Preparation of Anti-RANKL IgY for Chicken Bone Health

by

Kumakshi Sharma

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Abstract

Osteoporosis is one of the prevalent disorders that exist in both humans and chickens. The bone disorder is characterized by depletion of bone mineral content, leading to fragile bones and increases susceptibility to fractures. Cage layers are more prone to fragile bones and fractures, during the egg laying cycle due to lack of physical activity, nutritional factors and bone resorption. Receptor activator of nuclear factor kappa B Ligand (RANK-L) expressed on the surface of osteoblasts play a major role in bone resorption process. Interaction and binding of RANK-L to its receptor (RANK) on the surface of osteoclasts, results in differentiation and maturation of osteoclasts, hence, leading to osteclastogenesis. This thesis focuses on development of therapeutics that would inhibit the activity of RANKL and prevent bone breakdown in chickens. Immunoglobulins in chicken egg yolk, called IgY have been reported to be potential candidate against several bacterial pathogens. Health Canada approved Denosumab, which is a human monoclonal antibody that prevents RANK – RANK-L interaction, as a treatment for osteoporosis in humans. Based on the concept of denosumab, we propose that producing IgY against RANK-L would be an effective way to treat osteoporosis in chickens by binding to RANK-L, thereby preventing bone resorption caused by RANK – RANK-L interaction.

The aim of this project is to produce recombinant chicken RANK-L using *E. Coli* and *Baculovirus* expression systems. Because the expression of chicken RANK-L *E. Coli* expression system led to formation of insoluble inclusion bodies, there were challenges to solubilize and purify the proteins. However, *baculovirus*

expression system was successfully able to produce intracellular recombinant ligands. The chickens were immunized intramuscularly with chicken RANK-L, and then IgY specific to RANK-L was isolated and purified from egg yolk. The affinity and binding of specific IgY with respective RANK-L was investigated and showed by immunoasays. We did a preliminary *in vitro* study on murine marcrophage cells (RAW 246.7). The cells showed positive tartrate-resistant acid phosphatase activity when the marcrophages were treated with mouse and chicken RANK-L and there was no activity when cells were treated with RANK-L and their specific IgY.

Preface

This work was supported by a grant from the Alberta Livestock and Meat Agency.

No part of the thesis has been published to date in a journal. But, the work done in the thesis has been presented at Faculty of Pharmacy and Pharmaceutical Sciences Research Day.

The following manuscripts are in preparation:

- Comparative Insights: Osteoporosis in Humans and Chickens. Kumakshi Sharma, Advaita Ganguly, Hoon H. Sunwoo, Michael Doschak
- Comparative analysis of chicken RANK-L protein expressed by *Escherichia coli* (*E. coli*) and *Baculorvirus* expression systems. Kumakshi Sharma, Biwen Xu, Advaita Ganguly, Hoon H. Sunwoo, Michael Doschak
- Production of IgY against recombinant chicken RANK-L. Kumakshi Sharma, Biwen Xu, Advaita Ganguly, Hoon H. Sunwoo, Michael Doschak

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ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BM	Bone marrow
BMD	Bone mineral density
BMP 2/4	Bone morphogenetic proteins 2 and 4
BMPs	Bone morphogenetic proteins
BPs	Bisphosphonates
BSP	Bone sialoprotein
CD	Celiac Disease
CF	Cystic fibrosis
CFU-MG	Colony-forming units-granulocyte-macrophage
chRANK-L	Chicken RANK-L
CXCR4	Chemokine receptor type 4
E. coli	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent Assay
FGF	Fibroblasts growth factor
FPP	Farnesyl pyrophosphate
GIT	Gastrointestinal tract
GSK	Gushukang
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
HA	Hydroxyapatite

HEAL	His-Glu-Ala-Leu
hMAb	Human monoclonal antibody
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
LiCl	Lithium chloride
LIF	Leukemia inhibitor factor
LRP5	Lipoprotein receptor-related protein
MCSF	Macrophage colony-stimulating factor
mRANK-L	Mouse RANK-L
MSC	Mesenchymal stromal cells
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCN	Osteocalcin
OLC	Osteoclasts like cells
OPG	Osteoprotegerin
PA	*Pseudomonas aeruginosa*
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PTH	Parathyroid hormone
PTHrP	PTH related peptide
RANK	Receptor Activator of Nuclear factor kappa B
RANK-L	Receptor Activator of Nuclear factor kappa B Ligand
r-chRANKL	Recombinant chicken RANK-L

SR	Strontium Ranelate
SSCs	Skeletal stem cells
TGF-β	Transforming growth factor beta (β)
TNF	Tumor Necrosis Factor
TPO	Thrombopoietin
TRAP	Tartrate-resistant acid phosphatase
WHO	World Health Organization
WSF	Water soluble fraction

Chapter 1: Comparative Insights: Osteoporosis in Humans and Chickens

1.1. Abstract

Osteoporosis is a prevalent skeletal disorder in North America, characterized by progressive loss of bone mass with increased risk of fractures. The bone structure deteriorates, though the progression of bone loss is slower in men than women. Other factors that contribute to the rate of bone deterioration include nutrient deficiencies like calcium and vitamin D, lack of physical activity, and hormonal changes in post-menopausal women. Several drugs and biological agents including denosumab (a human monoclonal antibody) have been used to treat osteoporosis in humans. Osteoporosis is also very common in egg-laying hens that leads to increased mortality and thereby affects commercial production of eggs. As commercial chickens are housed in conventional cages, they develop cage-layer fatigue along with osteoporosis due to minimal physical activity and depletion in bone mineralization during the molting period. A strategy to prevent loss of bone mass, increase in mineral content without affecting the commercial production of eggs needs to be developed. The knowledge gained and strategies developed over the years to manage osteoporosis in humans, can be useful in development of a strategy to treat osteoporosis in chickens.

1.2. Introduction

There are studies from a number of years on osteoporosis and the factors that result in bone loss and osteoporosis in humans. Due to an increase in the risk of osteoporosis, scientists have been working on different strategies to manage osteoporosis and to reduce bone resorption in humans. Likewise, there are large volumes of research articles on morphology of bones, bone loss leading to osteoporosis and change in morphology of bones in chickens and hens, once their egg-laying period starts. But, there is no strategy or treatment yet to prevent bone loss or enhance bone resorption in chickens. The aim of the review is to examine the information on osteoporosis in humans that will help to identify possible solution for osteoporosis in egg-laying hens.

1.3. Osteoporosis in Humans

The progressive structural degradation of bone is a result of disproportionate bone resorption and bone replacement, which leads to a condition known as osteoporosis. Osteoporosis results in low bone mass, makes bone more fragile and then results in fractures of hip, spine and wrist (National osteoporosis foundation. http://www.nof.org. Accessed: 16 September 2015). According to an osteoporosis overview published by the United States National Institutes of Health in June 2015, more than 53 million people in the United States are at a high risk of osteoporosis or they already have it (Osteoporosis Overview, NIH. Accessed: December 2, 2015). As women have less peak bone mass and hormonal changes occurring after menopause, osteoporosis is three times more prevalent in women than men (Kanis and Pitt, 1992). Significant bone loss starts occurring in the case of men after the

age of 65; however, in women it typically starts around the age of 50 (Cheung and Wren, 1992).

Osteoporosis is categorized into four types:

a) "Primary" Osteoporosis: is known as "postmenopausal" osteoporosis, when a skeletal disorder occurs in women after their menopause, and is known as "senile" osteoporosis, when it occurs in older men and women (Marcus and Majumdar, 2001).

b) "Secondary" Osteoporosis: occurs when defined and clinical disorders such as thyrotoxicosis or hyperadrenocoticism results to a condition of bone loss (Riggs *et. al.*, 2001).

c) As deficiency in reproductive hormones is directly related to postmenopausal osteoporosis, such secondary forms of osteoporosis are termed as "variants of primary" osteoporosis. For example, osteoporosis caused by exercise-related amenorrhea and tumors that secrete prolactin (Marcus and Majumdar, 2001).

d) "Juvenile" Osteoporosis: is a rare form of the condition that is reported in children near puberty and young adults (both males and females) (Riggs *et. al.*, 2001).

Primary osteoporosis can have two individual entities, one relating to estrogen secretion during menopause and the second to aging, was first proposed by Albright and Reifenstein in 1948 (Albright and Reifenstein, 1948)**.** Then in 1982, Riggs *et al.* advanced this concept and developed two terms for different types of primary osteoporosis (Riggs *et al.,* 1982).

a) *Type I osteoporosis*: is a term that is implied as loss of trabecular bone after menopause and lack of endogenous estrogen is the direct cause.

b) *Type II osteoporosis:* is a term for loss of cortical and trabecular bone in both genders as a result of aging. The amount of dietary calcium and vitamin D, intestinal mineral absorption, renal mineral handling, the efficacy of long-term bone remodeling and parathyroid hormone (PTH) levels are factors that influence Type II osteoporosis (Riggs *et al.,* 1982).

Women who have an inconsistent menstrual cycle, eating disorder, or have been on bed rest for an extended period of time or had systemic illness during growing years of adolescence, might have achieved deteriorated bone mass in their adult life. But, even if they had normal rate of bone loss during their adult life, they would be still at risk for a deficiency in peak bone mass. It is appropriate to draw a conclusion from this that osteoporosis is a multifaceted process with single or combined effects of various factors like multiple genetic, hormonal and nutritional that led to reduced skeletal integrity (Appelboom and Body, 1993).

e) As osteoporosis, occurs in both sexes and is related to age, the authors Riggs, Khosla and Melton, have preferred to use “involutional” osteoporosis, instead of “Primary” Osteoporosis (Riggs *et. al.*, 2001). The two types of “involutional” osteoporosis are classified in Table 1.

	Type I	Type II
Age (years)	51 – 75	> 70
Sex ratio (F:M)	6:1	2:1
Type of bone loss	Mainly Trabecular	Trabecular and cortical
Rate of bone loss	Accelerated	Not accelerated
Major fracture sites	Vertebrae (crush) and distal radius	Vertebrae (multiple wedge) and hip
Parathyroid function	Decreased	Increased
Estrogen effects	Mainly skeletal	Extraskeletal
Main causes	Menopause plus individual predisposing factor (s)	Factors related to aging including late effects of estrogen deficiency

Table 1-1. Characterization of Two main Types of Involutional Osteoporosis (B. L. Riggs, S. Khosla and L. J. Melton. The Type I/Type II Model for Involutional Osteoporosis. Osteoprosis. 2001. Chapter 38, 2nd Ed. Vol.2).

1.4. Osteoporosis in Chickens (Hens)

As we had described earlier, osteoporosis is a prevailing disease characterized by increased probability of fractures due to loss of bone mass and strength. Among the many factors that lead to formation of osteoporotic bones (Brandenburger, 1993), lack of exercise is a factor that contributes to increase in probability of bone fractures in all vertebrates, including caged laying hens (Sherman *et al*., 1993).

It was initially noticed in caged egg-laying hens that they were not able to stand but still ate, by Couch *et al*. and Grumbles *et al.*, in the mid-20th century and they called it as caged-layer fatigue (Burnell *et al*., 1982; Vose and Kubala, 1959). In

1960, Urist *et al*. demonstrated and reported that treatment of exogenous estrogen to the normal laying chickens reduced the thickness of bone cortex, making bones more porous and hence making them fragile. It was reported in 1962 by Bell *et al.* that thin cortical bone and trabecular bone (both play a role in bone strength) were reduced in normal laying hens, which became more prominent and prevalent in hens suffering from cage layer fatigue. It was also reported by Bell *et al*. that certain genetic lines of hens were more susceptible to cage layer fatigue and also described two types of cage layer fatigue:

a) *Peracute cage layer fatigue*: in which the hens did not show any prior symptoms of the fatigue, but died suddenly.

b) *Acute cage layer fatigue*: hens suffered from paralysis of legs and then collapsed. They would be in danger of death, but if they survived for a few days and were given proper food and water and taken care of, could recover within two months (Burnell *et al.*, 1986).

Riddell *et al.* in 1968 described that birds affected from cage layer fatigue had fragile bones and cage layer fatigue was strongly related to bone pathology. Also, keel and ribs became deformed at the costochondral junction due to fractures that caused the bones to fold. While, these fractures would heal in surviving birds, they would form permanent skeletal abnormalities. Fractured thoracic vertebrae found in birds affected with cage layer fatigue would lead to compression of the spinal cord that may be sometimes penetrated by bone fragments. This resulted in further spinal cord degeneration that would produce the paralysis typical of cage layer fatigue. The compression fractures of the thoracic vertebrae would lead to internal

hemorrhage and hence leading to death of hens due to starvation if left in their cages (Crofts, Boyce and Bloebaum 1994).

Thus, these studies and subsequent research on bone strength and osteoporosis provided strong evidence that cage layer fatigue was the root cause for osteoporosis in hens (Leone *et al*., 1955). Osteoporosis has resulted in 20 % to 35 % of all mortalities during the egg laying cycle in white leghorns, this affliction has caused a huge economic loss to the commercial egg production worldwide (Richards *et al*., 1994; Raisz, 1999; Whitehead, 2004).

In cage-laying hens, by the end of lay period (71 to 80 weeks of age), approximately 30 % of the hens have one or more broken bones. As laying hens require calcium for eggshell formation, they experience continuous loss of bone strength and mass during aging (Sherman *et al*., 1993). During osteoporosis, there is an increase in medullary bone volume (Burnell *et al.*, 1986) that is used as a calcium reservoir for eggshell formation, when structural bone undergoes the process of resorption. Due to commercialization of egg production, limited amount of physical exercise in conventionally caged hens, have accelerated the process of structural bone loss (Dacke *et al*., 1993). However, non-caged housing increases the chance of physical activity and jumping for hens, which then produces the required bone strength (Sherman *et al*., 1993; Richards *et al*., 1994).

1.5. Composition of Mammalian Bone

Bone is composed of 60 % by weight of inorganic matter, 8-10 % water and the rest constitutes the organic matter (Gong *et al*., 1964). Naturally occurring calcium

phosphate, also known as impure form of hydroxyapatite, forms the inorganic matter. Type I collagen forms the predominant part of the organic phase (98% by weight) and the rest 2% of the organic matter is constituted by noncollagenous proteins and cells (Einhorn *et al*., 1994).

The organic phase of bone is known to play an important role in mechanical and biochemical properties of the tissue and hence significantly influences the structure of bone tissue. Growth factors, cytokines and extracellular proteins constitute a small portion of the tissue, but have a major role to play in its biological function. The mineral phase has a noncollagenous protein named osteocalcin, which is the most abundant protein and makes up for 10-20 % of all the noncollagenous proteins in bone. Osteocalcin plays an important role in regulating the activities of osteoclasts and their precursors, hence is very significant in bone formation and mineral maturation (Boskey *et al*., 1998).

Bone has matrix proteins (for example, osteopontin, bone sialoprotein, bone acidic glycoprotein, thombospondin and fibronectin), in addition to noncollagenous proteins. These matrix proteins contain cell-binding amino acid sequences, aginine-glycine-aspartic acid (RGD) that is characteristic of a family cell membrane protein called integrins. Integrins are present on the cell membranes of osteoclasts, osteoblasts and fibroblasts and, provides a connecting link to the extracellular matrix and the receptors can bind the cell membrane via integrins that aid in their biological function (Rusolahti, 1991).

The bone mineral apatite, contains impurities of carbonate instead of phosphate groups and 4-6 % of carbonate in apatite, makes it similar to dahllite, another name for carbonate apatite. In some cases, potassium, magnesium, strontium and sodium may substitute calcium ions, and fluoride may replace hydroxyl groups (McConnell, 1962). These substitutions make apatite highly impure, deteriorating its crystal structure (Ou-Yang *et al*., 2001) and hence hampering the solubility of the mineral (Kaplan *et al*., 1994). The solubility of bone mineral plays an important role in bone adaption and maintaining mineral homeostatis. The crystal size for bone mineral is reduced in diseases like Paget's disease (Siris, 1998) and diabetes (Einhorn *et al*., 1998), while it increases in osteopetrotic individuals (Boskey and Marks, 1985) and in in osteoprososis patients with bisphosphonate treatment (Fraztl *et al*., 1996)

1.6. Composition of Chicken Bones

Compact cortical bones, the outer shell surrounding the cancellous or trabecular bone and marrow space, is present and constitutes long bones in chickens, as it is in mammalian bones. It had been shown by Albright in 1987 that the trabecular bones show lattice organization along with high turnover rates and provides larger surface areas (Albright, 1987).

The strength of a bone is determined by the architectural organization of the lattice structure. Bone can be either lamellar or woven in their structural organization. Lamellar bone is a mature form, constituting cortical and cancellous bone. They deposit in a slow manner and have collagen arrangements in a well-organized, aggregated manner. Woven bones on the other hand deposit rapidly, during

intramembrane ossification or the fracture healing process. Collagen fibres are randomly organized and loosely packed in woven bones; with lower mineral density and high water content in woven bones unlike lamellar bones (Turek, 1984; Gorski, 1998). In chickens, during the time of maturation of ovarian follicles, medullary bone develops in the endosteal surface of long bones. Medullary bones are woven bones with an increased rate of remodeling, essentially providing calcium required for eggshell formation (Dacke *et al*., 1993).

