functional Apoptosome. That Activates Procaspase-9*," by H. Zou *et al.*, 1999, *JBC, 274 (17)*, p. 11555. Copyright 1999 by the American Society for Biochemistry and Molecular Biology.

v. The Current Tumour Treatments

a. Chemotherapy

Chemotherapy is one of the famous forms of cancer treatment. It is a pharmacotherapy of cancer, so alkylating agents, anti-microtubules agents, and anti-metabolites are primarily used for targeting the tumor cells. Sometimes, chemo-hormonal therapies are conducted for those who are suffering from neuroendocrine cancers such as breast and prostate cancer. Mostly, these drugs are DNA recombinants to help efficient apoptosis, and by inhibiting indiscreet mitosis, it can selectively kill the cancerous cells. However, it is a problem that there are many different types of tumor cells and various treatment methods are needed to treat cancer effectively. Moreover, as high doses of chemo-therapeutical medicine also alter the function of healthy cells; it can cause many adverse side effects such as

myelosuppression, immunosuppression, mucositis, and alopecia. (Lalla *et al.*, 2008; Ockerby *et al.*, 2012; Sengupta *et al.*, 2012)

Figure 47. The Current Medication for Cancer Chemotherapy.

Therefore, it can cause medical symptoms such as multiple sclerosis, vasculitis, systemic lupus erythematosus, rheumatoid arthritis, and vasculitis. (Ben-Ari, 2004) According to Partridge, Burstein & Winer (2001), standard short-term and long-term side effects are observed for the breast cancer patients treated with "cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) or the methotrexate and 5-fluorouracil (MF) regimens." (p. 135) As depicted in Figure 2, some patients suffered from premature infertility or cardiac dysfunction.

Furthermore, chemo-therapeutical medicine can build up the auto-immune system in the body. Therefore, it can cause medical symptoms such as multiple sclerosis, vascular cognitive impairment and leukemia after

undergoing long-term chemo-hormonal treatment. Consequently, it is crucial to find more natural and holistic treatment options, and we recommend AIC as a new treatment option for cancer. As it increases body metabolism by initiating calcium signaling and produces apoptosomes by modifying mitochondria, it can substitute the role of chemotherapy, meaning that patients with cancer can avoid the possible adverse effects associated with chemotherapy.

Short-term effects	Long-term effects
Emesis	Premature menopause/infertility
Nausea	Weight gain
Stomatitis	Cardiac dysfunction
Alopecia	Leukemia/MDS*
Myelosuppression	Congnitive dysfunction†
Thromboembolism	
Myalgias	
Neuropathy‡	
Fatigue‡	

*MDS = myelodyspastic syndrome.
†Possible long-term effect; studies are preliminary in nature.
‡May be both short-term and long-term effect.

Table 6. Side effects of chemotherapy. Adapted from "Side Effects of Chemotherapy and Combined Chemo-hormonal Therapy in Women with Early-Stage Breast Cancer," by A.H. Partridge, H.J. Burstein and E.P. Winer, 2001, *JNCI Monographs, 96 (8)*, p. 579. Copyright 2001 by the Oxford Academic.

b. Radiation Therapy

Radiation therapy is also one of the well-known forms of cancer treatment, which is synergetic when conducted alongside chemotherapy. It is applied to tumors, and it impedes the growth of cancer cells by damaging the DNA of tumor tissues. When performing, external beam radiation and x-rays are applied to the tumor cells, and as it breaks the cancer cells, it will lead efficient apoptosis to target the malignant tumor cells. However, the problem is that when radiation therapy is applied, physicians should include the margin of normal tissues that are near the tumor cells. By administering high doses of radiation therapy and since it has to include the normal cell regions, it can also kill the DNA of normal tissue, which can exacerbate the human body condition. Therefore, to avoid possible adverse effects such as

infertility, fibrosis, epilation, lymphedema, heart disease, cognitive decline and radiation enteropathy (Meek, 1998; Taylor *et al.*, 2017; "Late Effects of Treatment for Childhood Cancer", 2012; Hauer-Jensen, Denham & Andreyev, 2014), it is critical to find more holistic and palliative treatment options for treating cancer. For this reason, we recommend AIC as a new treatment option for cancer as it naturally treats cancer by increasing human body metabolism using a tiny amount of AIC solution.

Figure 48. The Current Method for Cancer Radiation Therapy.

c. Surgical Operation

The surgical operation is also one of the outstanding treatment options for cancer. Surgeons use scalpels to cut out the infected part of the body to get rid of tumor cells. They also use anesthesia to reduce pain during the surgery. For example, the most frequently performed treatment option for a brain tumor is a craniotomy. By cutting out the infected portion of the scalp, the tumor cells can be removed. However, surgical treatment has serious side effects as it can cause permanent disability after surgery. (Kaya *et al.*,2010) Moreover, Kaya *et al*. (2010) found that breast cancer patients who had undergone surgical intervention complained of "arm pain on motion,

anterior chest wall pain, loss of grip strength, and shoulder flexion." (p. 37) Therefore, AIC can be a new treatment option for treating cancer as it does not require any surgical procedures. Also, as it enhances the overall body metabolism by stimulating calcium homeostasis, it can modify mitochondrial activity, which helps to produce apoptosome enzymes.

