Dose Dependent Effects of Exogenous Vitamin D$_3$ Metabolite 1,25(OH)$_2$D$_3$ During Bone Development

Vivek Pande
B.V.Sc & A.H
M.V.Sc (Pathology)

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>1,25-dihydroxycholecalciferol</td>
</tr>
<tr>
<td>1α-hydroxylase</td>
<td>25-hydroxyvitamin D-1α-hydroxylase</td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>25-hydroxycholecalciferol</td>
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<tr>
<td>25-hydroxylase</td>
<td>Vitamin D 25-hydroxylase</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BrDU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoproteins</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>DiMethyl-SulphOxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GMEM</td>
<td>Glassgow Minimum Essential Medium</td>
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<tr>
<td>H &amp; E</td>
<td>Haematoxylin and Eosin</td>
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<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HVD</td>
<td>High vitamin D</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
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<tr>
<td>LVD</td>
<td>Low vitamin D</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>NC</td>
<td>Negative control</td>
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<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NT</td>
<td>No treatment control</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celsius</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pmoles</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferative activated receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume for volume</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<td>γ</td>
<td>Gamma</td>
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<td>μg</td>
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Certificate of Authorship

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Signature: 

Date: 

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Publications

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1. **Vivek Pande**, Steven Croom, Kapil Chousalkar, Marie Bhanugopan, Jane C. Quinn. Role of vitamin D₃ in osteogenic differentiation of mesenchymal stem cells. Research higher degree symposium, December 2011, Charles Sturt University, Wagga Wagga campus.

2. **Pande V**, Martin E, Chousalkar K, Bhanugopan M, Quinn J. Species-dependent effects of vitamin D₃ metabolite 1,25(OH)₂D₃ during osteogenic differentiation of mesenchymal stem cells *in vitro*. Combio 2012 September 23 to 27, Adelaide convention centre, South Australia.

Oral presentations

1. Investigating the role of vitamin D₃ metabolite, 1,25(OH)₂D₃ in bone formation in poultry. Research higher degree symposium, December 2011, Charles Sturt University, Wagga Wagga campus.

2. Species-dependent effects of vitamin D₃ metabolite 1,25(OH)₂D₃ during osteogenic differentiation of mesenchymal stem cells *in vitro*. Research higher degree symposium, December 2012, Charles Sturt University, Wagga Wagga campus.

3. Effects of 1,25(OH)₂D₃ during *in vitro* osteogenic differentiation of chicken mesenchymal stem cells. Research higher degree symposium, July, 2013, Charles Sturt University, Wagga Wagga campus.
Abstract

The biologically active form of vitamin D₃ is 1,25(OH)₂D₃ involved in mineral homeostasis and bone formation. Deficiency of vitamin D₃ is a major cause of bone disorders in poultry. The use of supplementary dietary 1,25(OH)₂D₃ to combat this problem in growing and adult birds is well documented however there is paucity of literature on how 1,25(OH)₂D₃ modulates the bone growth in avian species. In order to determine the role of 1,25(OH)₂D₃ during embryonic osteogenesis, this study has examined the effects of low and high doses of 1,25(OH)₂D₃ by in ovo administration on day 8 or day 11 of embryonic development and during in vitro osteogenic differentiation of chicken mesenchymal stem cells.

Body weight and bone length of chicken embryos was significantly reduced with low doses (0.12 μm/5ng) of 1,25(OH)₂D₃. No significant effect was observed by in ovo injection of exogenous 1,25(OH)₂D₃ on the length of different zones of both proximal and distal tibia and diaphysis width. The results of this study showed that in ovo administration of 1,25(OH)₂D₃ negatively affect the embryonic body weight and bone development suggesting additional doses of 1,25(OH)₂D₃ are not essential provided the eggs sourced from vitamin D fed parent flock.