Medullary bone has low collagen and high mineral, preoteoglcan and carbohydrate content, in comparison to cortical or cancellous bone (Dacke *et al*., 1993; Rath *et al*., 1999). That is why medullary bone is not intrinsically strong, but it influences the mechanical strength of cortical bone (Knott *et al*., 1995, Flemming *et al*., 1998). Collagen, a triple helical fibrous protein forms the primary scaffolding of skeletal tissues and is the major constituent of the organic matrix (80-90 %) of the bone. It contributes to the tensile and biomechanical strength of the bone and provides oriented support for the mineralization process. Post-translational modifications of collagen, for example, hydroxylations and intermolecular crosslinking; results in developing the ability to cope with physical stress and hence enhance the tensile strength (Eyre, 1996; Knott and Bailey, 1998; Riggs *et al*., 1993). The most well known types of cross-linking are pyridinium crosslinks, which are hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) (Eyre, 1996).

It was reported by Knott et al., in 1995 that another type of crosslink in avian bone collagen, called “pyrrole” crosslink is the contributing factor for the biomechanical strength of bones and osteoporotic avian bones are characterized by reduced level

of pyrrolic crosslinks (Knott and Bailey, 1998). The remaining 10-15 % of the organic matrix of avian bone are made up of proteoglycans, lipids, and noncollagenous proteins such as osteocalcin, osteonectin, and osteopontins. The noncollagenous proteins play a role in matrix stabilization, calcification, and other metabolic regulatory activities of the bone (Termine and Gehron Robey, 1996).

Calcium and phosphorous are in the form of hydroxyapatite and constitute the mineral matrix and ~ 60 to 70% of the bone weight and hence are responsible for stiffness and compressional strength to the bone. During the process of calcification, hydroxyapatite crystals deposit in the hole zones produced by repeating periodic segments of collagen fibres (Turek, 1984). Avian bone like mammalian bone also contains ~ 10 to 15% water of total weight, that contributes to its viscoelastic properties. The other structural complexities of bone are the osteoblasts, osteocytes, and osteoclasts; which are discussed in later sections.

1.7. Nature of Osteoporotic Bone and Factors affecting Bone Strength in Mammalian system

Osteoporosis has been defined as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to bone fragility and increased risk of fractures" (Consensus Development Conference, Prophylaxis and treatment of osteoporosis, 1991). Over the years, until the late 1980's and early 1990's there has been a consistent view that osteoporosis resulted from a deficiency in normal bone mass and defective residual bone, but is not due to a deficiency in composition of bone matrix and mineralization (Bounjour and Rizzoli 2001). It was in the mid-1990's that scientists brought the attention to

abnormalities in bone acquisition as a cause for fragility in bones that ultimately led to osteoporosis. McCabe *et al.*, 1991, provided the evidence in this support by developing a technique called Mechanical Resistance Tissue Analysis, which showed a decrease in ulnar bending stiffness in women that occurred with age, that was related to depletion in bone mineral and/or gross bone geometry (Kann *et al.*, 1994). In another study, it was reported that resonance frequency of cortical bone in women decreases with increase in age (Brandenburger, 1993). Bone material and its structural properties that degrade with age, pose a risk for fractures, and was for the first time related to transmission velocity of ultrasound (Sherman *et al.*, 1993).

Burnell *et al*. were the first scientists to make an attempt in a systematic manner to study the composition of osteoporotic bone by comparing iliac crest biopsies from osteoporotic postmenopausal women with vertebral compression fractures to biopsies with control groups that had normal bones. The study showed that bones in osteoporotic postmenopausal women were less dense and there was a decrease in fraction of mineral per gram of bone tissue was also reduced. It was found that carbonate and calcium-to-phosphorus ratio within the bone mineral reduced significantly, while sodium and magnesium content were increased. This revealed a deficiency of skeletal calcium in most patients but no evidence of osteomalacia was found in the biopsies (Vose and Kubala, 1959). Under-mineralization poses a serious consequence to the bone's mechanical properties. Bone mineralization forms the backbone of the structural strength of the skeletal system (Currey, 1969). Young's modulus of elasticity is a factor for determining fundamental bone

material properties. A 7 % under mineralization increases bone stiffness by three-fold and decreases in bone strength by two fold, which ultimately leads to increase in bone fragility (Burnell *et al.*, 1986).

In a further study, Burnell *et al*. reported that the calcium content of bone tissue showed an improvement with intake of daily supplements of calcium and vitamin D while simultaneously decreasing its sodium content. It was known that bone remodeling was a result of simultaneous turnover of matrix and mineral, but it was unclear earlier, as to why alterations in remodeling activity alone would result in a relatively undercalcified bone matrix. Burnell *et al.* proposed two reasons and explanations for this view. The first explanation was that bone would not reach its full mineral potential if the bone formation occurred in an environment of limited calcium supply. As a chemical equilibrium can be reached between extensive hydroxyapatite surface area with bone interstitial and marrow fluids, the second explanation is that a slight decrease in bone fluid calcium, which could be as a result of a decrease in efficiency of calcium absorption (due to minimal amount of Vitamin D) or decrease in dietary intake of calcium, would lead to a shift in chemical equilibrium that favors calcium loss (Burnell *et al.*, 1986).

Crofts *et al*. were the first to report heterogeneous mineralization within the femur due to age and locational differences in osteonal mineralization, using backscattered electron imaging (Crofts *et al.*, 1994). A decrease in ash and mineral content of bones to 12 % from the older to young group of adults has been reported to show substantial mechanical consequences (Currey, 1969).

There is a potential influence on age-related changes in bone mineral properties with life-long fluoride exposure, as incorporation of fluoride makes the hydroxyapatite crystal more brittle. The fluoride content in normal portable water ranges from 0-3 mg/L (ppm). Concentrations higher that 3 mg/L can lead to mottling of dental enamel and also increase the risk of skeletal fluorosis, particularly in hot weather conditions where there is rise in daily water consumption. That is the reason for the community water to be regulated to a level of approximately 1 mg/L of fluoride (Leone *et al.*, 1955). It is well established that bone mass and strength both decreases with age, but a study by Richards *et al*. revealed that bone fluoride content, increased more than three-fold from age 20 to 80 years. The authors concluded that the consumption of fluoride for a long period of time does not independently affect bone quality unless the effects on bone mass due to aging and gender are also considered (Richards *et al.*, 1994).

1.8. Factors affecting Bone Strength in Chicken

According to Whitehead *et al*. the development of medullary bone is associated with cessation of remodeling of structural bone (both cortical and cancellous or trabecular bone), which marks the beginning of sexual maturation in the chicken (Whitehead, 2004). The primary function of medullary bone is to store significant quantities of calcium and release it later when required for calcification of eggshell when sufficient amount of calcium is not available in the digestive tract (Etches, 1987).

a) *Calcium:* Many researchers have concluded that deficiency in intake of dietary calcium leads to low bone mineral content and is the underlying cause for fragile bones in egg-laying birds (Cheng and Coon, (1990a) (1990b); Douglas *et al*., 1972; Mehring *et al*., 1964). The requirement for calcium further increases if there is a rise in early egg production in commercial hens and there is a decrease in feed intake (Roland, *et al*., 1996) but bone strength can be increased early in egg production if intake of limestone (particles) is included in the feed (Fleming *et al*., 1996). The levels of calcium ions in the blood are affected by rise in temperature that can reduce bone integrity; hence extra intake of dietary calcium is required in such weather conditions (Koelkebeck *et al*., 1993).

b) *Phosphorous:* Bone mineral does not constitute calcium alone but calcium phosphate and 1.5-2 mmol/L of serum phosphorous concentration is required for formation of bone mass during growing stage (Nordin, 1988; Standing Committee on the Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine. Washington, DC: National Academy Press. 1997; Shapiro and Heaney 2003). An inadequate amount of phosphorous in the diet intake of egg-laying hens during the laying period can lead to reduced bone strength, cage-layer fatigue and severe osteoporosis (Bell and Siller 1962; Simpson *et al*., 1964; Harms *et al*., 1990).

c) *Vitamin D:* Deficiency of vitamin D effects the overall mineralization of the skeletal system and results in depletion in bone mineral density (BMD), higher bone turnover and high risk of fractures. 1,25

dihydroxycholecalciferol, is the active metabolite of Vitamin D, responsible for opening the calcium channels in the gut, stimulating the formation of calcium binding proteins and hence increases the absorption of calcium and phosphorous from the gut. Low vitamin D intake ceases the absorption of calcium intake and increasing the secretion of parathyroid hormone (PTH) that stimulates a rise in bone turnover and reduced bone mass, thereby leading to osteoporosis (Lips, 2001).

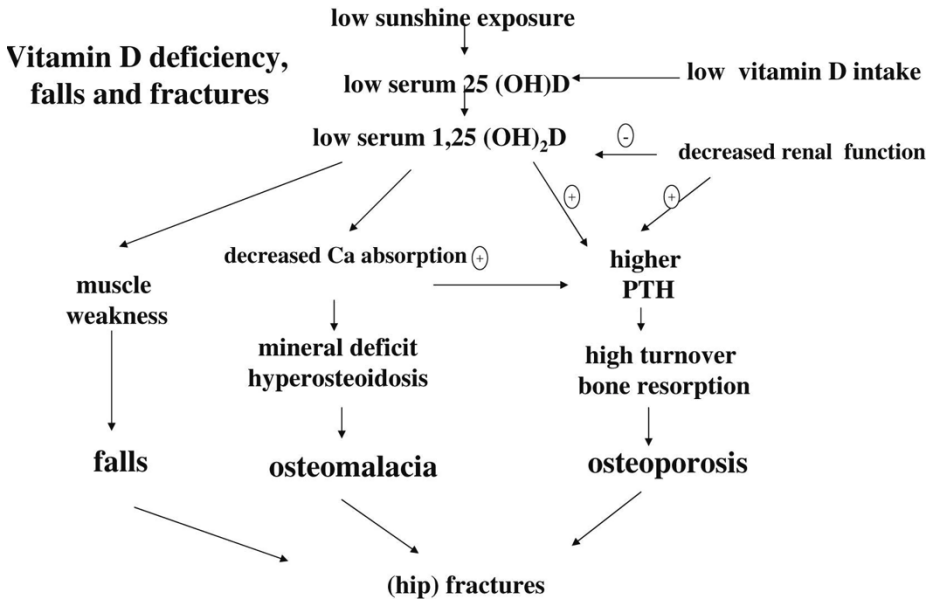

Figure 1-1. The pathophysiologic pathways from deficiency of vitamin D intake to osteoporosis, osteomalacia, falls and fractures (Reprinted by permission from Elsevier Limited. Lips P and van Schoor NM. 2011. *Best Practice & Research Clinical Endocrinology & Metabolism.* 25:585–591).

d) *Housing and Exercise:* We described earlier that cage layer fatigue and severe osteoporosis resulted in egg-laying hens, when they were housed in conventional cages that had minimal physical activity (Grumbles, 1959).

Many researchers have demonstrated that different types of housings for egg-laying birds such as floor pen, perchery, aviary, enriched or unenriched cage have a positive influence on the bone strength (Fleming *et al*., 1994; Gregory *et al*., 1990; Rowland *et al*., 1990).

1.9. Similarities and differences in Chicken and Human Osteoporosis

Bone metabolism is related to production of eggs in domestic chickens and the bone phenotype in turn is related to the fitness of chickens during domestication. The sexual ornament in both male and female chickens (Pizzari and Birkhead *et al*., 2000; Pizzari *et al*., 2003), known as the comb, has a relation with BMD. BMD is related to biological processes that form the underlying cause for osteoporosis in both chickens (Budgell and Silversides, 2004; Webster; 2004) and humans (Ralston and de Crombrugghe, 2006). Osteoblasts are the cells responsible for building new bone components and osteoclasts are the ones responsible for breaking of bone tissue; both play a major role in bone remodeling. The remodeling of bone in egg laying hens is associated with their egg-laying cycle and is faster than humans (Van de *et al*., 1984; Kerschnitzki *et al*., 2014). Medullary bones in hens are storehouses for calcium that are utilized for eggshell production, making bone metabolism unique in egg-laying hens (Bloom *et al*., 1958; Mueller *et al*., 1968).

Various studies have shown that osteoporosis in chickens is not only due to deficiency in dietary intake of calcium, but also due to some genetic effects involved with bone strength (Fleming *et al*., 2006; Rennie *et al*., 1997). Gene

variants such as LRP5 (Fleming *et al*., 2006; Rennie *et al*., 1997) and BMP2 (Ferrari, 2008) affects the bone density has been found by linkage mapping in humans. It was reported by Hiller *et al*. that recombination rate of various genes or variants in chickens are very high and have a very compressed genome as compared to mouse models (Hillier *et al*., 2004).

1.10. Physiology of Osteoporosis in Humans

In order to maintain and increase bone mass and strength, physical exercise is essential (Rutherford, 1990). Disuse or lack of physical activity is one of the reasons for stimulation of bone loss and thereby resulting in secondary osteoporosis (Howard, 2011). A regional trend is seen with disuse osteoporosis in the areas with tremendous decrease in weight bearing like lower limbs (Rutherford, 1990). An investigation on weight training exercise on BMD in postmenopausal women carried out by Milliken *et al*. showed that elevated levels of BMD of trochanter and femoral neck in women due to weight training exercise as compared to the ones without exercise (Milliken *et al*., 2006). During disuse osteoporosis bone loss, there is a sudden speedy loss of trabecular bone, while cortical loss occurs slowly but consistently. It was observed that although the ratio of bone mineral to collagen remains consistent, there was depletion in bone mass as compared to bone volume (Robling *et al*., 2006). It was further reported that bone loss occurs differently depending on its in anatomical location, quantity, velocity, and through different mechanisms that may be related to differential mechanical stimulations during regular life style. The three types of mechanical loadings reported in case of lower limbs are static gravity-related weight bearing, ground

reaction forces, and dynamic loading. As it was reported earlier long-term bed rest caused lack of ground force reaction and reduces muscle activity but according to the assessment conducted by Rittweger *et al*., bed rest has a minimal effect on balance of bone metabolism (Rittweger *et al*., 2009).

The second cause for disuse osteoporosis is paralysis. Sudden loss in bonemass in disuse osteoporosis has also been characterized by brain neurologic conditions and spinal cord injury (SCI) that further results in mitigated ground force reaction and muscle contraction in the lower limbs. Garland *et al*. carried out a cross-sectional study that reported an accelerated bone loss below the pelvis that became linear in its acute stage (Garland *et al*., 1992). They also showed that three months after SCI in post-traumatic paraplegic and quadriplegic patients more than 20% of the bone loss occurred at the distal femur (Kiratli *et al*., 2000). Smith *et al*. described that patients that suffered disability after neurological traumas, paralysis or SCI, would develop osteopenia and subsequently osteoporosis (Smith *et al*., 2000).

Reduced weight bearing and ground reaction forces led to bone loss, when exposed to microgravity (a third cause for disuse osteoporosis). Collet *et al.* confirmed this by studying effects of microgravity on the skeleton of astronauts after spaceflights. A significant amount of both trabecular and cortical bones loss was observed in the case of astronauts that stayed in space for 6 months as compared to the ones that stayed for one month (Collet *et al*., 1997). Vico *et al*., 2000, reported that the effect of microgravity was not consistent between different human skeletons, but showed a mean decrease in cancellous BMD that varied from 0.4 to 23.4 %.

1.11. Physiology of osteoporosis in Chickens

Decrease in tensile strength in cortical and cancellous bone was reported in humans (Wall *et al.,* 1979; Danielsen *et al.,* 1993), however very limited information is available for chickens or hens. In the study by McCoy *et al.* in 1996, the physical, compositional, and mechanical properties of tibial bones from 5- to 55-week-old chickens were compared. Even though the relative collagen content remained constant between 5 and 55 weeks of age, the other parameters such as allometric, biomechanical, and biochemical showed significant changes. It was reported in the same study that at 25 weeks of age, the weight, length, diameter, and pyridinium crosslink content reached a maximum unlike the mineral content, density, and the breaking strength of bones that did not reach maximum until 35 weeks of age. There were significant changes in the bone ash content between 25 and 35 weeks of age. This all gives an indication that it takes longer to attain maximal physical and functional potentials of bone than the growth process itself. Rath *et al*., 1999 showed earlier that the changes in the crosslink content of bones and the bone strength in birds of 7 and 72 weeks of age are extensively correlated (Rath *et al*., 1999). But, in chickens of or less than 5 weeks of age, bone strength did not show correlation to the collagen crosslink content due to the reason that 5-week-old chickens showed higher breaking strength and low content of pyridinium crosslink. Infact, they showed high stress and modulus values and, low strain values due to higher mineral matrix content relative to crosslink content indicating brittle bones. It was reported that there was a several fold increase in the crosslink content in comparison to bone ash content with the weeks of growth that decreased the

brittleness and made the bones tougher. High stress and modulus values and low strain values due to a higher mineral matrix content relative to crosslink content; are indicators of brittle bones (Rath *et al*., 2000).