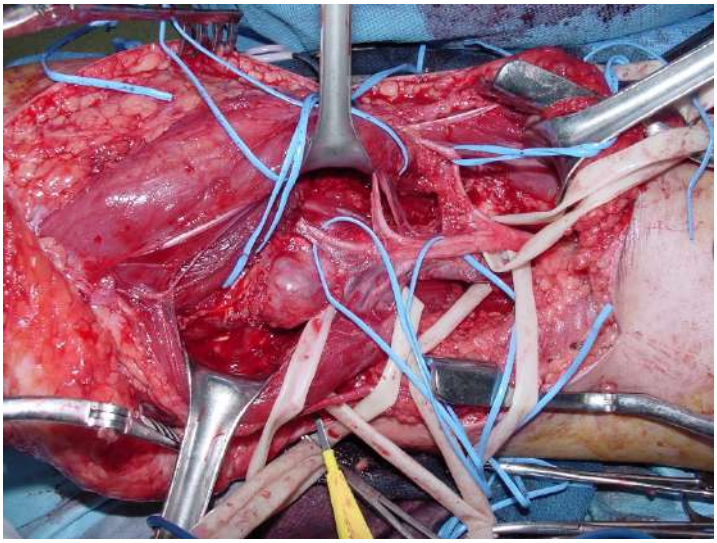


Figure 49. The Current Method for Surgical Operation to Remove Tumour Cells.

d. Chemotherapy and Radiation Therapy with AIC Therapy

For several years, CBHI researchers observed tumor patients who have been treated using chemotherapy and AIC treatment simultaneously. They found that ninety percent of the tumor patients are free from adverse effects of chemo and radiation therapy with the help of AIC treatment. This is because AIC treatment recovers cell viability through regeneration of calcium depolarization by increasing the permeability of calcium in mitochondrial matrix.

vi. Summary of Animal Study: Summary of Animal Study for Colon Cancer

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"Evaluation of AIC Calcium for Tumor Growth Inhibition in the COLO 205 Human Colorectal Adenocarcinoma Nude Mouse Xenograft model. June 7, 2017. Charles River Discovery Services."

CBHI Canada contracted Charles River Discovery Services North Carolina (CR Discovery Services, https://www.criver.com) to evaluate AIC Calcium on two dosing schedules for Tumor Growth Inhibition (TGI) in the COLO 205 human colorectal adenocarcinoma nude mouse xenograft model as a feel-good trial for 28 days only.

In the Colo205-e347 study, AIC Calcium was administered orally (p.o.) at 0.83 mg/kg to two groups. One received dosing twice daily, with one dose on the first day (bid to end, beginning day one dose) and the other received dosing three times daily, with two doses on the first day (tried to finish, beginning day two doses). Treatments started on Day 1 in mice with established subcutaneous COLO 205 tumors. The study included a vehicle control group, dosed bid to end, first day two doses. The study endpoint was a mean tumor volume of 2000 mm3 in the control group or 28 days, whichever came first. The study ended on Day 28. Treatment response was determined from an analysis of the percentage of tumor growth inhibition (% TGI), defined as the percentage difference between median tumor volumes (MTVs) of treated and control groups, with differences between groups deemed statistically significant at $p<0.05$ using the Mann-Whitney U Test. Tumor regressions, mean tumor growth, and treatment tolerability also were considered.

In Summary, the median tumor volume on Day 28 for the control Group 1 was 936 mm^3. Treatment of animals with AIC Calcium at 0.83 mg/kg, was administered bid to end, the first day. One dose and tried to stop, the first

day two doses were associated with 0% and 8%, respectively. Treatments administered in the study were acceptably tolerated based on the group mean BW measurement and clinical observations.

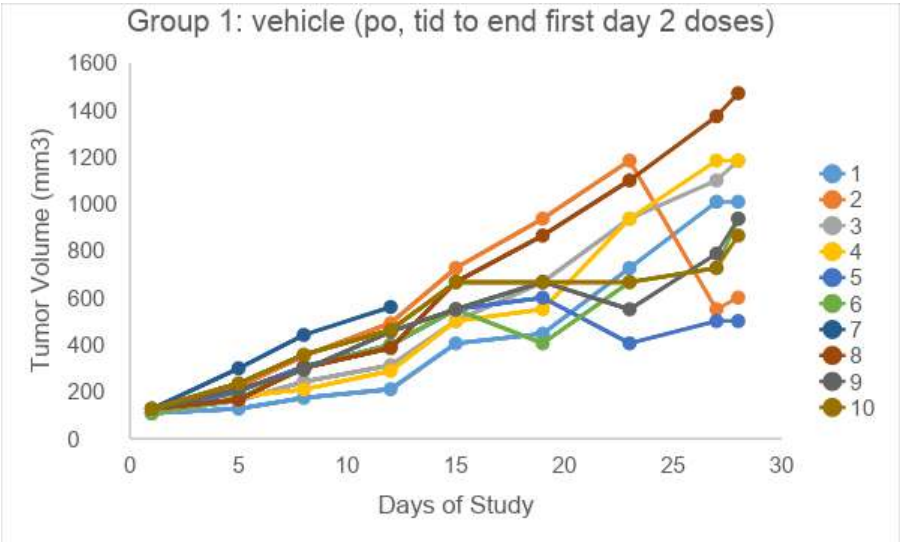


Figure 50. The Tumour Suppression After Treating with AIC. (Group 1)

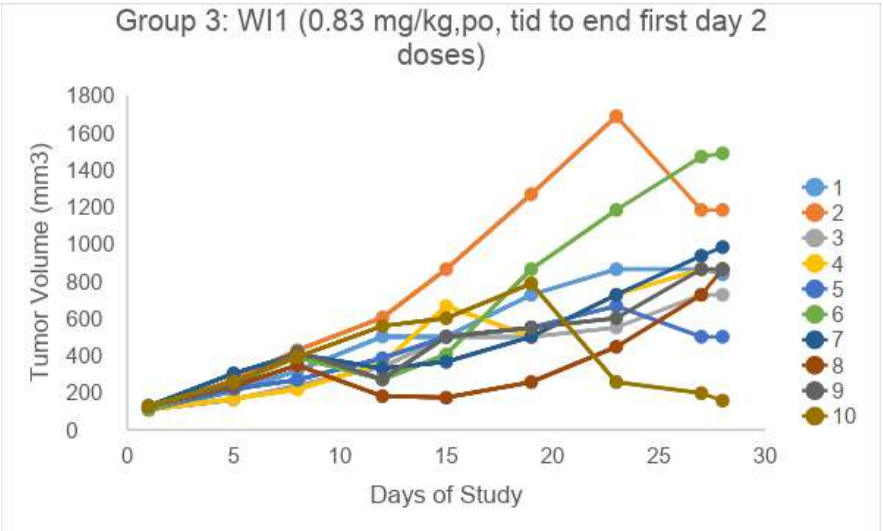


Figure 51. The Tumour Suppression After Treating with AIC. (Group 3)