Species specific differences were observed in osteogenic differentiation between avian and mammalian species in vitro. Mineralization was evident in chicken MSCs cultures in a dramatically shorter timeframe than in mammalian counterparts. High doses (24nM) of exogenous 1,25(OH)₂D₃ inhibited calcium deposition during osteogenic differentiation of MSCs in all species. However, some strain specific differences were observed. Layer
MSCs were more sensitive to increasing concentrations of exogenous 1,25(OH)2D3 than cells derived from broiler chickens. Early osteogenic differentiation and cell proliferation was inhibited by exogenous 1,25(OH)2D3 in dose dependent manner in both broiler and layer MSCs at all days examined.

Overall, the data suggests that increasing concentrations of exogenous 1,25(OH)2D3 has a detrimental effects on skeletal development in birds as well as mammals. Therefore, optimal species and strain specific concentrations need to be established in vivo to determine the critical levels of 1,25(OH)2D3 for optimal bone health and welfare of poultry and other production species.
CHAPTER 1

Literature Review

1.1. Introduction

There has been tremendous improvement in the growth potential of poultry over the decades; however this has compromised the bone development of young broilers and lead to many skeletal disorders (Julian, 1998; Lilburn, 1994). Leg disorders have become a major economic and welfare concern due to reduced performance, increase mortality and carcass condemnations. Proper nutrition is an integral component for optimum growth and development of chickens. Poor skeletal growth, lameness and increased susceptibility to diseases are a result of inadequate nutrition of poultry (Edwards Jr, 2000; Julian, 2005; Kidd, 2004). It is well known that nutritional factors play an important role to reduce many locomotion problems and vitamin D$_3$ is one of the key nutrients involved in calcium and phosphorous metabolism and therefore in bone formation. The biologically most active form of vitamin D$_3$ is 1,25(OH)$_2$D$_3$ involved in absorption of calcium and phosphorous in intestine and mineralisation of bone (DeLuca, 2004; Garcia et al., 2013).

Previous studies also demonstrated that dietary supplementation with 1,25(OH)$_2$D$_3$ is effective in preventing the incidence and severity of bone disorders such as rickets and tibial dyschondroplasia (Edwards Jr, 1989, 1990; Elliot & Edwards Jr, 1997; Rennie et al., 1993; Roberson & Edwards Jr, 1996). In the past, many studies have shown that in ovo administration of 1,25(OH)$_2$D$_3$ increased hatchability, body and bone weight; improved bone resorption and mineralisation of trabeculae (Narbaitz & Tsang, 1989) and
restored calcium and phosphate levels (Narbaitz et al., 1987). This confirms that vitamin D₃ metabolite, 1,25(OH)₂D₃ is not only required for calcium and phosphate metabolism but plays an important role during chick embryonic development.

Vitamin D has significant impact on bone health. Vitamin D deficiency leads to plethora of bone disorders both in animals and humans. The biologically most active form of vitamin D₃ is 1,25(OH)₂D₃, involved in absorption of calcium and phosphorous in intestine and mineralisation of bone (DeLuca, 2004). The shift in the levels of 1,25(OH)₂D₃ in systemic circulation is associated with poor utilisation of calcium and phosphorus and failure to effectively mineralise the developing bone. Therefore, the altered levels of 1,25(OH)₂D₃ are attributable to leg disorders in young broiler chickens (Vaiano et al., 1994).

MSCs provide a source of multipotent stem cells and capable to differentiate into osteogenic, chondrogenic and adipogenic lineage (Pittenger, et al., 1999). Osteogenic differentiation of bone marrow derived MSCs is well characterised in rat (Maniatopoulos et al., 1988), mouse (Song et al., 2003; Tropel et al., 2004) and human (Frank et al., 2002) however the effects of 1,25(OH)₂D₃ during osteogenic differentiation of chicken MSCs is not well defined and there is paucity of literature on how 1,25(OH)₂D₃ modulates osteogenesis and therefore how it is affecting bone growth in avian species.

Previous studies have shown that treatment of MSCs with 1,25(OH)₂D₃ inhibit (Atmani et al., 2002; Atmani et al., 2003) or stimulate (Beresford et al., 1994; van Driel et al., 2006) the osteogenic differentiation.
During embryonic life, intramembranous and endochondral ossification occurs by differentiation of mesenchymal progenitor cells directly into osteoblast (Ducy et al., 2000; Karsenty, 1999; Olsen, et al., 2000; Reddi, 1997). Since bone marrow derived MSCs differentiate into osteogenic lineage both in vitro and in vivo, MSCs are suitable tool to study bone development.