1.12. Pathophysiology of Osteoporosis

Disuse osteoporosis can be due to either the inability to acquire optimal peak bone mass and strength during the period of bone mass accumulation or an accelerated rate of bone resorption and decelerated rate of bone formation in adults (Raisz *et al*., 2008). Bone remodeling is a regulated process that maintains the normal level of bone mass and hence plays an important role in growth and repair of skeletal system (Komori *et al*., 1997). Bone remodeling involves two phases:

a. Bone resorption phase: in which osteoclasts get placed at the bone surface, where remodeling is required. After scraping off the damage part of the bone, osteoclasts move away from the site. This process is very regulated and takes about two to four weeks.
b. Bone formation phase: Osteoblasts precursors differentiate into osteoblasts after moving at the bone surface. After osteoblasts become mature, they start forming bone matrix and then mineralization occurs in the next two to four months (Martin and Rodan 2001).

1.12.1 *Osteoblasts*:

Bone forming cells, osteoblasts are mononucleate cells that arise from differentiation of mesenchymal progenitor cells. They are responsible for producing an unmineralized, organic portion of the bone matrix known as

osteoid. Mineralization of osteoids with calcium and zinc results in the formation of new bone tissue (Komori *et al*., 1997). Research studies done by Komori *et al.*, 1997 and then Otto *et al*., 1997, suggested that the gene necessary for osteoblast differentiation and bone formation is Cbfa1 (Runx2). As reported by D'Ippolito *et al*. that the rate of bone formation tends to decrease with age due to depletion in the number of osteoblasts, that becomes the ultimate cause for osteoporosis (D'Ippolito *et al*., 1999). A number of growth factors such as fibroblasts growth factor (FGF), transforming growth factor beta (TGF-β), and bone morphogenetic proteins (BMPs) are known to play a pivotal role in the differentiation of osteoblasts from mesenchymal progenitor cells (D'Ippolito *et al*., 1999). Another growth factor, the insulin-like growth factor (IGF) system had been linked to the process of skeletal acquisition by Rosen in a review (Rosen, 2004). IGF-1 has been reported to be a required growth factor for normal bone formation. This was confirmed by a study done by Sakata *et al*., in which they showed that integrin expression was affected due to anabolic actions of IGF-I on bone and hence may be an essential component in the mechanism of disuse osteoporosis (Sakata *et al*., 2004).

1.12.2. *Osteoclasts*:

Osteoclasts are multinucleated bone resorbing cells that differentiate from haemopoietic monocyte or macrophage cells (Narducci and Nicolin 2009; Baron *et al*., 1985). Recruitment of osteoclasts during bone resorption phase, to the bone matrix results in the formation of a deeply folded membrane. This is subsequently followed by secretion of protons and hydrolytic enzymes to the

lacuna. Organic components of the bones, such as collagen are hydrolyzed by hydrolytic enzymes on being exposed to acidic environment created by protons. This is called demineralization of lacuna (Horne *et al*., 2008). Although, bone resorption is physiologically important for bone remodeling, rise in number or accelerated activity of osteoclasts may lead to excess bone loss and disrupted skeletal structure, ultimately leading to skeletal fragility (Khosla, 2001). Bone resorption process by osteoclasts have been known to be a tightly regulated process that involves interactions between different growth factors and many cytokines such as interleukin -1 (IL-1), tumor necrosis factor alpha (TNF-α) and prostaglandin E2 (Kimble *et al*., 1995; Ammann *et al*., 1997). *Hughes et al*. established that osteoclasts formation and its activity had a negative impact due to stimulation of apoptosis of osteoclasts by estrogen in postmenopausal osteoporosis (Hughes *et al*., 1996).

1.12.3. *Tumor Necrosis Factor (TNF) Superfamily*

The TNF superfamily is comprised of ligands and their respective receptors; and is associated with pathways that regulate and modulate the immune system including cell death, cell survival, cytokine and chemokine expressions (Locksley *et al*., 2001). The members of TNF receptor superfamily are type I transmembrane proteins and are characteristic of presence of conserved cysteine-rich domains (CRD) in the extracellular ligand-binding region (Naismith and Sprang 1998). However, the ligands of TNF superfamily are mostly type II transmembrane proteins and have an extracellular proteolytic cleavage site to release a soluble protein from the membrane (Lam *et al*., 2001).

Analysis of chicken genome by Kaiser *et al*., showed a reduced repertoire of TNF superfamily in comparison to mammals (Kaiser *et al*., 2005). Further analysis concluded that there were 29 receptors and 19 ligands determined for TNF superfamily in mammals, while for chicken genome there were 15 receptors and 10 ligands identified (Kaiser, 2012).

1.12.4. *Receptor Activator of Nuclear factor kappa B (RANK):*

A major factor responsible for osteoclast differentiation, receptor activator of nuclear factor kappa B (RANK) is a transmembrane heterotrimer with three molecular intracellular domains (I, II and III). It belongs to the TNF (tumor necrosis factor) receptor (TNFR) superfamily with type I transmembrane protein (constituting 616 amino acids). It has the longest extracellular domain (residues 234–616) among all TNFR members, which contains four cysteine-rich domains (CRD). It is the only receptor for RANK ligand (RANKL) (Boyle *et al*., 2003; Fata *et al*., 2000; Hsu *et al*., 1999). The expression of RANK occurs on the surface of various cells that includes hematopoietic osteoclast progenitors, mature osteoclasts, chondrocytes, mammary gland epithelial cells, and trophoblast cells (Fata *et al*., 2000; Hsu *et al*., 1999).

Hsu *et al*. showed the binding of RANK with its ligand called RANKL (discussed in next section) in an *in vitro* study reportedly led to differentiation of monocyte/ macrophage progenitor to osteoclasts and further activation of mature osteoclasts, a process known as osteoclastogenesis (Hsu *et al*., 1999). In 1999, Wong *et al*. described the mechanism for activation of RANK and

osteoclasts. They reported that the interaction of intracellular I, II and III domains of RANK and adapter proteins, TNF receptor-associated factors (TRAF) made RANK receptor active on the osteoclast surface, hence resulting in intracellular signals (Wong *et al*., 1999).

1.12.5. *Receptor Activator of Nuclear factor kappa B ligand (RANK-L):*

Another member of TNF superfamily that is encoded by the rankl gene, RANK-L forms a functional molecule with 316 amino acids (38 kDa) (with three RANK-L subunits): a COOH-terminal receptor-binding domain, a hydrophobic transmembrane domain (constituted by 20 amino acids) and a relatively long extracellular domain that contains a TNF-homologous domain (the active receptor-binding site) (Lum *et al*., 1999). RANK-L is a cytokine released from the cell as a result of proteolytic cleavage by the metalloprotease desintegrin TNF-α convertase enzyme (Lacey *et al*., 1999; Ikeda at al., 2001). RANK-L are highly expressed on the surface of osteoblasts in various tissues such as lymph nodes, thymus, bones, and spleen. Their expression is stimulated by the factors that stimulate formation and activation of osteoclasts (Wada *et al*., 2005; Boyce and Xing, 2007). An interaction occurs between RANK-L and its receptors RANK on osteoclast precursors, thereby activating and differentiating osteoclast from their precursors for bone resorption (Khosla, 2001). RANKL increases the lifespan of osteoclasts by helping them survive from the anti-apoptotic effect by serine/threonine kinase Akt/PKB through a signaling complex involving c-Src and TRAf-6 (Simonet *et al*., 1997) and, thereby increasing osteoclastic activity (Lum *et al*., 1999; Lacey *et al*., 1999).

Murine RANKL constituting of 316 amino acids, has a transmembrane domain and a C-terminal extracellular domain. Murine RANKL is 83 % homologous with human RANKL (Wong *et al*., 1997). While chicken RANKL is 59-62 % identical to mammalian RANKL sequence, along with possesing a highly conserved sequence (residues 163-318) in the extracellular TNF domain that is crucial for interaction with its receptor (Lam *et al*., 2001).

1.12.6. *Osteoprotegerin (OPG):*

Another protein related to TNF receptor family but is secreted by activated osteoblasts is osteoprotegerin (OPG) (Simonet *et al*., 1997). Two research groups independently identified OPG, but in a different perspective. One research group from the USA, named The Amgen, Inc. group had a project based on characterizing cDNA *in vivo* that encode TNF receptor-related molecules and could be exploited for their application in therapeutics. Interestingly, they found one cDNA that was over expressed in transgenic mice and encoded for a TNF receptor-like protein without any hydrophobic transmembrane-spanning sequences. This gene when over expressed led to a change in skeletal phenotype and ultimately resulted in osteoporosis in mice. After further characterization, they came to a conclusion that osteoporosis was a result of the TNF receptor-like protein, that was called osteoprotegerin (OPG) (Simonet *et al*., 1997).

Osteoblasts played a pivatol role in regulating osteoclastogenesis and Rodan and Martin, first proposed subsequent bone resorption, in 1981 (Rodan and

Martin, 1981). Another research group from Japan, Snow Brand group was searching for inhibitory and stimulatory factors for osteoclasts. After the purification of osteoclastogenesis inhibitory factor (OCIF), they isolated cDNA clones that encoded OCIF, which was later found to be similar to the one reported by Amgen, Inc. group (Tsuda *et al*., 1997).

OPG inhibits maturation of osteoclast and its activation *in vitro* and *in vivo* as OPG being a receptor for RANKL, competes with RANKL for binding RANK (Simonet *et al*., 1997; Rodan and Martin, 1981; Tsuda *et al*., 1997; Kong *et al*., 1999; Anderson *et al*., 1997). Hence, the level of bone mass is associated with the level of OPG (Wong *et al*., 1997). Boyle *et al*. concluded that by maintaining a balance between RANKL-RANK signaling and the levels of biologically active OPG; bone metabolism and development and activation of osteoclasts are regulated (Boyle *et al*., 2003; Boyce and Xing, 2007).

1.12.7. *Role of RANK/RANKL/OPG and the Immune System*:

It was revealed in the work done by Anderson *et al*. and Wong *et al*. that RANK-L is expressed in various immune cells such as activated T-cells, lymph nodes, spleen thymus, intestinal lymphoid patches, and immature CD4/CD8 thymocytes and RANK is expressed on the surface of dendritic cells, mature T-cells and hematopoietic precursors (Wong *et al*., 1997; Josien *et al*., 2000). As shown in Figure 2, interaction of RANK with RANK-L can activate the dendritic cells to induce the proliferation of T cells (Josien *et al*., 1999). Bcl-XL expression, CD40 expression and IL-12 production in dendritic cells are

stimulated by RANK/RANK-L interaction (Josien *et al*., 1999), which also play an essential role in development of lymphoid tissue and T- and B-cell precursors maturation in bone marrow (Wong *et al*., 1997). OPG, being expressed on the surface of dendritic cells, regulates RANK/RANK-L interactions and influence these immune responses (Wong *et al*., 1997; Boyce and Xing, 2007).

Macrophage-colony stimulating factor (M-CSF) helps in the osteoclastic development by determining the precursor cells and binding to its receptor c-Fms on their surface (Udagawa *et al*., 1990). When RANKL interacts with its receptor RANK on the surface of preoteoclasts, it leads to differentiation of proteoclasts to multinucleated osteoclasts and further activating the osteoclasts for bone resorption (Udagawa *et al*., 1990). Pro-resorptive and calciotropic factors like, vitamin D, PTHrP (parathyroid hormone), PGE2, cytokines IL-1, IL-6, TNF, prolactin, corticosteroids, leads to induction of M-CSF, which further leads to stimulation of RANK-L to bind RANK and hence activation of osteoclasts (Boyle *et al*., 2003). OPG acts an inhibitor of RANK/RANKL interaction, by binding to RANK-L, hence it blocks the complete system for osteoclastic activity (Hofbauer *et al*., 1999). Anabolic or anti-resorptive factors like estrogen, BMP 2/4, TGF-β, TPO, PDGF and calcitonin, deactivates M-CSF, which then leads to binding of OPG to RANKL, inhibiting the differentiation of proteoclasts to multinucleated osteoclasts and hence blocking the osteoclastogenesis pathway (Boyle *et al*., 2003; Boyce and Xing, 2007).

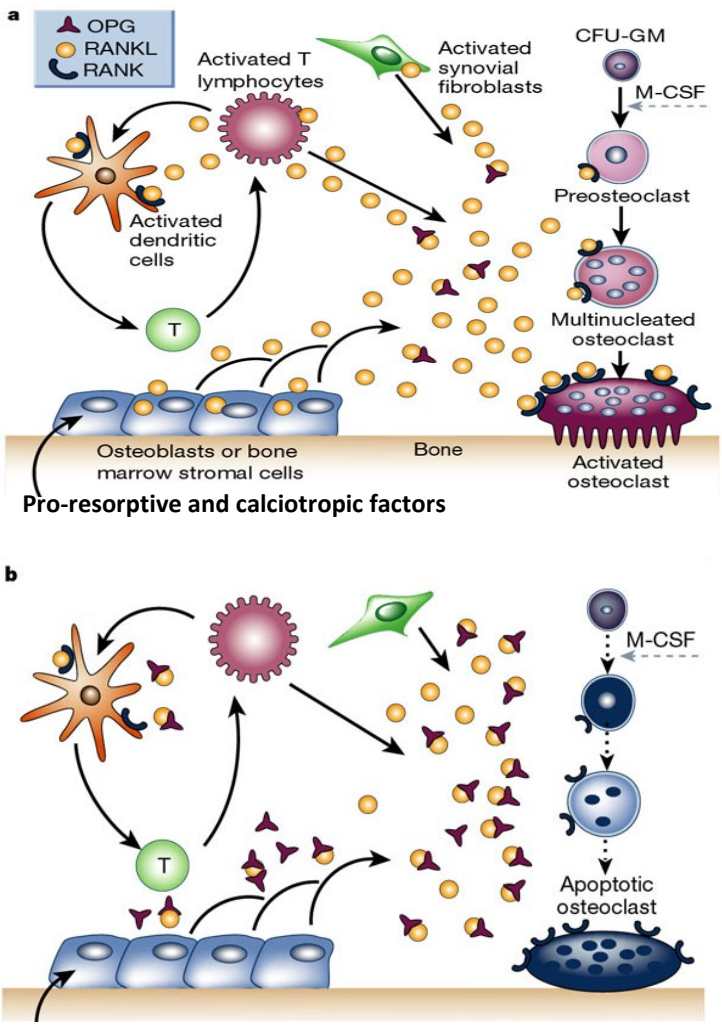

Figure 1-2. Hormonal control of bone resorption. CFU-MG = colony-forming units-granulocyte-macrophage; MCSF = macrophage colony-stimulating factor; PTH = parathyroid hormone; PTHrP = PTH related peptide; PGE2 = prostaglandin E2; IL = interleukin; LIF = leukemia inhibitor factor; BMP 2/4 = bone morphogenetic proteins 2 and 4; TGF-β = transforming growth factor β; TPO = thrombopoietin; PDGF = platelet-derived growth factor. (Reprinted by permission from Nature publishing group. Boyle, W. J., W. S. Simonet, D. L. Lacey. 2003. *Nature*. 423: 337-342).

1.13. Management of Osteoporosis

Many drugs and biological agents such as hormone analogs and monoclonal antibodies have been used to manage osteoporosis in humans. Some of the drugs and therapies are discussed in this section.

1.13.1. *Antiresorptive Drugs*:

Bisphosphonates (BPs) are a very common class of drugs that have been extensively used for bone-related disorders including post-menopausal osteoporosis in women (Wysowski and Greene, 2013). These drugs have a very strong affinity in order to bind to the bone mineral, hydroxyapatite crystals and on the other hand have an antiresorptive effect on osteoclasts in the region where there is a huge increase in bone mass (Rogers *et al*., 2000; Jung *et al*., 1973). Two classes of BPs have been reported:

a. *Nitrogen-containing BPs*: contains a nitrogen atom, some of the examples being, alendronate, ibandronate, pamidronate, risedronate, and zoledronate. They block the cholesterol synthesis pathway by inhibiting farnesyl pyrophosphate (FPP) synthase enzyme. This further hinders the prenylation of small guanosine triphosphate (GTP)-binding proteins, leading to inactivation of osteoclasts. (GTP)-binding proteins are known to be imperative for cytoskeletal organization and vesicular traffic in the osteoclast (Green, 2004; Rodan and Reszka, 2002).
b. *Non-Nitrogen-containing BPs*: as the name suggests are the ones without a nitrogen atom. For example, clodronate and etidronate. Unlike nitrogen

containing BPs, non-nitrogen-containing BPs are degraded into adenosine triphosphate (ATP) analogs that leads to apoptosis of osteoclasts by blocking the osteoclasts function and enhancing IL-6 production (D'Aoust *et al*., 2000; Giuliani *et al*., 1998).