vii. Case Study

After Treating with AIC for a certain period of time, patients have shown significant improvement, especially those who are suffering from bone-related cancer. Ms. Holt and Mr. Kim have shown notable improvement after treatment with AIC. They are completely recovered from cancer after only being treated with AIC. While the two cases are remarkable, we asked their permission to open their medical data to the public. As it is shown in the data, the white blood cell (WBC), hemoglobin, platelet and neutrophil concentrations have increased a lot in 4 years. For Mr. Kim's case, the WBC, RBC, neutrophils, lymphocytes and hemoglobin concentrations have been significantly increased within a month.

a. Ms. Holt (Multiple Myeloma, 78 years old, Caucasian, Female): Hematological Data Became Normal After Treating with AIC.

Date	WBC	Hemoglobin (g/dl)	Platelet	Neutrophil
10-25-13	2,590	10.1	79,000	1,350
11-27-13	2,190	9.4	35,000	890
12-27-13	3,150	9.9	62,000	1,560
01-21-14	3,650	10.9	89,000	1,710
02-18-14	3,370	11.3	55,000	1,540
03-18-14	3,260	10.8	55,000	1,670
04-08-14	2,670	10.0	67,000	1,270
05-13-14	3,520	9.9	89,000	1,760
06-17-14	3,190	10.0	90,000	1,400
08-05-14	4,160	11.5	101,000	2,160
10-07-14	4,300	10.5	114,000	2,230
12-09-14	4,700	11.2	145,000	2,290
01-27-15	4,760	11.4	152,000	2,560
05-31-17	4,530	12.0	169,000	2,280
09-29-17	4,000	11.9	166,000	2,090
11-27-17	4,190	12.3	171,000	2,100
Ref	**4,000/ml ~ 10,800/ml**	**M: 13~17g/dl F: 12~16g/dl**	**150,000/ml ~ 400,000/ml**	**2000/ml ~ 7000/ml**

Table 7. The Changes in Patient's' Hematologic Data After Treating with AIC.

Date	T Score	State
Nov.25.2015	**-0.9**	**Normal**
Nov.20.2013	**-3.6**	**Osteoporosis**

Table 8. The BMD Change for Ms. Holt After Treating with AIC.

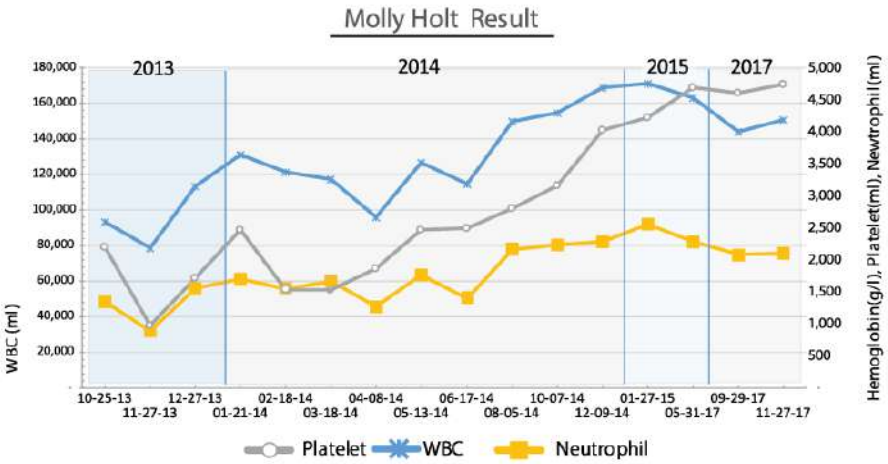
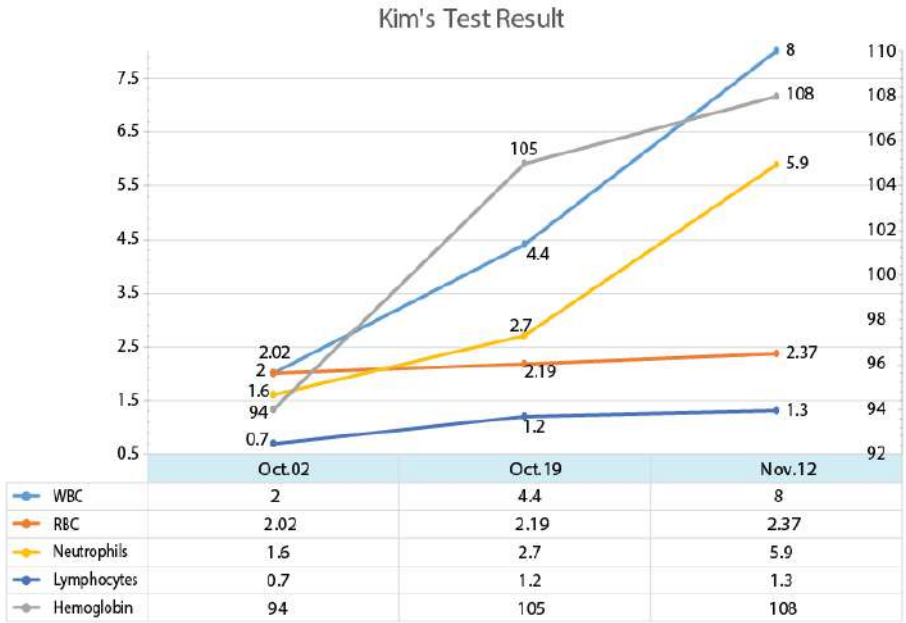


Figure 52. The Graphical Change of the Patient's Hematologic Data After Treating with AIC.

b. Mr. Kim (Chronic Leukemia, 72 years old, Male, BC. Canada): Hematological Data Became Normal After Treating with AIC.