The following section of thesis summarises the literature relevant to two important experiments examining the role of vitamin D₃ metabolite 1,25(OH)₂D₃ in bone development in ovo and during osteogenic differentiation in vitro.
1.1.1. **Overview of leg disorders in poultry industry**

Poultry is one of the most popular livestock due to fast growth and worldwide popularity for food (Knowles et al., 2008). Over the years there has been tremendous growth in production of modern broilers as a result of improved genetic selection, quality nutrition and supplementation with growth promoting antibiotics. Though the rapid growth has improved production and economy of the poultry industry, at the same time body has failed to keep pace with increased body growth. This has resulted in significant disturbances in the normal process of skeletal growth and homeostasis giving rise to many bone diseases and painful leg disorders in the modern poultry industry.

Leg disorders are not only a major economic problem but also a great welfare concern for the poultry industry (Bradshaw et al., 2002; Fleming, 2008). The economic losses associated with leg disorders are increase culling, condemnations of birds from septicemia-toxemia, increased susceptibility to various diseases and a downgrading of meat from the breast and leg (Morris, 1993). Leg disorders compromises the welfare of broilers due to the pain from the condition, an inability to walk and mortality from starvation and dehydration (Bradshaw, et al., 2002; Fleming, 2008). The specific aetiology of skeletal deformities in poultry is complex and not related to any single cause, however, there are many causes identified that can affect the skeletal and bone development such as nutrients, genetics, mycotoxins, pathogens, growth rate, body confirmation, exercise and stocking density (Bradshaw, et al., 2002; Cook, 2000). The most common forms of leg weakness in meat chickens in Australia are focal
osteodystrophy, osteomyelitis, synovitis, deviated toe, twisted leg and plantar pododermatitis (Nairn & Watson, 1972).

1.1.2. Classification of leg disorders

The leg disorders are classified into three different categories based on underlying pathological condition such as developmental, degenerative and infectious and are described below (Bradshaw, et al., 2002).

Classification of leg disorders

A. Developmental
   a. Varus valgus disease
   b. Rotated tibia
   c. Tibial dyschondroplasia
   d. Rickets
   e. Chondrodystrophy
   f. Spondylolisthesis

B. Degenerative
   a. Osteochondrosis
   b. Epiphyseolysis (Femoral head necrosis)
   c. Degenerative joint disease
   d. Spontaneous rupture of gastrocnemius tendon
   e. Contact dermatitis

C. Infectious
   a. Bacterial chondronecrosis with osteomyelitis or femoral head necrosis
   b. Tenosynovitis and arthritis
   c. Infectious stunning syndrome
   d. Virus induced neoplasia

1.1.3. Prevalence and economic loss due to leg disorders

The prevalence of leg problems has been evaluated via many surveys throughout the world. A recent survey of 176 commercial broiler flocks in UK indicated poor locomotion problems in almost 28% birds and 3.3% broiler birds were unable to walk (Knowles, et al., 2008). A survey conducted in United States that represented 55% of the poultry industry demonstrated an annual loss of $80 to $120 million due to leg problems
which caused increase mortality, culling and condemnation of birds. Altogether, 1.1% mortality was attributable to leg problems and 2.1% birds were downgraded due to leg disorders (Morris, 1993).

A survey in Western Canada revealed an average incidence of birds with skeletal deformities of 1.72%, out of which 1.10% of birds were culled in the field and 0.62% were condemned or downgraded as carcass (Riddell & Springer, 1985). It therefore appears that the leg disorders are worldwide concern and are responsible for huge economic loss to the poultry industry. Consequently, it is essential to understand the pathophysiology of bone development to improve bone quality in poultry.