Black *et al*. studied the long-term effect of BPs and concluded that use of BPs for the treatment of BPs for up to 10 years is safe (Black *et al*., 2006). But later, further studies conducted by Rizzoli *et al*. and Shane *et al*. claim that BPs can pose a risk for osteonecrosis of the jaw and fractures to the femur [Rizzoli *et al*., 2008; Rizzoli *et al*., 2011; Shane *et al*., 2010].

Strontium Ranelate (SR) is another drug, containing two strontium cations (active part) and a ranelate anion (carrier) used for osteoporotic treatment (Cesareo *et al*., 2010; Roux *et al*., 2008; Marie, 2005). Unlike other drugs, SR offers an advantage of having a dual role in bone remodeling; by inducing proliferation and differentiation of pre-osteoblasts to osteoblasts and enhancing their activity; and de-activating bone resorption process (Fonseca, 2008; Hofbauer and Schoppet 2004; Brennan *et al*., 2009; Marie, 2007). Bonnelye *et al*. revealed after *in vitro* studies in primary murine osteoblasts that SR also induces the markers for differentiation of osteoblasts, namely alkaline phosphatase (ALP), hydroxyapatite (HA) deposit formation, bone sialoprotein (BSP), and osteocalcin (OCN) (Bonnelye *et al*., 2008).

1.13.2. *Biological Agents*:

Denosumab is a human monoclonal antibody (hMAb), IgG2 that inhibits the interaction of RANKL to RANK, resulting in apoptosis of osteoclasts, thereby reducing bone resorption. Administration of 60 mg of denosumab via subcutaneous injection in every 6 months was approved by the FDA (Breda: Amgen Europe B.V 2010). The efficiency of denosamab was evaluated in clinical trials. Phase 2 clinical trial (Miller *et al*., 2008; McClung *et al*., 2006; Lewiecki *et al*., 2007) [159-161] studies conducted at the end of 1 (McClung *et al*., 2006), 2 (Lewiecki *et al*., 2007), and 4 (Miller *et al*., 2008), years suggested that there was a considerable increase in BMD levels in a dose-dependent manner in the denosumab group. No serious adverse effects were observed in denosumab group in 48 months clinical trail other than serious reactions at the site for injections (Miller *et al*., 2008; McClung *et al*., 2006; Lewiecki *et al*., 2007). Although denosumab was considered well tolerated, cellulitis and eczema was found in some of the cases. The explanation for these effects can be that denosumab inhibits the activity and binding of RANK to RANKL expressed on the surface of immune cells like dendritic cells and monocytes/macrophages. This would significantly affect the inflammatory immune responses mediated by T-cells, hence increasing the risks for various infections and cancer (Miller *et al*., 2008; McClung *et al*., 2006; Lewiecki *et al*., 2007). Brown *et al*. demonstrated that no case of hypocalcemia and osteonecrosis of the jaw were found in phase 3 trials of denosumab at the end of 36 months of study (Brown *et al*., 2009).

An analog of Parathyroid Hormone (PTH), teriparatide, or PTH_{1-34}, is a human recombinant PTH peptide 1-34 fragment. It has a similar binding affinity for PTH receptor-1, as that for PTH and is the first molecule that was approved by the FDA to be used as an anabolic factor for treatment for osteoporosis (Neer *et al*., 2001). Parathyroid gland secretes PTH, and its level is regulated by calcium ions (Brown *et al*., 1993) PTH stimulates bone formation and enhances BMD both *in vivo* and *in vitro* by inducing anabolic effects in osteoblasts, when PTH is artificially given in low doses (Neer *et al*., 2001; Brown *et al*., 1993). Many scientists have proven that in rats, PTH_{1-34} plays a significant role in skeletal repair by increasing osteogenesis and chondrogenesis along with bone remodeling (Bukata and Puzas, 2010; Andreassen *et al*., 1999). Administration of PTH_{1-34} leads to enhancement in the proliferation of osteoblasts, acceleration of their activity; rise in cortical thickness and trabecular bone volume; and ultimately leading to improvement in bone structure (Compston, 2007). Some clinical studies have reported that PTH therapy have great positive effects like increased bone mass and reduced bone fragility on osteoporotic treatment (Hodsman *et al*., 2005). But some studies have shown contradictory results of toxicity and development of a condition called osteosarcoma (in animals and not in humans) with high doses of teriparatide or PTH for long term-treatment (Subbiah *et al*., 2010). For this reason, FDA has approved administration of teriparatide, only between 18-24 months and in European countries, it is approved for use not exceeding 18 months in duration (Ryder *et al*., 2010; Losada *et al*., 2009).

1.13.3. *Lithium Chloride:*

As described earlier that osteoblasts and osteoclasts are the cells that play a crucial role in bone formation and the process is highly regulated by hormones, growth factors and signaling pathways. A critical role in bone formation is played by a signaling pathway called Wnt/β-catenin and can hamper the ability of osteoblasts to produce bone matrices (Canalis, 2010; Lewiecki *et al.,* 2007). When Wnt glycoproteins bind with lipoprotein receptor-related protein (LRP5) or LRP6 and a 7-transmembrane serpentine Frizzled (Fz) receptor in the Wnt/β-catenin signaling pathway (Canalis, 2010; Tamura *et al*., 2010; Boyce *et al*., 2005), hypophosphorylated β-catenin gets accumulated in the cytosol of the cell (Piters *et al*., 2008). The products of transcription of target genes produced by reaction of a lymphoid-enhancer binding factor and a T-cell specific transcription factor with β-catenin, on being translocated into the nucleus leads to stimulation of bone growth (Vestergaard, 2008). Glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin, which leads to accumulation of β-catenin in the cytosol (Piters *et al*., 2008; Clement-Lacroix *et al*., 2005). Lithium (Li) can lead to an increase in the number of β-catenin molecules in the cell by inhibiting the activity of GSK-3β, and hence the transcription in Wnt target genes increases (Clement-Lacroix *et al*., 2005; Hedgepeth *et al*., 1997). A study of oral administration of lithium chloride (LiCl) in mice by Clement Lacroix *et al*., in 2005 reported a 35 % of increase in trabecular bone volume to total bone volume fraction (BV/TV) and an increase in mineral apposition rate by 130 % via the Wnt/β-catenin signaling pathway, showed anabolic effects of

LiCl on bone (Clement-Lacroix *et al*., 2005; Hedgepeth *et al*., 1997; Vestergaard, 2008; Loiselle *et al*., 2013; Satija *et al*., 2013). It was further reported by Vastergaard in 2008 that the risk of bone fractures was reduced to an extent in humans when Li was administered as a psychotropic drug (Vestergaard, 2008). Based on these findings, where LiCl have shown a positive effect on bone strength and quality, Harvey *et al*. in 2015 conducted a study where growing broilers were administered with LiCl (a dose similar to that was supplemented to mice) (Clement-Lacroix *et al*., 2005), at the beginning of 1 week and 3 weeks of age and growth, bone mass, bone strength and muscle quality was measured at six weeks of age (Harvey *et al*., 2015). The results of this study were different from the administration of LiCl in mice by Clement Lacroix *et al*., in 2005 as it did not impact bone volume or trabecular thickness and number, but it did positively affect the bone stiffness in the femur and energy in 3-point bending of tibiae. Further studies using different doses and different time points of treatment of LiCl need to be conducted to draw firm conclusions about the effect of LiCl on broilers and chickens (Harvey *et al*., 2015).

1.13.4. *Letrozole*

Cage-layer fatigue and development of osteoporosis is due to a decrease in bone mass and estrogen is a hormone that maintains bone mass (Whitehead, 1992). It is a known fact that synthesis of estrogen is inhibited by a third generation aromatase inhibitor; known as letrozole (Dowsett *et al*., 2005) A study be Deng *et al*., shows that estradiol levels were reduced to one-third to half the value of

control levels in prelay pullets by letrozole (Lewiecki *et al.,* 2007). It was also reported that bone growth and osteogenesis of medullary bone was inhibited by letrozole in prelay pullet by decreasing the level of estrogen and estrogen receptor (Deng *et al*., 2010).

1.13.5. *Gushukang*

A traditional Chinese herbal medicine, called Gushukang (GSK) has been widely used in China as an effective treatment for postmenopausal osteoporosis in women (Song *et al*., 2000; Wang *et al*., 2006; Shi *et al*., 2008). GSK is comprised of four active constituents: Herbal Epimedii, derived from the leaves of the plant. Rhizoma Drynariae, Rhizoma Atractylodis, and Radix Astragali, all derived from the roots of the plants (Deng and Hou, 2003). In a study by Zhou *et al*., the effect of GSK on was studied on bone characteristics of 55-week egg-laying hens, when osteoporosis is very common (Zhou *et al*., 2009). In a study by Zhou *et al*., the effect of GSK was studied on bone characteristics of 55-week egg-laying hens (Zhou *et al*., 2009). It was reported that egg laying hens that were fed with a diet supplemented with GSK for 10 weeks showed a considerable increase in egg production and reduced the number of cracked eggs in the post-peak period of egg laying in hens. GSK also provided some evident effect on structural bone resorption and bone metabolism. This study suggested that GSK could result in prevention of osteoporosis in egg-laying hens but the mechanism of action associated with it and what component the

GSK formula plays a role in prevention of structural bone loss remains unclear and would require some future studies (Zhou *et al*., 2009).

1.13.6. *Mesenchymal Stem Cells*

Most pharmacological regimens focus on decreasing the bone resorption activity rather than increasing the bone formation process. Recent studies have shown that age-related (type II) osteoporosis arises due depletion of bone marrow (BM) mesenchymal stromal cells (MSC) that is a heterogenous pool of skeletal stem cells (SSCs), osteoblastic cells and fibroblasts; which results in reduced bone formation (Muschler *et al.*, 2001; Majors *et al.*, 1997; D'Ippolito *et al.*, 1999). A number of preclinical animal studies on some murine models have been conducted to study MSC based transplantation to stimulate osteogenic differentiation. For example, in a study, human MSCs were engineered to express 33 CXCR4 (chemokine receptor type 4), along with RANK-Fc (non-functional fusion protein comprised of RANK receptor and Fc region of human IgG) on the cell surface. This led to inhibition of normal osteoclastogenesis by antagonising of normal RANK-RANK-L signaling between osteoblasts and preosteoclasts. Furthermore, CXCR4 increased the retention of MSCs in the bone marrow, hence maintaining the bone mass. RANK-Fc mediated antagonism of osteoclastogenesis could be increased by CXCR4 by enhanced interaction of RANK-Fc with RANK-L when more MSCs are drawn to the bone surface (Cho *et al*., 2009).

1.13.7. *Acetylsalicylic Acid (Aspirin)*

Aspirin, a known cyclooxygenase inhibitor (Raisz, 2001) and also prototype drug for nonsteroidal anti-inflammatory drugs (NSAIDs) (Fuster and Sweeny, 2011); has an effect on bone cells in two ways. One, aspirin showed inhibitory effects on osteoclast cells by suppressing phosphorylation and degradation of signalling molecules in nuclear factor kappa-B (NF-κB) system in macrophage cells (Zeng *et al*., 2016). The second effect aspirin could have is on the promoting the progenitor of osteoblasts, i.e. BM-MSC, to survive by (a) increasing the telomere length and its function in BM-MSC and (b) preventing apoptosis of BM-MSC due to Fas/Fas ligand (FasL) interaction, (FasL expressed by T cells and Fas expressed by osteoblasts) (Yamaza *et al*., 2008).

1.14. Summary

Osteoporosis is a common bone disorder and its etiology stems from multiple factors including changes in postmenopausal hormones, dietary deficiency of vitamin D, calcium and phosphorous, and lack of physical activity. Type I and Type II are the two main types of osteoporosis, however, a rare form of osteoporosis called juvenile osteoporosis also occurs. Osteoporosis is also very common in egg-laying caged commercial chickens due to their sedentary life style. As most of the dietary calcium stored in the chicken's body is used for eggshell formation, deficiency of calcium is another main cause of bone fragility and bone fractures in chicken. We further discussed that bone remodelling occurs in two phases: bone resorption phase (destruction of damaged bone by osteoclasts) and bone formation (bone reformation by

osteoblasts). RANK, RANK-L and OPG, the members of TNF superfamily, play a significant role in bone remodelling and are also associated with the immune response system (Boyce and Xing, 2007). A number of strategies such as using anti-resorptive drugs, PTH analogs, monoclonal antibodies are in place to deal with and manage osteoporosis in humans, which may have some disadvantages on their prolonged use. A novel system is essential that will target RANK-RANKL system and improves bone formation and structure both in chickens and humans.

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Chapter 2: Production of chicken RANK-L protein by *Escherichia coli* (*E. coli*) and *Baculorvirus* expression systems

2.1. Abstract

Mammalian receptor activator of nuclear factor kappa B (RANK) is the receptor expressed on the surface of bone forming progenitor cells. A receptor activator of nuclear factor kappa B ligand (RANKL) is a transmembrane protein of tumor necrosis factor (TNF) family that is secreted by osteoblasts. Interaction of RANKL with RANK leads to activation of the NF-κB pathway leading to differentiation of bone-forming progenitor cells to osteoclasts and hence playing an important role in bone resorption. The main objective of the current study is to produce recombinant chicken RANKL (r-chRANKL) via *E. coli* and *Baculovirus* expression systems and purify them using affinity chromatography.

Key Words:

Receptor activator of nuclear factor kappa B ligand – RANKL; chicken RANKL – chRANKL, recombinant chRANKL – r-chRANKL; mouse RANKL – mRANKL; Tumor necrosis factor – TNF.

2.2. Introduction

Chickens are susceptible to cage-layer fatigue and develop brittle or broken bones in over time during the egg-laying cycle (Couch, 1955; Grumbles, 1959). Osteoporosis is defined as structural loss of bone mineralization in egg-laying hens that result in fragile bones, making them more prone to fractures. Although chicken feed formula provides enough calcium and Vitamin D, lack of physical activity in the cage is the primary factor resulting in the bone loss (Whitehead and Fleming, 2000). It was

reported in 2004, 80-89 % of the commercial egg-laying hens develop osteoporosis, resulting in 20-30 % of caged hens mortality (Webster, 2004). The commercial egg industry suffers an enormous economic loss due to osteoporosis in white leghorns (Whitehead and Fleming, 2000; Anderson, 2002).

During the egg-laying period, a high absorption rate of calcium is required for calcification of eggshells. Most of the calcium from the feed is accumulated (or deposited) into medullary bones as reservoirs of absorbed calcium. In nocturnal periods, The medullary bones, femur, ribs, tibia, release a significant amount of calcium for eggshell calcification, causing a depletion of depleting calcium in bones, hence making them weak and fragile (Etches, 1987). Osteoporosis or bone brittleness is a result of the perturbed bone regeneration process. Bone remodeling in chicken is characterized by two phases: bone matrix formation by osteoblasts and bone resorption process by osteoclasts (Theill *et al*., 2002; Boyle *et al*., 2003; Karsenty and Wagner, 2002).

Receptor activator of nuclear factor kappa B ligand (RANKL) is a member of tumor necrosis factor (TNF) family. RANKL is a transmembrane protein constituting of 316 amino acids; that includes a hydrophobic domain (20 amino acids), a COOH-terminal receptor-binding domain, and a long extracellular domain with an active receptor-binding site (Anderson *et al*., 1997; Lam *et al*., 2001). The RANKL is known to play a crucial role in the conversion of osteoblasts into osteoclasts associated with bone metabolism (Theill *et al*., 2002; Boyle *et al*., 2003; Karsenty and Wagner, 2002; Khosla, 2001). In general, RANKL released from osteoblasts

binds with its receptor RANK on the surface of bone forming progenitor cells (precursors of osteoclasts) derived from bone marrow, leading to maturation of progenitor cells to multinucleated osteoclasts and hence facilitates bone resorption process. An imbalance between osteoclastic and osteoblastic activities results in fragile bones, where a new bone matrix does not replenish bone breakdown.

Although there is information on RANK being a major trigger for osteoporosis, limited information is available in the immune response of chicken by injecting recombinant mouse and chicken RANKL (chRANKL). In the present study, the main objective was to produce recombinant mouse and chicken chRANKL by the baculovirus expression system, postulating exogenous RANKL to trigger an immune response of chicken to produce IgY antibody specific to chRANKL.

2.3. Methods

2.3.1. Materials

Restriction enzymes were procured from New England Biolabs (Mississauga, Canada). The anti-His6 was purchased from Novagen Inc (Madison, USA). Hybond ECL nitrocellulose membrane, X-ray film, and the ECL Western blotting reagents were obtained from Amersham Pharmacia Biotech (Baied Urfe, Quebec, Canada). Ni-NTA agarose, plasmid DNA isolation kit and gel extraction kit were obtained from Qiagen (Mississauga, Canada). Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG, rabbit anti-chicken IgG conjugated with horseradish peroxidase and streptavidin-HRPO were purchased from Sigma (St. Louis, MO,

USA). Tetramethylbenzidine (TMB) was purchased from Fisher Canada (Ontario, CA) and KPL (Maryland, USA), respectively. Bio-Rad protein assay kit, glycine, Precision Plus Protein Standard, sodium dodecyl sulfate (SDS), and precast gels (4–20% Tris-Glycine, 16.5% Tris-tricine) were purchased from Bio-Rad Laboratory (Ontario, CA). The ELISA Vmax kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA). Slide-A-Lyzer® was purchased from Thermo Scientific Pierce Protein Research Products (PA, USA). All analytical grade chemicals and solvents were from commercial sources.