	Oct.02	Oct.19	Nov.12
WBC	2	4.4	8
RBC	2.02	2.19	2.37
Neutrophils	1.6	2.7	5.9
Lymphocytes	0.7	1.2	1.3
Hemoglobin	94	105	108

Figure 53. The Graphical Change of the Patient's Hematologic Data After Treating with AIC. (Dose: 2 times a day; 5 mL with 500 mL of Water)

VII. Conclusion

By analyzing the current trend of treatments for ageing-related chronic degenerative diseases including osteoporosis and cancer, it became clear that all these diseases are related to the dysfunction of calcium influx and efflux. As the flow of ionized calcium is altered, it is calcified in the mitochondrial matrix, which results in secretion of Reactive Oxygen Species (ROS). Moreover, as the calcium homeostasis system is deconstructed, Bone Mineral Density (BMD) and the bone turnover rate are altered, which ultimately causes the deterioration of bone health.

Through the research, we have found that AIC can be a practical treatment option for treating chronic degenerative diseases as it balances calcium homeostasis, and initiates calcium signaling at both of the extracellular and intracellular levels. As Choi *et al.* (2011) have investigated, AIC has a notable efficacy in elevating BMD as they prove it with their animal experiment. As the BMD rate has been highly increased when treating with AIC for ovariectomized rats for 12 weeks, it became clear that AIC is useful as a treatment for osteoporosis. Moreover, AIC is also used to treat chronic neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's Disease and Alzheimer's Disease. As calcium modifies mitochondrial function by balancing up the intracellular calcium level, it can decrease ROS production.

Moreover, it has proven that AIC helps to initiate the activity of Mesenchymal Stem Cells (MSC) by regenerating mitochondrial function. As more ATP is produced and MSC is commenced by the depolarization caused by the influx of calcium ion, it can stimulate the cell division of MSC. As MSC is initiated, it can be the key to treating chronic diseases. For example, as MSC in bone marrow is commenced, it can transform to chondrocytes, which can help to manage osteogenic arthritis.

Furthermore, as calcium enhances the activity of mesenchymal stem cell, it helps to develop the dental bone health; which is helpful to rebuild dental

bone after dental implants. Moreover, as calcium deficiency and unbalanced level of ionic calcium can contribute the risk of developing dental gum and periodontal diseases. Therefore, as the efficacy of AIC has been proven to balance extracellular and intracellular calcium, the intake of AIC calcium is recommended to prevent the growth of bacteria in the gums and improve gingivitis and periodontal health.

Also, as AIC modifies mitochondrial function, it can adjust protein synthesis, which helps to produce p53 protein. Also, by secreting apoptosomes such as cytochrome c, it can help to effectively target the cancer cells when it is introduced into the cell. Moreover, as AIC neutralizes pH level by forming calcium lactate and calcium ammoniate, it can prevent cancer cells from being proliferated in the cell. Furthermore, as calcium reduces TXNIP protein expression by inhibiting the production of glutamine, AIC can be a treatment for cancer.

The case studies have shown that AIC has notable efficacy in treating cancer. Ms. Holt and Mr. Kim's data demonstrate the likely effects of AIC. As they are completely recovered from cancer by taking AIC, it is hard to deny the efficacy of AIC while the clinical data provides proof of its effects. Moreover, as their hematological data has been significantly improved, it would be a wise idea to try AIC as a cancer treatment option. While currently used cancer treatments have many adverse side effects, it is essential to find an alternative option that can enhance overall body metabolism.

Through the thoughtful, systematic literature review, it became more evident that AIC can be an innovative way of approach to treating chronic degenerative diseases. Even though there has been limited research conducted to date around the efficacy of AIC, it is crucial to investigate more about the effects of AIC while it shows significant improvements when applied to patients with chronic degenerative diseases, such as osteoporosis and cancer.

VIII. Appendix A

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Laboratory Animal Research
http://submission.kalas.or.kr

Effects of Anti-orbital Ionic Calcium Carbonate on bone turnover and calcium balance in ovariectomized rats

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This study was conducted to evaluate the effect of Anti-orbital Ionic Calcium Carbonate (AIC) as therapy for ovariectomy-induced osteoporosis in rats. Three weeks after surgery, fifteen ovariectomized Sprague-Dawley rats were divided randomly into 3 groups: sham-operated group (sham), ovariectomized group (OVX) and AIC-treatment group (OVX+AIC). The OVX+SAC group was given drinking water containing 0.0012% SAC for 12 weeks. Bone breaking force and mineralization as well as blood parameters related to the bone metabolism were analyzed. In OVX animals, blood concentration of 17 β-estradiol decreased significantly, while osteocalcin and type I collagen C-terminal telopeptides (CTx) increased. Breaking force, bone mineral density (BMD), calcium and phosphorus in femurs, as well as uterine and vaginal weights, decreased significantly following OVX. However, AIC treatment (0.0012% in drinking water) not only remarkably restored the decreased 17 β-estradiol and increased osteocalcin and CTx concentrations, but also recovered decreased femoral breaking force, BMD, calcium and phosphorus, although it did not reversed reproductive organ weights. It is suggested that AIC effectively improve bone density by preventing bone turnover mediated osteocalcin, CTx and minerals, and that it could be a potential candidate for therapy or prevention of menopausal osteoporosis.

Key words: Osteoporosis, bone mineral density, Aiti-orbital Calcium Carbonate, 17 β-estradiol, osteocalcin, type I collagen C-terminal telopeptides

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Osteoporosis is defined pathologically as a skeletal disorder characterized by absolute decrease in the amount of bone, leading to fractures after minimal trauma [1]. Also, osteoporosis is a major age-related health problem for men and women who often have a negative calcium balance due to decreased intestinal calcium absorption, insufficient dietary calcium intake, as well as increased urinary Ca loss associated with estrogen deficiency during menopause [2]. The main underlying cause of fractures in osteoporosis is increased bone fragility as a result of bone loss. Fracture risk is determined by absolute bone mineral density (BMD), regardless of age [3-6]. Low BMD is realized as the most important risk factor that can lead to osteoporosis [7,8].

In humans, osteoporosis was classified as 3 types; postmenopausal osteoporosis (Type I), age-related osteoporosis (Type II) and secondary osteoporosis (Type III). Postmenopausal osteoporosis typically affects women by estrogen deficiency within 15 to 20 years after menopause [9,10]. BMD of bilateral ovariectomized women is significantly lower than similarly aged women with normal ovarian function [11]; 50-year-old women who were ovariectomized at about 30 years old have similar bone density as 70-year-old women who become menopausal at the age of 50. This fact indicates that the decline of ovarian function is more important than age [12]. Therefore, postmenopausal administration of estrogen decreases the occurrence of fractures associated with

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osteoporosis by about one half [1].

In patients with osteoporosis, estimation of biochemical markers indicating bone turnover and bone quality has been known to be useful in predicting the rates of bone loss in postmenopausal women [13-15]. Bone quality is determined by structural and material properties that are influenced by bone turnover rate [16]. Histomorphometry is a method of estimating method of bone quality in addition to newer imaging techniques such as microcomputed tomography and magnetic resonance microimaging of bone non-invasively, but the use of these techniques is restricted due to their limited availability, high cost and relatively high radiation exposure [16]. Accelerated bone turnover leads to the irreversible loss of some trabeculae, resulting in weaker bone and increased risk of fracture [16]. Therefore, bone turnover is most commonly assessed in clinical practice by the measurement of biochemical markers of bone turnover. Detectable bone turnover markers are products of osteoblast/osteocyst and osteoclast type I collagens (CTx) breakdown reflecting bone formation and bone resorption, respectively [13,16]. CTx, and bone type I collagen C-telopeptides, are products of collagen degradation and therefore mirrors bone resorption; elevated CTx levels generally mean accelerated bone turnover [16]. Therefore, estimation of BMD and CTx may be helpful for predicting fracture risks.

Drugs used in the treatment of osteoporosis can be classified in two groups; antiresorptive or stimulators of bone formation. Antiresorptive drugs include calcium, estrogen, calcitonin and bisphosphonates [17-20]. Plant estrogen for osteoporosis treatment has been used for prevention of bone resorption [21]. But, discontinuation of estrogen administration leads to the recurrence of bone resorption and the degree is similar with bone resorption after ovariectomy. Also, severe side effects of estrogen treatment are endometrial carcinoma, venous thrombosis and pulmonary embolism [1]. Bisphosphonates are generally well tolerated, with few side effects, which include gastrointestinal symptoms such as nausea, vomiting and diarrhea. However, recent findings suggest that bisphosphonates induce osteonecrosis of the jaw, atrial fibrillation, acute phase response and renal insufficiency, raising safety and ethical concerns around the use of bisphosphonates [20]. Therefore, new drugs need development for use in the prevention of osteoporosis, particularly in postmenopausal women.

Anti-orbital Ionic Calcium Carbonate (AIC; activated ionic calcium) harvested from oyster shells as the raw material, results in production of calcium oxide; this calcium has a weak bonding force with other molecules, radicals or atoms. Therefore, this treated calcium can easily be absorbed into the cell, increasing an animal's metabolic system. Also, AIC helps growth and increases endogenous growth hormone by effectively supplying calcium into the cell which is needed for metabolism.

Therefore, this study was performed to evaluate the effect of AIC as a therapy for osteoporosis in an osteoporotic rat model induced by ovariectomy. Also, we investigated the effects of the AIC on BMD, bone and mineral metabolism as well as biochemical markers of bone turnover in osteoporotic rats.

Materials and Methods

Chemicals

AIC was obtained from NTS Research & Inc. (Coquitlam, BC, Canada). The composition of AIC (above 98% in purity) is shown in Table 1. Specific gravity (at 20°C) was 7.0-7.5 and heavy metals (As, Pb, Cd and Hg) were <1 ppm.

Animals

Twelve-week old female SPF Sprague-Dawley rats (250 g body weight; n=15) were purchased from the Daehan Laboratory Animal Center (Eumseong, Korea). They were housed in stainless net cages (800W × 900L × 650H mm) or fenced rooms (1,300W × 3,720L × 1,250H mm) and 5 rats were contained in each place. The housing conditions as follows: temperature, 23 ± 3°C; relative humidity, 55 ± 10%; lighting time, 12 h (08:00-20:00); ventilation frequency, 10-20 changes/h; illuminance, 150-300 lux. The temperature and humidity was measured hourly by a thermo-hygrometer and there were no variables affecting results of the experiments. The animals were fed a standard rodent chow and purified water ad libitum. All experimental procedures were approved and carried out in accordance with the Institutional Animal

Table 1. Composition of Anti-orbital Ionic Calcium Carbonate

Contents	Concentration (weight %)
$CaCO_3$	98.5
Na_2O	0.56
MgO	0.49
SiO_2	0.22
Fe_2O_3	0.02
K_2O	0.04
MnO_2	0.001
TiO_2	0.002
Al_2O_3	0.012
Cl	0.02
S	0.09
P	trace
Cu	trace
Ni	trace

Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University, Korea.

Grouping and treatment

Experimental animals were divided randomly into 3 groups of 5 female SD rats. The acclimatized rats underwent either bilateral laparotomy (sham, n=5) or bilateral ovariectomy (OVX, n=10). Three weeks after recovering from surgery, the OVX rats were randomly divided into two groups: vehicle-treated (OVX, n=5) and AIC-treated (OVX+AIC, n=5). Only OVX +AIC rats were given drinking water containing 0.0012%AIC for 12 weeks following 3 weeks post-operation. Changes in body weight and food and water intakes were measured at a regular time. The food and water intakes were calculated by differences between previous intake and the daily measured value.