2.3.2. Production of chRANKL by *E. coli* Expression System - Construction of plasmid vectors (pET22b(+)-chRANKL)

The nucleotide sequence of chRANKL (Sutton *et al*., 2015) was codon-optimized for *E. coli* expression and synthesized by GeneArt Inc. (Life Technologies). The chRANKL fragments amplified by PCR were digested and ligated into Nco I-Xho I sites of the pET22b (+) expression vector.

The *E. coli* Rosetta 2 (DE3) competent cells were transformed with the pET22b (+)-chRANKL expression vectors. Transformants were grown on LB plate with kanamycin/chloramphenicol (34 µg/mL). A single colony was picked up and cultured in 2XYT medium containing kanamycin (5 µg/mL) at 37 °C with shaking overnight. Fresh medium was inoculated with the overnight culture and continued to grow until they attained an optical density (OD_{600}) = 0.8. The chRANKL expression was induced by adding 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG).

The chRANKL expression level was optimized by the cell density, IPTG concentration, induction temperature and time, and monitored by running SDS-PAGE and detected by the Western blot.

2.3.3.1. Purification of inclusion bodies (IB)

The purification of inclusion bodies was done according to the previously published method (Ganguly *et al*., 2013). The bacterial wet pellets from 4 L *E. coli* cultures were suspended in phosphate buffer saline (PBS) at pH 7.4 (10 mL of PBS per g of pellet) and lysed by passing through a French Press (20,000 psi). The cell lysates were centrifuged at 27,000 x g for 30 min at 4 °C and supernatants were collected as total soluble proteins. The pellets were resuspended in a lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl and 1 mM EDTA) and then 2 % sodium deoxycholate was added to the mixture. The mixture was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000 x g for 30 min at 4 °C. The pellet was obtained, again suspended in lysis buffer and then washed at 27,000 x g for 20 min at 4°C (washed three times).

2.3.3.2. IB solubilization and immobilized metal affinity chromatography (IMAC) purification

Inclusion bodies were solubilized in denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) for 1 h at room temperature with occasional shaking. Solubilized denatured chRANKL proteins from insoluble materials were separated by centrifugation at 27,000 x g, 4 °C for 30 min. A Ni–NTA column was prepared

by loading the Ni–NTA agarose on a plastic column (Bio-Rad). The column was equilibrated with 5-bed volumes of denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8). The solubilized denature protein chRANKL was loaded on the column. Non-specific bound protein was removed by a wash step (washing buffer, 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3). Bound protein was eluted and analyzed by SDS–PAGE. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to 100, 75 and 50 μg/mL in Tris Arginine (TA) buffer (50 mM Tris, pH 8.0, 0.4 M L-arginine) and refolding was done by dialysis in TA buffer in the presence of 1.0 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized) for 3 days with two changes at 4 °C. Final dialysis was done in PBS, pH 7.4 at 4 °C.

2.3.4. Production of mouse RANK-L by *E. coli* Expression System

The production and purification of mRANKL were done in a similar way as described for chRANKL (section 2.3.2) except that the nucleotide sequence for mRANKL was codon-optimized for *E. coli* expression and synthesized by GeneArt Inc. (Life Technologies).

2.3.5. Production of soluble chRANK-L by *Baculovirus* Expression System - Construction of recombinant transfer vector

The chRANKL gene that contains a Honeybee Melittin signal (HMS) sequence at 5' end and a sequence coding for 6XHis at 3' end was designed and synthesized by the GeneArt (Life Technologies). A transfer vector, pVL1393, can be used for

expression of targeting protein in the polyhedrin site of the Autographa California nuclear polyhedrosis virus DNA. The chRANK-L gene and the upstream polyhedron promotor DNA fragments on pVL1393 were amplified by polymerase chain reaction (PCR) and seamlessly fused by overlap extension PCR. The extended product was cloned into vector pVL1393 at NgoM I / Bgl II sites to generate the final desired baculovirus transfer vector pVL1393-chRANKL. There are two transfer vectors, one with the HMS coding sequence at the 5' end of the chRANK-L gene (vector C) and another without the signal sequence (vector B). All the protocols were derived from Current Protocol in Molecular Biology (2004).

2.3.5.1. Generation of recombinant *Baculovirus* chRANK-L

Frozen Sf9 (Spodoptera frugiperda) cells were initially cultured in a monolayer and then suspended in an ESF921 medium at 37 °C with shaking at 120 rpm for 4-5 days. During the incubation period, host insect Sf9 cells were co-transfected with the BestBac 2.0 v-cath/chiA, deleted virus DNA and the baculovirus transfer vector pVL1393-chRANK-L. After incubation, the recombinant virus in the culture medium was collected by centrifuge (27,000 x g for 30 min at 4 °C) as P1 stock. The virus P1 stock was amplified by infecting new Sf9 cells to generate P2, and in the same way for P3. Plaque assay was performed to determine the titer of each virus stock. Two different recombinant virus stocks were produced from each transfer vector (vector B and vector C). Sf9 cells were cultured in an ESF921 medium till the mid-logarithmic phase of growth at a density of 2.0 - 2.5 X 10^6 /mL. The virus P3 stock of high titer (1 - 5 X 10^8 pfu/mL) was used to infect the Sf9

culture. The optimal multiplicity of infection (MOI) was determined for every batch of virus stock by infecting culture using MOI of 1, 2, 5 and 10 and a time course was performed to determine the expression kinetics of the recombinant protein.

2.3.5.2. Purification of recombinant chRANKL (r-chRANKL)

Intracellular and secreted chRANK-L were collected from the infected Sf9 cell pellets and suspensions after centrifuging the culture at 27,000 x g for 30 min at 4 °C, respectively. The intracellular chRANK-L was purified from the supernatant of cell lysate by Ni-NTA agarose affinity chromatography (Bio-Rad) by loading 9 mL of Ni–NTA agarose on a column under denaturing conditions denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8). The secreted chRANKL was purified from the culture medium by a combination of ion exchange SP-sepharose column (Bio-Rad) and Ni-NTA column, all under native conditions, followed by dialysis against PBS (pH 7.4) The protein concentration was determined by micro BCA protein assay (Thermo Fisher Scientific). The proteins were characterized by SDS-PAGE and western blot with anti-6XHis mAb and anti-chRANKL polyclonal IgY.

2.3.6. Characterization of intracellular and secreted r-chRANKL proteins

Both expressed chRANKL, intracellularly and secreted into the cell culture medium for each transfer vector (Vector B and vector C, respectively) were analyzed by western blot. Sf9 cells infected with baculovirus-derived from vector B and vector C at a different multiplicity of infection (MOI) or infection time were tested. Samples

were taken and a time course analysis was performed to determine the expression kinetics of the recombinant protein. The cell pellet from infected cell culture was collected by centrifuge (27,000 x g for 30 min at 4 °C) as non-secreted or intracellular protein sample and the supernatant was obtained as secreted protein sample. Uninfected Sf9 cells were used as a negative control, and partial purified 6XHis-tagged chRANKL was used as a positive control. Protein samples were electrophoresed and detected by Western blot (as described in section 2.4.2) with anti-6XHis mAb HRP conjugated, and further verified by detection with anti-chRANKL polyclonal IgY and rabbit anti-chicken IgG HRP conjugated. For the intracellular chRANKL derived from vector C, the protein expression and purification were up-scaled. Two batches of the final purified chRANKL were performed using SDS-PAGE and Western blot.

2.3.6.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified samples were run on Mini-PROTEAN® TGXTM Any kD Precast Gels in Tris-Glycine-SDS buffer (Bio-Rad). Gels were stained with a solution of 0.05 % Coomassie Blue R-250 (Sigma-Aldrich Ltd., Oakville, ON), 0.1M aluminum nitrate, 25 % isopropanol, 10 % acetic acid, and 1 % TritonX-100. Gels were destained with 7 % acetic acid. Precision Plus Protein Standards (Bio-Rad) were loaded as molecular weight markers. Images of gels were scanned in the Gel DocTM EZ Imager (Bio-Rad).

2.3.6.2. Electrophoresis and Western Blot

Electrophoresis was performed in 15 % acrylamide in Tris buffer, pH 6.8. Samples were dissolved in deionized water. Two slabs were generally run at the same time, one for staining with Coomassie brilliant blue and the other for Western blot with IgY antibodies against to chRANKL and mRANKL. Electrophoretic transfer to nitrocellulose was accomplished in Tris-borate (gel electrophoresis buffer) without sodium dodecyl sulfate at 40 volts for 2 h. Nitrocellulose sheets were then soaked in 2 % BSA in PBS (pH 7.2) for 1 h at room temperature. After washing with PBS (three times for 5 min each), nitrocellulose sheets were incubated with anti-chRANKL and mRANKL IgY antibodies (in PBS containing 1 % BSA) for 1 h at room temperature. The incubation was followed by washing in PBS and 1 h incubation with rabbit anti-chicken IgG conjugated with HRPO (diluted 1:3,000 in PBS-Tween). Colour was developed by incubating in 0.05 % diaminobenzidine tetra-hydrochloride in PBS containing hydrogen peroxide (0.01 % w/v) and cobalt chloride (0.033 % w/v) for 5 min. Stained blots were then washed several times with water and dried.

2.4. Results

2.4.1. Expression of chRANK-L in *Baculovirus* Expression System

An *E. coli* expression system with vector pET22b (+) was first used to express chRANKL. The high level of expression led the majority of the expressed protein in the form of inclusion body. Purification of a small amount of native protein from inclusion body wasn't successful in this case.

In order to produce native soluble proteins of chRANKL, we attempted to use the baculovirus expression system for producing recombinant chRANK-L. Western blot with anti-6XHis mAb demonstrates intracellular and secreted chRANK-L produced from vector B and vector C (Figure 2-1). For vector B, only one band appears with 6XHis tag detection. The molecular weight was slightly lower than the theoretical size of 21 kDa, meaning it might be truncated at the N-terminal. As expected, no secreted protein was observed in the culture medium. Intracellular proteins expressed from Vector C show at least five closely sized bands from 18 - 28 kDa, and secreted protein shows one band at 25 kDa. Clearly, the recombinant protein with a secretion signal was destined toward the secretory pathway. The signal peptide was then cleaved during the process. The signal peptide present at the N-terminus in the intracellular recombinant protein was properly processed on the secretory pathway. Some proteins were over or incorrectly processed, resulting in variable lengths of truncation at the N-terminal. The recombinant chRANKL would be 21 kDa if expressed in *E. Coli*. The increased molecular weight suggests translational modifications of the protein in the

baculovirus expression system. Only a small amount of expressed chRANKL was secreted into a culture medium. The yield was insufficient to obtain a significant amount of soluble proteins. Therefore, chRANKL used for any further experiments should be purified from the intracellular source for efficiency.

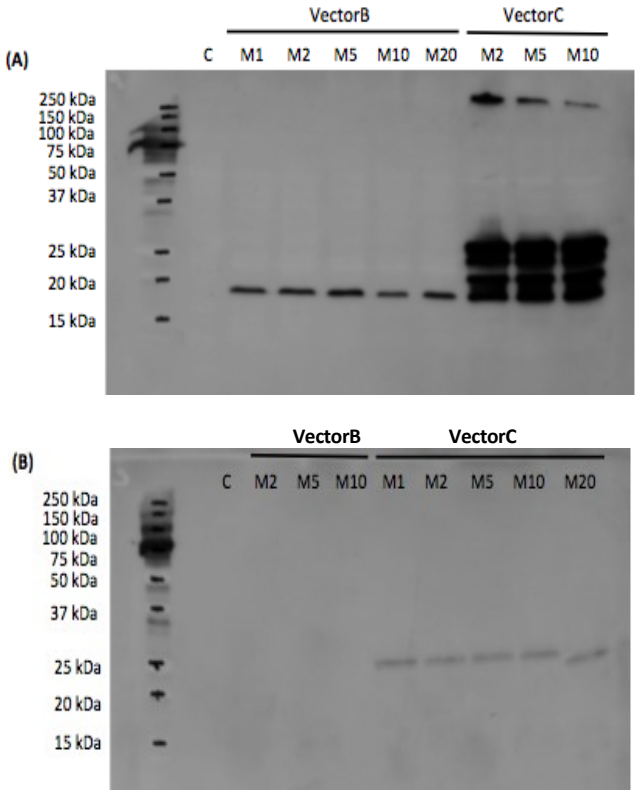

Figure 2-1. Western blot of chRANKL expressed by *Baculovirus* expression systems. Proteins were detected with HRP-conjugated anti-6XHis mAb.

C: uninfected Sf9 control. M: multiplicity of infection.

(A) Intracellular chRANKL: Samples were collected from cell lysate.

(B) Secreted chRANKL: Samples were collected from the culture medium

2.4.2. Characterization of Purified Intracellular chRANKL from *Baculovirus* expression system

The intracellular chRANKL from the *baculovirus* expression system was purified by using affinity chromatography method under denaturing conditions. Figure 2-2 illustrates that the protein was soluble after dialysis with PBS.

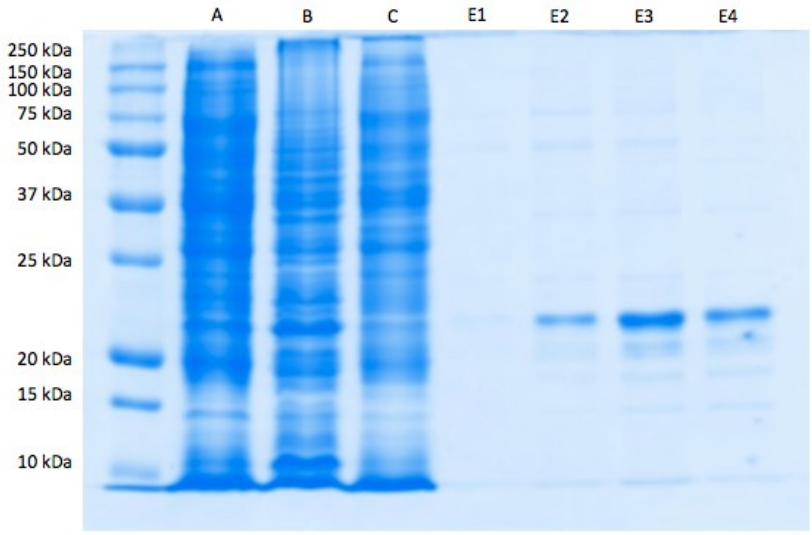

Figure 2-2. SDS-PAGE of Intracellular chRANKL purification from *Baculovirus* Expression system under denaturing conditions. A: supernatant of cell lysate. B: pellet of cell lysate. C: Ni-NTA unbound. E1, E2, E3, E4: Elution fractions.

Samples from two batches (500 mL of cell culture for each) of purified chRANKL were further characterized by SDS-PAGE (Figure 2-3A) and western blot (Figure 2-3B and C). The purified intracellular chRANKL from Vector C showed all the fragments of chRANKL. The concentration of protein was determined by micro-BCA assay, 0.5-0.8 mg/mL. Purified chRANKL is soluble and also stable at -20 °C. Samples were tested again on SDS-PAGE

after six months; the results showed no change compare to the previous SDS-PAGE analysis.

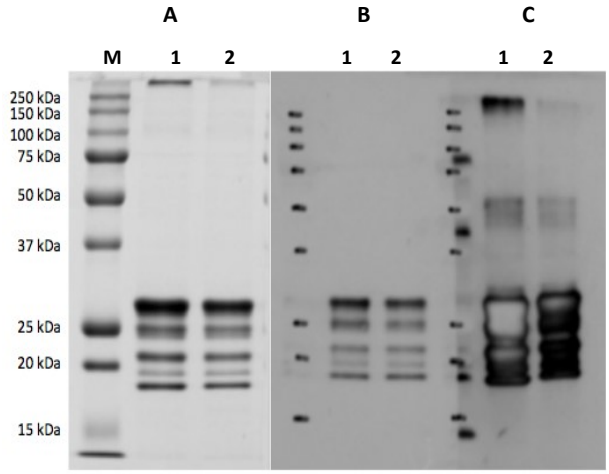

Figure 2-3. Characterization of purified intracellular chRANKL from *baculovirus* vector C expression system. Two samples of each analysis were from two different batches of purification (Lane 1 is batch 1, Lane 2 is batch 2). A: SDS-PAGE B: Western Blot, detected with anti-6XHis mAb/HRP, C: Western blot, detected with anti-chRANKL polyclonal IgY and anti-chicken IgY/HRP.

For the protein secreted into the culture, medium purification was performed by a combination of anion exchange SP-Sepharose column and Ni-NTA column, all under native conditions. Final protein was dialyzed against PBS buffer and protein concentration was determined by micro BCA protein assay. Immunoblotting with anti-6XHis mAb and anti-chRANK-L polyclonal IgY showed that the recombination successfully incorporated the chRANKL-6XHis

construct into the Baculovirus genome.