Blood collection

Blood samples were collected 12 weeks after surgery. Before the blood collection, animals were fasted for 16 h and anesthesia (Zoletile 50®, Virbac, Korea) was given. After an incision in the inguinal region, 0.5 mL of blood was collected from the abdominal artery and kept in a refrigerator (4°C). A part of the blood was used for hematology. Remaining blood was centrifuged at 3,000 rpm for 10 min to obtain serum. The serum was used for blood biochemistry, and for analyses of 17 β-estradiol, osteocalcin and CTx which are bone metabolism indicators.

Hematology and blood biochemistry

Hematologic tests were performed using T-540 Coulter Counter (Coulter Electronics Inc, Hialeah, USA) and ADVIA120 Hematology System (Bayer Healthcare LLC, Tarrytown, USA). White blood cells (WBC), differential leukocyte counts, hematocrit, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets were analyzed.

The following parameters were analyzed for blood biochemistry (Ciba-Corning 644 Na/K/Cl Analyzer, Ciba-Corning, Medfield, USA): alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), glucose, triglycerides (TG), total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), total proteins (TP), albumin, calcium and phosphorus. As parameters of bone metabolism, serum 17 β-Estradiol concentrations were analyzed using a 17 β-estradiol kit (Coat-A-Count® Estradiol: Diagnostic Products Corporation, Los Angeles, USA). Osteocalcin levels were determined using a sandwich ELISA kit (Biomedical Technologies Inc., Stoughton, USA), and CTx levels were measured using a serum RatLaps ELISA kit (IDS Inc., Fountain Hills, USA).

Measurement of bone density and minerals

Bone density of the femur was measured using dual energy X-ray absorptionmetry (DEXA; Prodigy Advance, Donga imaging, Korea). Just after BMD measurement, mechanical testing of the femur was performed at room temperature using a materials testing machine (MZ500D; Maruto, Tokyo, Japan). For stabilization, the mid-diaphysis of the femur was placed on two supports of the test apparatus that were 4 mm apart. The load of a three-point bending test was applied in the anteroposterior direction midway between the two supports. The breaking force was calculated using software that measures bone strength (CTR win, Ver 1.05). After breaking force measurement, the femurs were dried at 120°C for 6 h in a muffle furnace and the dry weights were recorded. The femurs were then incinerated at 800°C for 16 h and the ash was weighed. One hundred milligrams of bone ash was then dissolved in 2 mL of 37% HCl and diluted with distilled water. Calcium and phosphorus contents were determined by atomic absorption spectrophotometry (PerkinElmer; A Analyst 100 Spectrophotometer, Boston, USA).

Statistical analysis

Mean and standard deviation of all measurement values were computed by Microsoft Excel. The significance of differences in the groups was evaluated by ANOVA using SAS (Statistical Analysis System) and were considered significant for values of $P<0.05$. Post hoc analysis was carried out using Duncan's multiple-range test.

Results

Body weight gain and feed/water intakes

Up to 2 days after the operation, a decrease in body weight was noted in both the OVX and OVX+AIC groups due to post-operative stress but there was consistent increase in body weight and food intake afterwards (data not shown). The increase in body weight after ovariectomy was slightly higher in the ovariectomy group (0.98 g/day) compared to the sham group (0.84 g/day) (Table 2). Notably, AIC treatment further increased the body weight gain, although there was no statistical significance. In regards to feed intake, significant changes ($P<0.05$) in daily feed intake was seen in the OVX group (20.53 g/day) compared to sham group (19.15 g/day). In addition, AIC further enhanced the feed intake, leading to an increased feed efficiency ratio (FER). It is considered

Table 2. Body weight gain, feed intake and feed efficiency ratio (FER)

Treatment	Body weight gain (g/day)	Feed intake (g/day)	FER
Sham	0.84 ± 3.76	19.15 ± 0.98[a]	0.04 ± 3.8
OVX	0.98 ± 6.06	20.53 ± 0.81b[b]	0.04 ± 7.48
OVX+AIC	2.38 ± 6.74	21.59 ± 1.39[b]	0.11 ± 4.84

Means with different superscript letters are significantly different at P<0.05. Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate treatment after ovariectomy.

that such a difference in feed intake following AIC treatment had an influence on the change in body weight. On the other hand, there were no differences in water intake among groups (data not shown).

Organ weights

The absolute organ weights are shown in Table 3. Weights of the reproductive organs (uterus and vagina) were decreased significantly by ovariectomy, which were not recovered by AIC. Other organs including femurs decreased slightly after ovariectomy but there were no statistically significant changes following AIC treatment. It was found that AIC did not have a big influence on organ weights.

Hematology and blood biochemistry

In hematological analyses,, a statistically significant increase in eosinophils in the AIC treated group after ovariectomy was noted, although there was a big deviation (Table 4).

There were no significant changes in the blood parameters related to the hepatotoxicity (AST, ALT and ALP), hepatic

Table 3. The absolute organ weights of ovariectomized rats treated with AIC for 12 weeks

Organs	Sham	OVX	OVX+AIC
Body weight (g)	310.84 ± 11.14	353.75 ± 10.18	371.36 ± 9.97
Uterus (g)	0.189 ± 0.050[a]	0.038 ± 0.011[b]	0.044 ± 0.020[b]
Vagina (g)	0.113 ± 0.0303[a]	0.050 ± 0.021[b]	0.058 ± 0.020[b]
Liver (g)	2.34 ± 0.15	2.17 ± 0.10	2.18 ± 0.15
Kidneys			
Right (g)	0.324 ± 0.021	0.268 ± 0.003	0.263 ± 0.021
Left (g)	0.329 ± 0.033	0.271 ± 0.012	0.269 ± 0.011
Spleen (g)	0.184 ± 0.022	0.169 ± 0.020	0.186 ± 0.021
Adrenal glands			
Right (g)	0.0127 ± 0.0024	0.0093 ± 0.0012	0.0094 ± 0.0014
Left (g)	0.0128 ± 0.0020	0.0092 ± 0.0008	0.0094 ± 0.0014
Brain (g)	0.656 ± 0.031	0.604 ± 0.031	0.553 ± 0.022
Pituitary (g)	0.0055 ± 0.0001	0.0038 ± 0.0002	0.0042 ± 0.0010
Femurs			
Right (g)	0.330 ± 0.021	0.296 ± 0.023	0.290 ± 0.021
Left (g)	0.352 ± 0.030	0.293 ± 0.012	0.294 ± 0.021

Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate.

Table 4. Hematological values of ovariectomized rat treated with AIC for 12 weeks

Parameters	Sham	OVX	OVX+AIC
White blood cells ($10^3/\mu L$)	4.95 ± 3.15	4.69 ± 1.79	4.84 ± 1.17
Neutrophils (%) Eosinophils	22.28 ± 4.48	23.4 ± 7.18	21.21 ± 6.19
(%) Basophils (%)	1.00 ± 0.52[a]	1.16 ± 0.27[a]	5.04 ± 3.88[b]
Lymphocytes (%)	0.04 ± 0.05	0.04 ± 0.05	0.02 ± 0.04
Monocytes (%) Hematocrit	73.72 ± 3.97	72.54 ± 6.96	70.36 ± 8.78
(%)	2.68 ± 0.78	2.58 ± 0.33	2.50 ± 1.44
Red blood cells ($10^2/\mu L$)	44.16 ± 2.47	45.30 ± 1.23	43.94 ± 2.18
MCV (fL)	7.95 ± 0.64	8.26 ± 0.24	8.23 ± 0.55
MCH (pg)	55.68 ± 2.11	54.82 ± 0.82	53.44 ± 1.24
MCHC (g/dL)	32.26 ± 1.36	30.94 ± 0.91	30.50 ± 0.75
Platelets ($10^3/mm^3$)	57.94 ± 1.88	56.40 ± 1.33	57.14 ± 0.76
	1,440.2 ± 153.8	1,234.8 ± 82.4	1,297.0 ± 91.3

MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate treatment after ovariectomy.

Table 5. Blood biochemical values of ovariectomized rats treated with AIC for 12 weeks

Parameters	Sham	OVX	OVX+AIC
AST (IU/L)	159.8 ± 37.8	163.0 ± 20.3	148.0 ± 30.4
ALT (IU/L)	19.2 ± 4.5	18.6 ± 4.3	22.4 ± 4.0
ALP (IU/L)	108.6 ± 29.7	112.6 ± 17.9	120 ± 21.9
Glucose (mg/dL)	109.8 ± 7.4	115.0 ± 17.7	112.4 ± 7.7
Triglycerides (mg/dL) Total	11.8 ± 6.1	19.8 ± 6.6	20.6 ± 7.7
cholesterol (mg/dL) HDL	59.6 ± 4.8	60.2 ± 24.3	73.0 ± 17.3
(mg/dL)	20.8 ± 2.5	19.8 ± 6.8	23.8 ± 5.2
LDL (mg/dL)	10.2 ± 4.1	8.9 ± 5.3	8.0 ± 5.2
Total proteins (mg/dL)	5.0 ± 0.2	4.7 ± 0.2	4.7 ± 0.2
Albumin (mg/dL)	3.0 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
BUN (mg/dL)	9.7 ± 2.3	10.2 ± 2.4	9.2 ± 2.4
Calcium (mg/dL)	6.8 ± 0.3	6.7 ± 0.2	6.7 ± 0.4
Phosphorus (mg/dL)	4.4 ± 0.4	4.3 ± 0.3	4.1 ± 0.6

AST; aspartate transaminase, ALT; alanine transaminase, ALP; alkaline phosphatase, HDL; high-density lipoproteins, LDL; low-density lipoproteins, BUN; blood urea nitrogen. Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate after ovariectomy.

Table 6. 17 β -Estradiol concentration and bone metabolism markers of ovariectomized rats treated with AIC for 12 weeks

Treatment	17 β -Estradiol (pg/mL)	Osteocalcin (ng/mL)	CTx (ng/mL)
Sham	4.536 ± 0.343^{a}	3.398 ± 0.294^{a}	4.751 ± 0.244^{ab}
OVX	3.920 ± 0.277^{b}	4.047 ± 0.501^{b}	5.082 ± 0.462^{b}
OVX+AIC	4.217 ± 0.247^{ab}	3.558 ± 0.154^{a}	4.360 ± 0.495^{ac}

Means with different superscript letters in the same column are significantly different at P<0.05 by Duncan's multiple-range test. Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate treatment after ovariectomy, CTx; C-terminal telopeptides.

Table 7. Breaking force and values of ash, Ca and P of ovariectomized rats treated with AIC for 12 weeks

Treatment	Breaking force (kg)	BMD (mg/cm^2)	Ash (mg/dL)	Ca (mg/dL)	P (mg/dL)
Sham 정상쥐	7.113 ± 0.035^{a}	0.2276 ± 0.011^{a}	428.2 ± 17.34	140.8 ± 2.48^{a}	138.0 ± 3.16^{a}
OVX 골다공증쥐	7.002 ± 0.106^{b}	0.1965 ± 0.012^{b}	409.5 ± 8.39	136.0 ± 3.10^{b}	130.3 ± 7.72^{b}
OVX+SC 골다공증쥐 +BAC calcium	7.1220 ± 0.064^{a}	0.2276 ± 0.012^{a}	419.8 ± 8.76	147.5 ± 5.33^{c}	138.1 ± 5.39^{a}

Means with different superscript letters in the same column are significantly different at P<0.05. Sham; sham operation, OVX; no-treatment after ovariectomy, OVX+AIC; Anti-orbital Ionic Calcium Carbonate treatment after ovariectomy, BMD; bone mineral density.

energy storage and pancreatic injury (glucose), hepatic lipid metabolism (triglcerides, total cholesterol, DHL and LDL), hepatic protein synthesis (total proteins and albumin), renal injury and electrolyte balance (BUN, calcium and phosphorus) (Table 5). Especially, however, ALP, a bone growth marker, increased moderately in AIC-treated animals.