2.4.3. Characterization of secreted chRANK-L from *Baculovirus* vector C expression system

Secreted chRANK-L from Vector C was purified using an SP ion-exchange column and a Ni-NTA affinity column. Western blot with anti-chRANK-L polyclonal IgY confirmed chRANKL secreted into the culture medium (Figure 2-4). Western Blot for the purified secreted chRANK-L proteins from vector C (medium) showed single homologous chRANK-L bands, proving that secreted chRANKL from this vector are easy to purify.

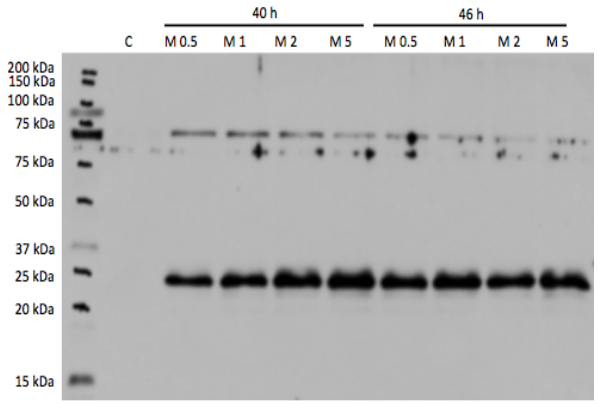

Figure 2-4. Western blot of secreted chRANKL, detected with anti-chRANKL polyclonal IgY. Samples were collected from culture medium 40h and 46h post-infection. C: uninfected Sf9 control. M: multiplicity of infection.

Western blot of intracellular proteins from baculovirus expression systems gave similar results as secreted proteins in terms of reactivity to anti-chRANKL, MOI, and expression kinetics. No significant difference was observed between

40 h and 46 h of infection time. Similarly, a slight increase in protein production is observed as MOI increases from 0.5 to 5, but quantitative measurement is necessary for confirmation. Further quantitative analysis and optimization are required to determine the optimal MOI and the expression kinetics.

2.5. Discussion

Chickens lay eggs almost daily during the egg cycle. Eggshell is formed overnight and composed of approximately 15 grams of calcium that must be supplied from feed and internal sources of bone in chickens by the interaction of cells between osteoblasts and osteoclasts known as bone remodeling. Interaction of RANK and RANK-L plays one of the key roles *in vivo* by regulating the number of osteoclasts and hence the bone turnover. The activated osteoclasts are also important in supplying of sufficient calcium from bones to form eggshells. Osteoclastogenesis is a multi-step process that leads to differentiation and activation of pre-mature osteoblasts and immature osteoclasts, respectively (Yavropolou and Yovos, 2008). The role of chRANKL in the activation of osteoclastic cells in chickens has been debatable for a long time, as chicken osteoclasts have been considered to be different from mammalian osteoclasts due to non-recognizable calcitonin receptor on their surface. To understand the role of RANK-L in bone remodeling mechanism, a mice model study showed that depleted or defective RANK or RANK-L genes develop a severe form of osteoporosis (Kim *et al*., 2000; Dougall et al., 1999; Li *et al*., 2000; Kong et al., 1999). Many studies on the mode of actions of RANK

and RANK-L to induce osteoporosis have been reported in a mammalian model. *In vitro* study shows that a recombinant chRANK-L using vector pET-32a(+) in *E. coli* activated osteoclasts in chicken bone marrow cells and thus induced bone resorption (Wang *et al*., 2008).

Another role of RANK-L is involved in the inflammatory immune system in mammals. The chRANK-L is a member of chicken TNF superfamily that plays a key role in activation and upregulation of pathways in the immune system (Sutton *et al*., 2015). In the recent study, recombinant chRANK-L and its receptor chRANK were also developed and characterized to study their roles in avian antigen presenting cells (APC) showing that chRANK-L induces proinflammatory reaction (Sutton *et al*., 2015). In an *in vivo* study RANK-L binds to the extracellular domain of RANK to stimulate pro-inflammatory cytokines such as TNF-α and IL-1β, resulting in an induction of a humoral immune response in chickens (Hsu *et al*., 1999).

It has also been reported that chicken RANK-L shares 59-62 % homology with mammalian RANK-L, along with the expression of the highly conserved extracellular TNF domain (residues 163-318) in the chicken molecule that is essential for interacting with their receptor (Lam *et al*., 2001). A bone stimulating effect of recombinant chicken RANK-L was examined in an *ex-vivo* culture system (Wang *et al*., 2008).

In this chapter, we aim to develop recombinant chicken RANK-L by *E.coli* and *baculovirus* systems and analyzing the pros and cons of both methods and

challenges encountered in both of the methods utilized.

Using *E.coli* as a host organism in expression system offers some well-known advantages, the major one being producing recombinant proteins in large quantities due to easily attainable high cell density cultures. However, the exponential growth in complex media does not lead to density close enough to estimated theoretical density limit of *E. coli* liquid culture (about 200 g dry cell weight/l or roughly 1×10^{13} viable bacteria/ml) (Lee, 1996; Shiloach and Fass, 2005). As cultivation of *E. coli* at 37 °C using LB media in a simple laboratory set-up is less than 0.1 % of the theoretical limit (Sezonov *et al*., 2007), methods with high cell-density culture were designed to enhance *E. coli* production and hence increase recombinant protein expression (Choi *et al.,* 2006). Inexpensive components are readily available to create rich complex media for protein expression. The other advantages that an *E.coli* expression system offers are fast and easy transformation with exogenous DNA and, a very short time (> 5 min) for plasmid transformation (Pope and Kent, 1996).

Due to fast growth kinetics of *E.Coli,* in glucose-salts media and under optimal environmental conditions, Sezonov *et al*., 2007 reported that it's doubling time is about 20 min (Sezonov *et al*., 2007). This would lead the culture to its saturation only within a few hours of inoculation with $1/100^{th}$ of its started culture. The metabolic burden on the microorganism may increase as a result of recombinant protein expression, leading to a significant decrease in generation time (Bentley *et al.,* 1990). Many other studies have reported that methods used

to increase the yield of recombinant proteins using *E.Coli* expression system subsequently resulted in formation of inclusion bodies in the cytoplasm (Colleluori *et al*., 2005). This commonly occurs during expression of eukaryotic proteins in bacterial hosts when proteins are over-expressed (De Marco, 2009). Inclusion bodies are formed as a result of aggregation and accumulation of newly synthesized polypeptides and further procedures such as, *in vitro* protein refolding are required to recover proteins from inclusion bodies (Cabrita *et al*., 2006). For example: artificial production of anti-HIV cyanobacterium lectin, cyanovirin N (CVN), (Colleluori *et. al*., 2005). Lotfi *et al*. (2018) used the conventional method to refold and ion-exchange chromatography to purify the recombinant CVN (rCVN). Production of inclusion bodies was a limitation as it also produced heteromeric isomers (including full-length monomeric, dimeric and N-terminal deleted residue) of rCVN, which resulted in the final low yield.

In the current study, chicken RANK-L expressed in *E. Coli* with vector pET22b(+) resulted in the formation of aggregation of expressed protein molecules into insoluble inclusion bodies due to a high level of expression (Chrunyk *et al*., 1993; Freedman *et al*., 1992). Reducing environment of bacterial cytosol and post-translational machinery can also result in partially folded or misfolded proteins to aggregate to form inclusion bodies (Carrió et al., 2000). The recombinant protein (r-chRANKL) had a 6X-His tag coding sequences at the C-terminal. It has been well reported that production and purifying of native recombinant proteins using *E. Coli* as a host, from inclusion bodies pose a great challenge and; hence require further processing,

solubilization and refolding to get functionally active proteins (Marston, 1986; Rudolph *et al*., 1996; Burgess, 2009). Purifying a small amount of native protein using Ni-NTA affinity purification, from inclusion body was not successful. So, several different denaturing conditions were used during Ni-NTA affinity purification that ultimately led to small amount of the purified intracellular proteins in soluble form at a large scale. After refolding, the proteins still retained their soluble form (Huston *et al*., 1995). It was proposed in 2000, by Nakashima, T *et al.* soluble forms of RANKL, designated as sRANKL, have greater potential activity for stimulation of osteoclastogenesis (Nakashima, T. *et al*., 2000).

Because of challenges faced in producing and purifying r-chRANKL using an *E.coli* expression system, we used a baculovirus expression system to produce r-chRANKL.

The *baculovirus* expression system is one of the most common insect cell expression systems that is used to express recombinant proteins in a soluble, stable and translational active state in large amounts (Hasemann and Capra, 1990). Therefore, the system has been widely used because of its ability to process proteins similar to other higher eukaryotes. Compared with the *E.coli* system, *Baculovirus* frequently gives a high-level expression of recombinant proteins with full biological activity as a result of being folded properly and post-translational modifications (similar to mammalian cells), which includes glycosylation and phosphorylation (Luckow *et al*., 1991). *Baculoviruses* have

other advantages over other expression vector systems. The important one being that because *baculoviruses* have a limited host range, they are non-pathogenic to mammals and plants, hence they are utilized within minimal containment conditions (Ignoffo, 1975). In 2015, van Oers *et al*. reviewed the developments and improvements in *baculovirus* expression technology in the last thirty years. A wide variety of applications for recombinant *baculoviruses*; categorically, *baculovirus* particles as antigen carriers, viral vectors for gene delivery, gene delivery vehicles for mammalian cells and, synthesis of single and multi subunit recombinant proteins; has been established (Airenne *et al*., 2013).

In this study, we first developed the *baculovirus* expression system to clone, express recombinant chRANK-L, and generated a soluble, stable and translational modified chRANK-L. Uniqueness of the system is that we are using the final desired baculovirus transfer vector pVL1393-chRANKL obtained by insertion of chicken RANK-L gene downstream of the first nucleotide in the polyhedron initiation codon, resulting in high expression levels of the recombinant proteins and preservation of transcription and translational sequences at 5' end of non-coding gene (Matsuura *et al*., 1987; Possee and Howard, 1987; Luckow and Summers, 1989). Since the two transfer vectors, as described in methods, are commonly available; vector C (with the HMS coding sequence at the 5' end of the chRANKL gene) (Tessier *et al*., 1991) and vector B (without the signal sequence) were used for generating the recombinant chRANK-L. We found that only one band of recombinant intracellular proteins shows slightly lower molecular weight than the theoretical size of 21 kDa by

using vector B, due to truncation at the N-terminus, and hence no secreted recombinant protein was observed in the culture medium.

However, at least five closely sized bands from 18 - 28 kDa appeared in SDS-PAGE for intracellular proteins expressed from Vector C. The intracellular proteins expressed from vector C appeared as a band at 25 kDa in SDS-PAGE, indicating that the secretory HMS signal led chRANKL protein toward the secretory pathway. The increase in molecular weight indicates the post-translational modifications of the protein (for example, phosphorylation, acylation, etc.) in the baculovirus expression system (Miyamoto *et al*., 1985; Jeang *et al*., 1987; Nyunoya *et al.,* 1988). As the yield of expressed chRANK-L was low in the culture medium, there was an insufficient amount of soluble proteins after purification; therefore, intracellular chRANK-L was purified for efficiency.

We successfully expressed recombinant chRANK-L in *Baculovirus* expression systems both as intracellular and secretory proteins. The exogenous chRANK-L can induce a chicken immune response to produce specific IgY antibodies during the immunization period.

2.6. Acknowledgements

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Chapter 3: Production of IgY against recombinant chicken RANK-L

3.1. Abstract

Osteoblasts secretes transmembrane protein, RANK-L (receptor activator of nuclear factor kappa B ligand), interacts with its receptor, expressed on the surface of bone forming progenitor cells, known as RANK (receptor activator of nuclear factor kappa B). These interactions result in activation of the NF-κB pathway, which leads to differentiation of bone forming progenitor cells to osteoclasts and therefore playing a key function in bone resorption. The main objective of the current study is to produce polyclonal chicken antibodies (anti-chRANK-L IgY) against r-chRANKL produced in large quantities via the *Baculovirus* expression system, to study the immune response in chickens. The second objective is to study and compare the cross-reactivity of anti-chRANK-L IgY against r-chRANK-L and mouse RANK-L. The purified proteins were injected in chickens to obtain the specific IgY in egg yolk. Indirect ELISA proved that the total content of anti-chicken and anti-mouse IgY in egg yolk were similar to each other. Anti-chicken IgY showed affinity and bound to both recombinant mouse and chicken RANKL, showing the cross-reactivity and homology between the two recombinant proteins.

Key Words:

Receptor activator of nuclear factor kappa B ligand – RANK-L; chicken RANKL – chRANK-L, recombinant chRANK-L – r-chRANKL; mouse RANK-L – mRANK-L; Tumor necrosis factor – TNF.

3.2. Introduction

The chicken immune system has been well studied and is very similar to the mammaliam immune system in two basic ways: (a) constituted by primary and secondary lymphoid organs (b) there are two types of immunity in birds – (i) non-specific innate immunity, which is a first line of defense when an antigen enters a body and; (ii) specific acquired immunity characterized by specific response based on memory if the antigen attacks the body for a second time (Sharma, 1997; Janeway, 2002).

The primary lymphoid organs that forms the immune system are: (a) thymus, located in the neck, is the organ where T-cell differentiation occurs and; (b) the organ which is unique in birds and located adjacent to the cloaca, called Bursa of Fabricius, is known to secrete the antibody-synthesizing B-cells. Spleen, lymph nodes, bone marrow, caecal tonsils, Harderian gland, and various lymphoid tissues e.g. lymphoid tissues associated with mucosal surfaces (MALT) are secondary lymphoid organs (Glick *et al*., 1956; Sharma, 1997). Spleen is the site for plasma cell proliferation and houses memory B-cells (Carlander *et al*., 1999).

Acquired or specific immunity are categorized into two types (a) cellular response, where there is highly specific response to specific antigens by immune cells such as T-cells (excluding the cells that produce antibodies) and (b) non-cellular or humoral response, comprises the immunoglobulins (Ig) in blood or lymph or B-lymphocytes, which on activation produce antibodies (Janeway, 2002; Chalghoumi, *et al*., 2009).

There are three isotypes of immunoglobulins present in chicken egg yolk – IgY, IgA and IgM; out of which IgY contributes to 75% of immunoglobulins in egg yolk. Chicken IgA and IgM resemble in molecular weight, electrophoretic mobility and structurally with mammalian IgA and IgM (Leslie and Clem, 1969; Leslie and Martin, 1973). However, chicken IgY that is analogous to mammalian IgG share a functional homology, but they have differences in their molecular weight, structure and biochemical functions. The similarities of IgY and IgG resides in the fact that they both have identical two heavy and light chains, connected by disulphide bridge, have an antigen binding variable region, and a highly conserved constant region. The differences are that IgY has a higher molecular weight than IgG because of larger heavy chain. Also, the hinge region of IgY is shorter and less flexible than IgG (Warr *et al*., 1995). IgY is more hydrophobic in nature and has a low isoelectic point as compared to IgG (Dávalos-Pantoja, *et al*., 2000). In terms of antibody functions, IgY unlike IgG does not activate the complement system or interact with rheumatoid factors (Carlander *et al*., 2000; Dávalos-Pantoja, *et al*., 2000).

Recent studies have drawn attention to chickens in various treatment and therapies, as a source of large quantitates of IgY antibodies, deposited in egg yolk (Touchette *et al*., 2003; Kovacs-Nolan and Mine, 2004). For example: Bovine group A rotaviruses (RVA) induces malabsorptive and secretory diarrhea in neonantal calves, by infecting the enterocytes in the tip of villi of small intestine (Bridger, 1994; Malik *et al*., 1995). IgY has been reported to be effective treatment for neonatal calves from bovine RVA diarrhea (Ikemori *et*

al., 1992, 1997; Kuroki *et al*., 1994, 1997).

In chickens, the bones become fragile with the start of the egg laying cycle and leads to osteoporosis in chickens (Van de *et al*., 1984; Kerschnitzki *et al*., 2014). When bone resorption exceeds the extent of bone formation (negative bone balance), it leads to structural damage and bone loss (Seeman, 2003). The reason for negative bone balance is secretion of RANK-L, by osteoblasts and osteocytes; which leads to activation of osteoclasts precursors and maturation of osteoclasts, resulting in enhanced bone resportion and a decrease in bone mass (Manolagas, 2000; Weitzmann and Pacifici, 2006). A fully human monoclonal antibody, Denosumab that is approved as an antiresorptive agent, binds to RANK-L, thereby preventing RANK-L to activate RANK, receptor on surface of osteoclast. Therefore, Denosumab reduces osteoclast formation, which leads to decreased bone resportion and an increase in bone mass (Amgen Canada Inc., 2011; Lipton and Goessl, 2011; Bone *et al*., 2011).

Based on the similar strategy, we aim to develop IgY specific for chicken RANK-L (chRANKL) for treatment of osteoporosis in chickens.