Blood markers of bone metabolism

Blood 17 β -estradiol concentration significantly decreased following ovariectomy, and the decrease in 17 β -estradiol due to ovariectomy was significantly restored by AIC treatment (Table 6). Such a result indicates that the decrease in estrogen concentration caused the acceleration of osteoclast generation, accelerating bone resorption, which might be prevented by AIC. Increases in osteocalcin and CTx were found in the ovariectomy group compared to sham group. But such increases in the bone metabolic factors were recovered to their normal levels following treatment with AIC.

Breaking force and bone mineralization of femurs

The breaking force of femurs of ovariectomized significantly decreased, indicative of the weakened bone integrity (Table 7). In parallel with the alteration of breaking force, BMD as well as the concentrations of ash, calcium and phosphorus also reduced by ovariectomy. Interestingly, however, the breaking force and related ingredients (BMD, calcium and phosphorus) were fully recovered by AIC treatment.

Discussion

Osteoporosis has been studied by many health professionals and nutritionists in order to prevent the disease. Two major methods are used to diagnose osteoporosis including radiology and biochemistry. Compared to radiology which is a static

analysis of bone condition, biochemical diagnosis is able to analyze the dynamic state of the bone by comparing the mutual relationship between markers of bone [22].

When the weight changes of ovary-removed rats were investigated, the OVX group showed significantly high weight gain compared to the control (sham) group [9,23,24]. Although there was a weight decrease during the first 1-2 days post-surgery caused by stress, weight increased over the 12-week period [25,26]. Similar to these results, when we removed both ovaries of the rats, body weights significantly increased in the experimental group. On the other hand, the food and water intake of the control group slightly increased, but was not significantly different. Such results show similarity with previous studies [25,26].

It has been attempted to investigate the form of osseous tissue and the kinetics of calcium metabolism by using calcium isotopes. However, because of the complexity of tests and the clinical limitation, a biological marker which has high sensitivity and applicability is needed to measure the bone turnover in osteoporosis. In addition, to evaluate the formation and absorption of the bone matrix, measuring the activity of alkali or acidic oxidase of osteoblasts and osteoclasts can be performed. Also, there is a way of measuring bone matrix components circulating in the system which are released during bone formation and absorption processes.

ALP increased in the serum depending on osteoblastic activity. ALP in the serum indicates positive correlation with other biological markers but has a negative correlation with bone density and it reflects increased bone turnover by bone loss after ovariectomy. However, because the rate of bone turnover can be increased by other diseases such as hyperthyroidism, a true correlation between ALP and osteoporosis may be skewed.

According to Delmas [27], ALP can be a possible marker for bone turnover but not suitable for bone loss because of the production of the enzyme is not only limited to the osseous tissue; the liver produces an isoenzyme of ALP which reduces the sensitivity and the specificity of the test. In our study, the concentration of the enzyme in serum increased, but did not show relevance with osteoporosis.

Known as a compound closely related with bone metabolism, 17 β -estradiol is mainly produced by the ovary, corpus luteum, placenta, adrenal glands and testis. Remarkable fluctuations occur during the menstrual cycle and pregnancy in women. Dr. Fuller Albright first mentioned that sex hormone deficiency can cause osteoporosis. Wroolie et al [28] and Durador et al [29] reported ovariectomy-induced osteoporosis and menopause induced osteoporosis with significant reductions of 17 β -estradiol in the blood. Also in this study, compared to the control group, the experimental group showed significant 17 β -estradiol reductions which is consistent to previous studies [28,29]. Bone turnover rate is increased by reductions in estrogen, and bone loss rapidly progresses by increased bone absorption compared to bone formation.

Osteocalcin exists in bone and dentin and accounts for about 20% of non-collagen proteins in bone. It was reported that osteocalcin limits mineralization in in vitro experiments and bone density in mice without the osteocalcin gene is increased [18]. The concentration of osteocalcin is increased due to the rapid bone turnover rate after menopause but is decreased by injection of estrogen. Therefore, the increase of osteocalcin will likely increase bone turnover rate [27]. In this experiment after ovariectomy, the concentration of osteocalcin increased, and after the AIC treatment, the concentration remarkably decreased, indicating the changes of bone turnover rate; this suggests the the possibility of AIC as a treatment for osteoporosis.

According to a 2 year period follow-up study among the women who had femoral fractures, the baseline of CTx was increased compared to women without femoral fracture [13,16,30]. Same as in this study, CTx remarkably increased after ovariectomy, followed by a significant decrease with AIC treatment.

Low bone density is a risk factor and tends to progress into decreases in femoral bone density after menopause [9,31]. In this study, bone density of the OVX group showed low values compared to the sham group and AIC treatment increased BMD after ovariectomy.

The primary change after estrogen deficiency is an increase in the outflow of calcium from the skeleton. Due to the rapid bone loss, the secretion of parathyroid hormone decreases, causing a decrease in the calcium absorption in the intestine and finally resulting in the loss of calcium from the body and bone [2,32]. It was recently reported that over 2 years administration of calcium was more effective in decreasing bone loss of the placebo group in the study of effect of calcium supplement on bone density and fracture in women after menopause with 15 meta-analysis [17,18].

In this experiment, compared to the sham group, OVX had a significant decrease in calcium while the OVX+AIC group was not significantly different to the sham group, demonstrating decreased progression to osteoporosis. In summary, osteoporosis induced by ovariectomy could be alleviated by AIC treatment.

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