3.3. Methods

3.3.1. Materials

Restriction enzymes were procured from New England Biolabs (Mississauga, Canada). Hybond ECL nitrocellulose membrane, X-ray film and the ECL Western blotting reagents were obtained from Amersham Pharmacia Biotech (Baied Urfe, Quebec, Canada). Ni-NTA agarose, plasmid DNA isolation kit and

gel extraction kit were obtained from Qiagen (Mississauga, Canada). Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG, rabbit anti-chicken IgG conjugated with horseradish peroxidase and streptavidin-HRPO were purchased from Sigma (St. Louis, MO, USA). Tetramethylbenzidine (TMB) was purchased from Fisher Canada (Ontario, CA) and KPL (Maryland, USA), respectively. Bio-Rad protein assay kit, glycine, Precision Plus Protein Standard, sodium dodecyl sulfate (SDS), and precast gels (4–20% Tris–Glycine, 16.5% Tris-tricine) were purchased from Bio-Rad Laboratory (Ontario, CA). The ELISA V_{max} kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA). Slide-A-Lyzer® was purchased from Thermo Scientific Pierce Protein Research Products (PA, USA). All analytical grade chemicals and solvents were from commercial sources.

3.3.2. Production of anti-chRANK-L and mRANK-L polyclonal IgY

Production of polyclonal IgY directed against chRANK-L and mRANK-L was followed by chicken immunization according to CCAS guideline. Proteins were suspended in sterilized PBS at a concentration of 100 μg/mL and emulsified with an equal volume of Freund's incomplete adjuvant. Twelve 23-weeks-old single comb white leghorn chickens were intramuscularly injected with the emulsified saline with or without the proteins at four different sites (0.25 mL per site) of breast muscles (two sites per left or right breast muscle). A booster immunization was given after two weeks of the initial immunization. Eggs were collected daily and stored at 4 °C until the extraction of the antibodies.

3.3.3. Purification of anti- chRANKL and mRANKL IgY

The egg yolk from immunized hens was physically separated from egg white by mixing gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give a pH of 4.0) to avoid possible disruptions of egg yolk granules due to the presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to a pH of about 5.0 to 5.2 and incubated at 4°C for 12 h. Water soluble fraction (WSF) was obtained by centrifugation (3125 × g) at 4°C for 20 min. The supernatant was collected as the IgY rich WSF and titrated by indirect ELISA (described in the following section) using pure chRANKL and mRANKL as coating antigen. The fractions found to have high titers, particularly the fractions collected during weeks 4^{th} to 6^{th} following immunization, which was further, purified by ammonium sulfate (40 %) followed by Sephacryl S-300 gel filtration column. Control IgY antibody was extracted from eggs obtained from non-immunized chickens.

3.3.4. Titer of anti- chRANKL and mRANKL IgY by Indirect ELISA

Microtiter plates were coated with 100 µL per well of proteins in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at a concentration of 8 µg/mL and incubated at 37°C for 1 h. The plates were then washed four times with PBS containing 0.05 % Tween 20 (PBS-Tween). After washing, 100 µL of 1 % BSA solution (w/v) in PBS-Tween was added to each well and the plates were incubated at 37°C for 30 min. The BSA solution was then discarded, and the

wells were washed four times with PBS-Tween. The water soluble fraction (diluted 1:1000 in PBS-Tween) containing specific IgY against chRANK-L and mRANK-L or non-immunized egg yolk IgY antibody as a control was added to each well (100 μL per well) and the plates were further incubated at 37°C for 1 h. After washing the plates with PBS-Tween for four times, 100 μL of rabbit anti-chicken IgG conjugated with horseradish peroxidase (HRPO, diluted 1:5000 in PBS-Tween) was added to each well and incubated at 37 °C for 1 h. The plates were washed four times with PBS-Tween, followed by the addition of 100 μL of the freshly prepared substrate solution, tetramethylbenzidine (TMB). OD_{650} was taken after 30 min using an ELISA V_{max} kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the control antibody from that of specific antibody.

3.3.5. Assays

3.3.5.1. Total IgY concentration

To quantify the total IgY concentration in WSF, ELISA was performed as mentioned above. A microtiter plate was coated with 100 μL per well of rabbit anti-chicken IgG at a final concentration of 2 μg. Samples of the chRANK-L and mRANK-L specific IgY rich WSF were diluted 1:10000 in PBS. Specific and non-specific IgY rich WSF were reconstituted and serially diluted in PBS (2 to 0.125 μg/mL). Two-fold serial dilutions of purified chicken IgG in PBS (0.5 to 0.031 μg/mL) were used as the reference antibody to prepare a standard curve.

Total IgY concentrations in lyophilized WSF were determined using the standard curve.

3.3.5.2. Cross-Reactivity of IgY

The cross-reactivity of IgY between chRANKL and mRANKL was determined by using the above-mentioned western blot method. Nitrocellulose sheets transferred by the proteins and egg proteins from electrophoresis were incubated with anti-RANK-L IgY antibodies (in PBS containing 1 % BSA) for 1 h at room temperature, followed by 1 h incubation with rabbit anti-chicken IgG conjugated with HRPO. The cross-reactivity of anti-chRANK-L and mRANK-L IgY against proteins was determined by comparing activities against chRANK-L and mRANK-L.

3.3.5.3. Total protein assay

The Bio-Rad protein assay, based on the method of Bradford, was performed using purified chicken IgY (1 mg of protein/mL) as the reference protein. Serial dilutions of the purified IgY and the reference protein in PBS (0.5 to 0.0625 mg/mL) were analyzed using the microtiter plate. OD_{595} was taken after 5 min using an ELISA V_{max} kinetic microplate reader. The student t-test (one-tailed t-test) was used to analyze the significant difference ($p < 0.05$) between the groups.

3.3.5.4. Biological activity of IgY (*In vitro* studies) using RAW 264.7 cells

RAW 264.7 cells from liquid nitrogen were thawed and 8 mL of the media [DMEM (1X) (500 mL, from Gibco, Life Technologies), with 10% FBS (50mL), 1% PSG (5mL)] was added and centrifuged for 5 mins to remove DMSO. 15 mL of fresh media was added to the cells and were added to 75 cm^2 flask and were incubated at 37 °C and 5% CO_2. After cells were grown for 3 passages, cells (10^6/ mL) were seeded on a 6-well plate at a confluency of 30 % and were allowed to grow to the confluency of 70 %. 0.15 µg of RANK-L, 0.15 µg of anti-mRANKL IgY, 0.1.5 µg of anti-mRANKL IgY + RANK-L were added in duplicates wells. Cells were incubated at 37 °C and 5 % CO_2 for 4 days. Acid Phosphatase, Leukocyte Kit (purchased from Sigma Aldrich) was used to perform TRAP (tartrate resistant acid phosphatase) assay.

Same experiment was conducted with 1.5 µg of anti-chRANKL IgY and 1.5 µg of chRANKL.

3.4 Results

3.4.1. IgY Production

The variation in anti-chRANKL and mRANKL IgY activity from the egg yolks of the immunized chickens against chRANKL and mRANKL was determined by indirect ELISA method as shown in Figure 3-1.

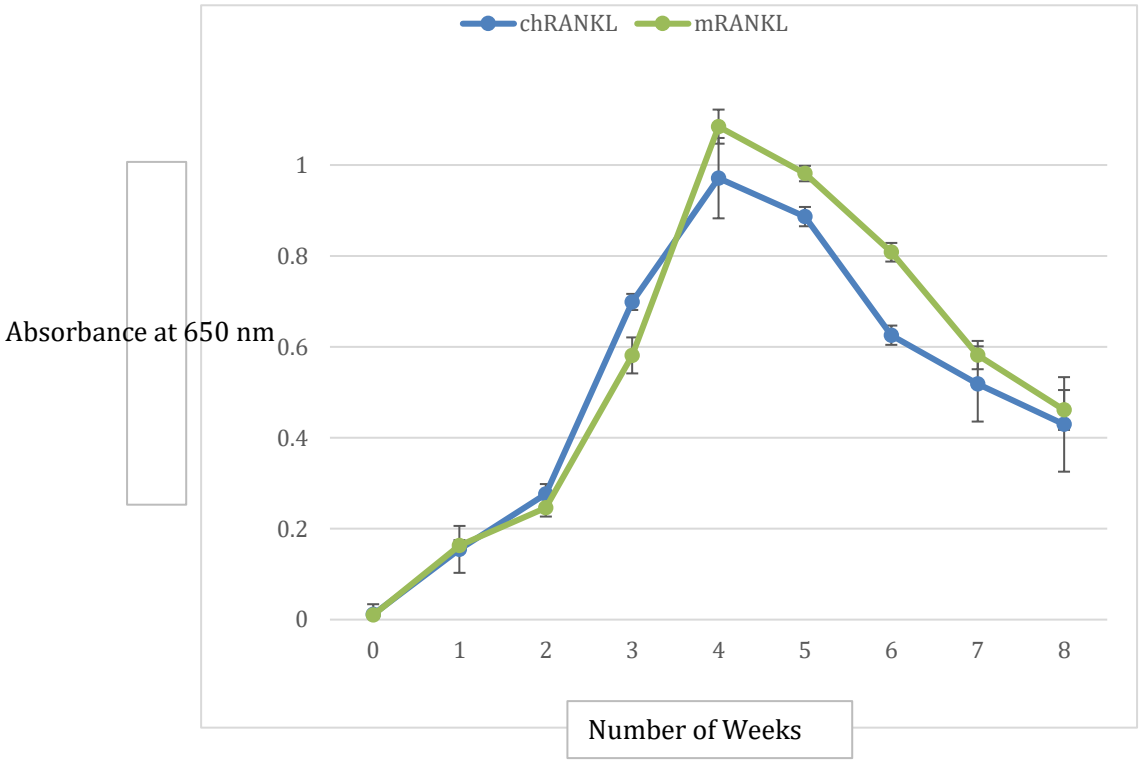

Figure 3-1. Absorbance of anti-chRANKL and mRANKL IgY from egg yolk, versus the number of weeks of the immunized chickens against r-chRANKL and mRANKL determined by indirect ELISA. Values are the mean of triple samples $\pm$ SD. $p > 0.05$.

The activity of anti-chRANKL and anti-mRANKL IgY was undetectable right

after immunization (on day 0), then rapidly increased from week 2 to week 4 and, thereafter remained relatively constant up to 8th week. The eggs obtained from 4th to 7th weeks were found with high levels of anti-chRANKL and mRANKL IgY activity (not significant difference between anti-chRANKL and mRANKL IgY activity, $p > 0.05$). Chicken can produce a high titre of IgY antibody against a wide range of proteins including highly conserved mammalian proteins.

The present study shows that chickens can produce specific IgY antibody against the self-antigen chRANK-L that is a recombinant protein. During the period of immunization, there were no adverse effects on daily egg production, egg yolk weight of laying hens. IgY egg yolk antibody can be obtained without bleeding or sacrificing the animal but by a simple collection of eggs on a daily basis. The yield of IgY antibodies can be compared to that of IgG antibodies obtained by conventional immunization methods; 200 mg of sera IgG can be obtained once, constituting of approximately 5 % specific IgG. In the case of chicken, approximately 3,000 mg of IgY can be harvested from egg yolks each month, with 5 to 10 % specific IgY.

3.4.2. Total and Specific IgY contents

The content of total IgY in the egg yolk was found to be constant (~ 9.0 ± 1.1 mg/mL) among the chickens immunized with RANKL during the experimental period as previously reported.

Table 3-1. Total IgY content and OD value of specific IgY from hyperimmunized egg yolk.

IgY	*Total IgY (mg/mL)*	*Specific IgY titre* $OD_{650\ nm}$
Anti-chRANK-L	10.2 ± 0.12	0.68 ± 0.05
Anti-mRANK-L	10.9 ± 0.15	0.83 ± 0.08
Non-specific IgY	9.4 ± 0.14	0.04 ± 0.00

The results showed that both the protein specific IgY titre of the defatted egg yolks, collected from 4 to 6 weeks after the immunization, were significantly higher ($p < 0.05$) than the control group (non-immunized chickens). However, the total IgY content does not show considerable difference ($p > 0.05$) between RANKL immunized and non-immunized groups. As expected, the chRANK-L and mRANK-L specific IgY was not detected in the control group (Table 3-1). There is limited information available on the quantification of chRANK-L and mRANK-L specific IgY. Therefore, this study introduces, for the first time, a simple and reliable method to obtain recombinant RANK-L specific IgY antibodies.

3.4.3. Cross reaction of IgY

To further investigate the polyclonal specific IgY against chRANKL and mRANKL, the cross-reactivity of IgY between chRANKL and mRANKL was examined by Western blot assay.

Western blot analysis of Sigma chicken IgY standard (non-specific) and anti-chRANKL polyclonal IgY (Figure 3-2), against chRANK-L and mRANK-L was conducted using HRP-conjugated rabbit anti-chicken IgG as the secondary antibody. In this experiment, no band appeared for non-specific Sigma IgY for both mRANKL and chRANKL (Figure 3-2A), but anti-chRANKL IgY showed a band against both mRANK-L and chRANK-L (Figure 3-2B), indicating the cross-reaction of the anti-chRANKL IgY to mRANKL.

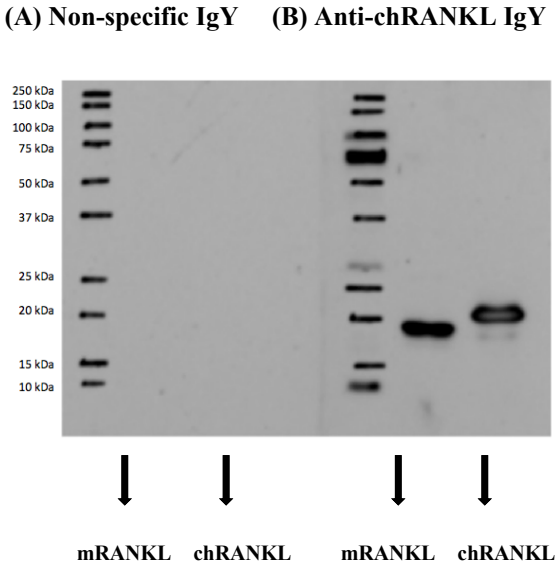

Figure 3-2. Western Blot of anti-chRANKL polyclonal IgY against chRANKL and mRANKL. (A) Left; detection with Sigma chicken non-specific IgY standard. (B) Right; detection with anti-chRANKL polyclonal IgY. HRP-conjugated rabbit anti-chicken IgG was used for secondary antibody for both gels. Left lane; Sigma mouse RANK-L. Right lane; chRANK-L.

3.4.4. Biological activity of IgY "*in-vitro studies*"

RAW 264.7 cell line is commercially available murine monocyte/macrophage cell line, widely used for *in vitro* research for over four decades now. We used RAW 264.7 cells to study *in vitro* effect of mouse and chicken RANKL and their respective RANKL IgY (Taciak *et al*., 2018). Chicken macrophages are not commercially available and would require a procedure to be isolated from chicken bone marrow cells.

Tartrate-resistant acid phosphatase (TRAP) activity is an important cytochemical marker of osteoclasts, osteoclast-like cells and precursor monocytes. TRAP activity appears as purplish to dark red in and around the cell.

TRAP-positive multinucleated cells were observed when RAW 264.7 cells were treated with RANKL (Figure 3-3 B), and no TRAP-positive cells appeared in the absence of RANKL (Figure 3-3 A), or in presence of IgY (Figure 3-3 C).

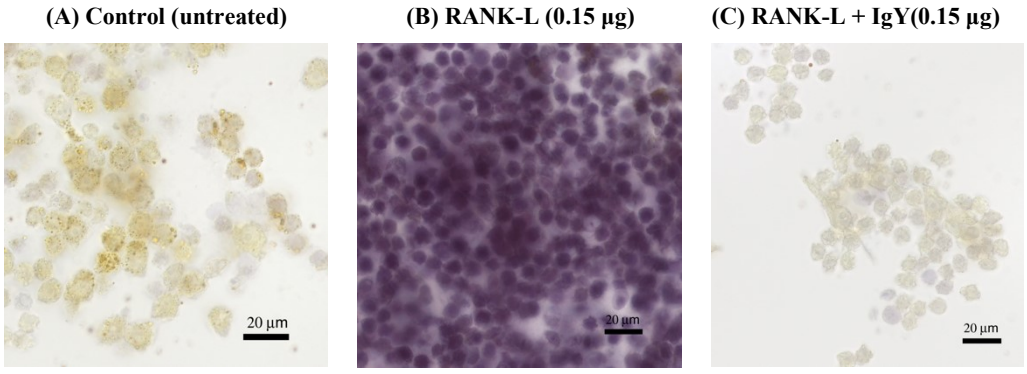

Figure 3-3. TRAP activity of RAW 264.7 cells treated with (B) 0.15 µg RANKL and (C) 0.15 µg each of RANKL and anti-RANKL IGY. (A) Untreated

(control group).

Likewise in a similar experiment, 10^6/mL of RAW 264.7 cells seeded per well into a 6-well plate. Two wells were treated with 1.5 µg of chRANKL and 1.5 µg each of chRANKL and anti-chRANKL IgY. Two wells were kept as untreated (i.e. control group). For four days, cells were incubated at 37 °C and 4% CO_2. After 4 days, acid phosphatase kit was used to stain cells and analyze TRAP activity.

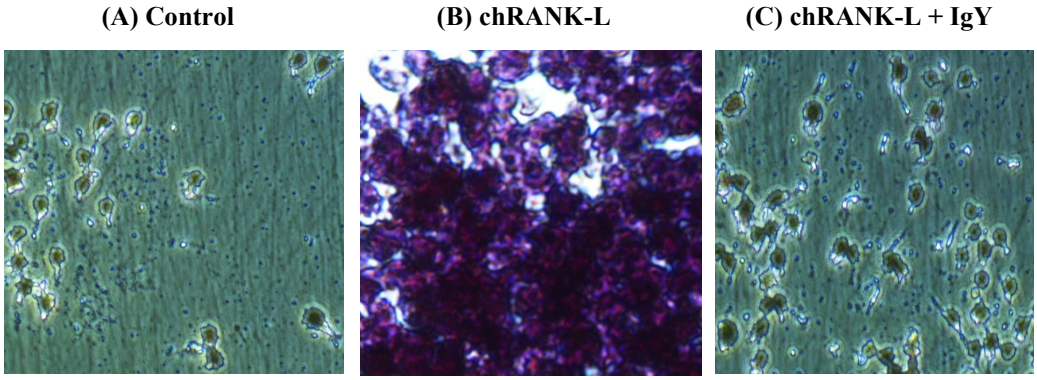

Figure 3-4. TRAP activity of RAW 264.7 cells treated with treated with (B) 1.5µg chRANKL and (C) 1.5 µg each of chRANKL and anti-chRANKL IgY (A) was untreated (control group).

TRAP-positive multinucleated cells were observed when RAW 264.7 cells were treated with chRANK-L (Figure 3-4 B). No TRAP-positive cells were observed in the absence of chRANK-L (Figure 3-4 A), or in presence of IgY (Figure 3-4 C).

3.5. Discussion

With the onset of sexual maturation in chickens, the development of medullary bone is followed and associated with discontinuation of remodeling of trabecular i.e. structural bone (Whitehead and Fleming, 2000). Medullary bone is the storehouse for calcium reserves in chickens and during the egg-laying cycle, these reserves are used for the calcification of eggshells (Etches, 1987). The continuity of calcium supply for remodelling of medullary bone, is associated with resorption of structural bone that makes egg-laying chicken susceptible to osteoporosis and; the depletion in bone strength increases with high egg production (Rennie *et al*., 1997). Osteoporosis is the condition characterized by progressive loss of mineralized structural bone in egg-laying chicken during the egg laying period, with increased fragility to bones and ultimately leading to bone fractures (Whitehead and Fleming, 2000). This is most common in caged egg laying hens, which leads to deformed and fragile skeletal bones due to minimal physical activity and depletion of calcium reserves (Webster, 2004). This is one of the greatest challenges faced by commercial egg industry (Sandilands, 2011).

Two types of functions maintain the normal bone metabolism that is, bone resorption and bone formation. The two activities are associated with bone-resorbing osteoclasts and bone-forming osteoblasts, respectively (Roodman, 1996; Suda *et al*., 1997a). Osteoclasts precursors (monocyte macrophages) mature and differentiate into multinucleate cells called osteoclasts, the process

regulated by receptor activator of nuclear factor-κB ligand (RANK-L), a transmembrane protein, expressed by pre-osteoblasts and osteoblasts (Collin-Osdoby, 2004). Murine RANKL shares 83 % homology with human RANKL (Wong *et al*., 1997) and chicken RANKL is 59-62 % homologous to mammalian RANKL (Lam *et al*., 2001).

Interaction and binding of RANK-L with its receptor RANK on the surface of osteoclasts results in activation of osteoclasts (Nardone *et al*., 2014). In 2008, Wang *et al*., investigated the effect of chicken RANK-L (chRANKL), in activation and stimulation of formation of osteoclast like cells, from chicken bone marrow cells. But, there is no treatment developed for managing or preventing osteoporosis in chickens. *E. coli* and *baculovirus* expression systems were used to express chRANK-L, as described in chapter 2.

The application of IgY technology in the field life sciences and biotechnology is rapidly growing, as chicken IgY have several advantages over mammalian antibodies. Use of IgY causes minimal assay interference in mammalian serum sample, leading to an increase in sensitivity and a decrease in background signal, hence serving as an ultimate reagent in immunoassays (Dias da Silva, Tambourgi, 2010). IgY technology has been used to develop therapeutics to treat various diseases (Spillner *et al.,* 2012). The major reason being is that IgY does not lead to activation of complement system in mammals and does not lead to interaction with rheumatoid factors or Fc receptors in bacteria or humans (Dias da Silva, Tambourgi, 2010). Good diagnostic techniques are available to

detect bacterial infections and; specific antibiotics regimes to treat those infections are readily available in the market (Müller *et al*., 2015). However, the World Health Organization (WHO) in 2014 reported that antibiotic or antimicrobial resistance is increasing not only in developing countries but also all over the world. According to several studies, specific IgYs had shown a positive impact to counteract bacterial infections and intoxications (Müller *et al*., 2015). Because of the advantages of ease of administration and reduced cost, passive oral antibody immunotherapy is of great significance as it holds the ability to treat gastrointestinal tract (GIT) infections and conditions (Reilly *et al*., 1997). Using IgY as passive immunotherapy in response to gut pathogens, such as E. *coli O157:H7* (Sunwoo *et al*., 2002), *Salmonella enteritidis*, *Salmonella typhimurium* (Lee *et al*., 2002), *Clostridium perfringens* (Song *et al*., 2002), etc., has been reported. In 1988, treatment of neonatal piglets suffering from enteric infections was conducted using specific IgY against *E. coli* K88+, which were tested in clinical trials (Marquardt *et al*., 1999). Evidences from this study prove that development of IgY technology may lead to a reduction or elimination of the possibility of the use of antibiotics for GIT infections (Müller *et al*., 2015).

In cystic fibrosis (CF) patients, protection from chronic pulmonary *Pseudomonas aeruginosa* (PA) infections has been demonstrated by oral administration of specific anti-PA IgY and hence reduces the use of antibiotics by CF patients (Nilsson *et al.*, 2007; Nilsson *et al.*, 2008). Gluten free diet is the only available and acceptable treatment of Celiac Disease (CD), characterized by intestinal damage due to loss of absorptive villi and hyperplasia of the

crypts, caused by wheat gluten and alcohol soluble proteins (Chand and Mihas, 2006). Gujral *et al.*, 2012, demonstrated that specific IgY antibodies against gliadin (with sugar protectants), led to neutralization and the adsorption of gliadin in both *in vitro and in vivo.* White leghorn chickens at 23 weeks of age, were immunized intramuscularly with chRANK-L, as per the procedure described in Gujral *et al*., 2012.

Antibodies in maternal blood or serum in mammals are known to cross the placental barrier to the foetus. An equivalent phenomenon of transfer of IgY from serum to the egg yolk is known to occur in hens and chickens (Rose *et al*., 1974). IgY antibodies pass across the yolk sac membrane during the later stage of embryonic development to the embryonic bloodstream (Kowalczyk *et al*., 1985). Mohammad *et al*., in 1998 and Morrison *et al*. in 2002; conducted studies to report that IgYs are selectively transferred from serum to egg yolk in a receptor-mediated process. Transport of IgY to egg yolk roughly takes about 4-6 days, post-immunization. In another study, it was reported that an intact hinge region and amino acid sequence of His-Glu-Ala-Leu (HEAL) in the Fc region are the specific requirements for IgY transport. IgY transport to egg yolk fails to occur, if there is any change in the amino acid sequence (Patterson *et al*., 1962; Woolley and Landon, 1995). IgY specific for gliadin was extracted and purified from egg yolk using the same procedure as established in Gujral *et al*., 2012 (b).

Purified IgYs have been reported to show long lasting stability from few months

to several years under specific storage conditions (Schade *et al*., 2005). The other advantages of using IgY's are that it is inexpensive to keep chickens for egg production and easy to handle. This would avoid an extensive procedure for bleeding of animals and the pain that laboratory animals have to go through would be eliminated as well. It been also reported that the IgY titre in egg yolk tends to remain high for a very period of time, hence offering stability in the IgY content in immunized chickens (Gassmann *et al*., 1990). About 250 eggs are laid by egg lying hens in a year, i.e. that accounts for approximately 4000 g of egg yolk. This is huge amount in comparison to 40 mL of serum derived from one rabbit. Roughly 10 mg of IgY can be recovered from about 1 gram of egg yolk from immunized hens/ chickens (Hatta *et al*., 1993). The specific IgY, anti-RANKL IgY is polyclonal and can bind to more than one epitope; hence would improve the binding efficiency to RANKL. The production of polyclonal IgY from chicken egg yolk is inexpensive as compared to production of monoclonal antibodies from animal blood or serum (Gujral *et al*., 2012, Gujral *et al*., 2015).

A preliminary *in vitro* study in RAW 246.7 murine macrophage cells was conducted to investigate the effect of RANKL and anti-RANKL IgY. RAW 246.7, has been a widely used murine macrophage cell line, utilized to study the bioactivity of various natural products on primary cells and provide an estimate of its effect *in vivo* (Merly and Smith, 2017). Tartrate-resistant acid phosphatase (TRAP) is recognized, as a histochemical marker, expressed on the surface of osteoclasts; along with having important functions to play both in skeletal

development and immune system (Hayman, 2008). Suda, *et al*. (1997b), described earlier in 1997 that cells showing positive stain for TRAP were determined to be osteoclast like cells (OLCs). To summarize, cells that were incubated were RANKL, showed positive stained TRAP activity (Wang *et al*., 2008). But, on incubating macrophages cells with RANKL and IgY, the cells showed no activity for TRAP, hence indicating that IgY blocked formation of OLC. To summarize, anti-RANK-L IgY has a great potential to be an effective and efficient treatment for osteoporosis in chickens.

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Chapter 4 – Discussion and Future Studies

To summarize, this thesis aimed to develop a method for the production of an anti-RANKL IgY therapeutic, for use to potentially find a solution to osteoporosis in chickens, and to improve chicken bone health with the onset of the egg-laying cycle. In the first chapter, we reviewed the condition of osteoporosis in humans and chickens and, the various factors that lead to fragile bones and fractures as a result of osteoporosis. We also studied bone remodelling and the vital cytokine factors and TNF members (e.g. RANK and RANKL) that play a role in bone resorption and reformation process. Osteoporosis in chickens become prevalent during the egg laying cycle, when minerals from bones are extracted and used for egg-shell formation, hence leading to reduced bone mineral density. The bone resorption process is initiated by the activation and maturation of osteoclasts due to interaction of RANKL (on the surface of osteoblasts) with RANK, expressed on the surface of osteoclasts. Bone remodelling is a modulated process, constituted of balanced bone resorption and bone reformation. Certain hormonal and other factors lead to increased secretion of RANKL by osteoblasts and osteocytes, which mean that it will lead to activation of precursors of osteoclasts, thereby causing maturation of osteoclasts. This leads to increased bone breakdown (resorption) and hence osteoporosis (Boyce and Xing, 2008). Mammalian and chicken RANKL are similar in two ways: they are 59-62% homolgous and have highly conserved extracellular TNF domain (residues 163-318) that is crucial for interaction with RANK (Lam *et al*., 2001).

For humans, a number of antiresorptive therapeutics, bisphosphonates (such as,

Alendronate, Risedronate, etc.) that induce apoptosis in osteoclasts, have been developed and used to manage osteoporosis in humans. Denosumab is a human monoclonal antibody that acts as an antiresorptive agent, by binding to RANKL and hence inhibiting its activity (Hanley *et al*., 2012).

There have been studies reported to produce recombinant chRANKL (Wang *et al*., 2008). However, there is no treatment to reduce or eliminate bone deformations and fractures as a result of osteoporosis in chickens. Based on the concept of denosumab, we propose that development of specific IgY against chRANKL from egg yolk can be a potential candidate for as an immunotherapeutic for osteoporosis in chickens.

In chapter 2, recombinant RANKL was produced using two expression systems – *E. Coli* and *Baculovirus*. The high level of expression from an *E. Coli* expression system resulted in the formation of inclusion bodies that are aggregates of protein molecules. As inclusion bodies are themselves insoluble, there were some challenges to solubilize and purify inclusion bodies to form soluble active proteins. Therefore, *Baculovirus* system was used to produce recombinant chRANKL as it is a widely used expression system as they have the ability to produce proteins and can be used for a number of applications. *Baculovirus* expression system successfully yielded soluble intracellular proteins.

In the subsequent study (Chapter 3), we described the production of IgY specific to chRANKL. Eggs were obtained from chickens that were immunized

intramuscularly. Anti-chRANKL IgY were isolated and purified from chicken egg yolk (Gujral *et al*., 2012). The binding of anti-chRANKL IgY with chRANKL was also tested. A preliminarily study to investigate the effect of chRANKL and specific IgY was conducted on the murine macrophage cell line RAW 246.7. The results of these studies investigate the effect of specific IgY on RANKL activity.

Whether chRANKL can induce differentiation of chicken macrophages to osteoclasts and if specific IgY against chRANKL has the ability to inhibit the formation of osteoclasts like cells (OLC), need to be investigated in chicken bone marrow cells or macrophages. Similarly, it would be advantageous to study the effect of anti-chRANKL IgY on the formation of OLC from chicken bone marrow cells by conducting a TRAP assay (Wang *et al*., 2008).

Further, *in vivo* experiments on 50-60 week old caged layers or chickens kept on low calcium diet, can be conducted to study the effect of specific IgY against chRANKL by analyzing calcium ion levels in blood. This would be similar to previous studies done by immunizing chickens with chRANKL in PBS buffer and collection of blood samples to analyse the level of calcium ions using an auto analyzer. It has been shown that there is a dose-dependent relationship between chRANKL and the blood level of calcium ions of 58-week-old cage layers that were subjected to a low calcium diet for 48 hours. This suggests possible activation of osteoclasts due to chRANKL *in vivo* that can lead to bone resorption (Wang *et al*., 2008). It would be interesting to see the dose response

relationship of blood calcium levels after injecting anti-chRANKL IgY in chickens.

During the egg laying cycle in the chickens, mineral content is extracted from the bones that compromises the bone structure and bone strength. Therefore, studies can be conducted to investigate (a) the effect on chicken bone strength and structure, and (b) the effect on eggshell formation, after immunizing chickens with specific IgY against chRANKL.

Histological analysis of chicken bones (femur and tibia) can be done to analyze the morphology of bones after injecting anti-chRANKL IgY intramuscularly and comparing it with non-immunized chicken femur and tibia. Analyzing bone mass and bone density in the femur of osteoporotic egg laying chickens would be the way to measure dose and efficacy of IgY using bone-imaging technology. The parameters for quality of bone density (weight of femur bone, femur bone height, and femur bone density) can be investigated using quantitative computed tomography (CT) or micro-CT, for white leghorn chickens at 65 or 70 weeks, vaccinated with IgY and for the control group (Aguado *et al*., 2015).

Because osteoporosis in chickens, widely affects the commercial egg industry, we propose to investigate egg production rate, egg weight and IgY content in egg yolk in white leghorn chickens from 50 to 70 weeks, after vaccinating chickens with specific IgY against chRANKL and compare it with non-vaccinated chickens. The egg weight is calculated as average grams per each cage (Pohle and Cheng, 2009). As chickens extracts calcium and other mineral

content from the bones to form eggshells, it is really crucial to check the effect of injecting anti-chRANKL IgY on eggshell formation and egg quality production. This study would be an important evidence for checking the safety of specific IgY. Hence, investigation of the other important parameters including eggshell thickness, Haugh Unit and Albumin Height, would give the effect on eggshell formation, post-immunization. Eggshell thickness can be determined as used in previous studies. (Schreiweis *et al*., 2006; Jendral *et al*., 2008). After discarding the yolk and albumin from the cracked eggs at first; the eggshells are dried in an oven. The thickness of the eggshell can be calculated by taking the average of the shell thickness measured at three sites (at the equator, at the blunt end and at the pointed end), using an Ames micrometer (Pohle and Cheng, 2009). Haugh unit and albumin height are indicators of internal egg quality (albumin quality). Albumin height is calculated by taking and average of the measurement of the height of albumin at the highest points on either side of egg yolk (away from chalazae). Haugh unit can be calculated from the thickness of the egg white and the initial egg weight as below (Haugh, 1937 a and b).

$$\text{Haugh units} = 100 \times \log (T - 1.7 \times W^{3.7} + 7.57),$$

Where, T is thickness of thick-white layer (mm) and W is egg weight (g).

Comparison of internal egg quality parameters that includes Haugh unit and albumin height, and eggshell thickness; between IgY vaccinated and non vaccinated groups of white leghorn chickens from 50 to 70 weeks, would be

important to investigate if immunization with specific IgY will affect any of the egg quality parameters.

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