Improvement in bone growth and strength during the early period of life may counteract the leg problems and skeletal disorders (Kim et al., 2011). Proper skeletal development and lameness in poultry is under the influence of many nutrients and nutrient balance is important for skeletal development. Nutrition is one of the most important and relevant components of bone integrity in poultry. Vitamins are crucial ingredients of poultry feeds and the role of vitamin D₃ and its metabolites in bone development have been studied in detail in growing birds, however their involvement in embryonic bone development is ill studied.

1.1.4. Vitamin D

Nutrition plays a crucial role in maintaining skeletal integrity. The optimum concentrations of minerals and vitamins in commercial poultry diets are essential not only for preventing deficiency signs but also for good health and the productive performance of poultry (Edwards Jr, 2000; Zhu et al., 2010). Vitamin D₃ has a major role in calcium metabolism and is required
for bone development and egg shell formation (Ameenuddin et al., 1987; Shen et al., 1982). Vitamin D$_3$ is essential for the development and maintenance of a mineralized skeleton and its deficiency results in abnormal bone formation (Matsumoto et al., 1991; Riddell, 1981; T. Suda et al., 2003).

Vitamin D is a fat soluble vitamin and present in two forms; vitamin D$_2$ (ergocalciferol) and D$_3$ (cholecalciferol). Vitamin D$_3$ (cholecalciferol) is formed in the skin from 7-dehydrocholesterol upon exposure to ultraviolet light (290 to 315nm). Vitamin D$_3$ is then transported from the skin to general circulation by vitamin D binding proteins for further hydroxylation in liver and kidney to become biologically active (Holick, 1981; Holick et al., 1980; MacLaughlin et al., 1982).

Vitamin D$_3$ in liver is converted to 25-OHD$_3$ by the enzyme cholecalciferol 25 hydroxylase and in kidney 25-OHD$_3$ is converted to 1,25(OH)$_2$D$_3$ by 25-OH-1α hydroxylase enzyme (Figure 1.1). The biologically most active form of vitamin D$_3$ is 1,25(OH)$_2$D$_3$ involved in absorption of calcium and phosphorous in intestine and mineralisation of bone (DeLuca, 2004).
The biological effects of 1,25(OH)\(_2\)D\(_3\) are mediated through vitamin D receptor (VDR) in the target tissues. VDR is a member of the nuclear receptor superfamily and forms a heterodimeric complex with retinoic X receptor. This complex then interacts with vitamin D response elements that results in either activation or repression of transcription of genes and mRNA synthesis for proteins (Haussler et al., 1998; Jones et al., 1998). Additional recruitment of co-regulators controls the rate of transcription of genes by the VDR (Bikle, 2009, 2012).

Vitamin D\(_3\) (cholecalciferol) is supplied to poultry mainly through dietary sources, ultraviolet light or comes from yolk in case of young chicks. However, in modern poultry farming birds are reared indoors and devoid of exposure to UV light and hence conversion of 7 dehydrocholesterol to cholecalciferol may not occur at the required level leading to insufficiency.
of vitamin D$_3$ in birds maintained in environmentally controlled houses (Edwards Jr, 2000; Norman & Hurwitz, 1993; Panda et al., 2006; Soares et al., 1995). Vitamin D$_3$ is an important factor in calcium homeostasis and required for bone metabolism. It is well known that vitamin D$_3$ is critical for bone formation and deficiency of vitamin D$_3$ leads to rickets and osteomalacia (Reinhold & Naski, 2007).

1.1.5. **Role of vitamin D$_3$ and calcium metabolism in embryo**

During 6 to 8 days of incubation the vitamin D endocrine system tightly regulates calcium homeostasis in the embryo. The embryo absorbs vitamin D$_3$ from the yolk with activating hydroxylation occurring in the mesonephric kidney. In the initial stages of development 1,25(OH)$_2$D$_3$ mediates the uptake of yolk calcium by the yolk sac membrane. The demand of calcium for skeletal development increases as the incubation progresses. At this time, 1,25(OH)$_2$D$_3$ promotes the absorption of calcium from the shell via the chorioallantoic membrane. At the end of embryonic development excess calcium mobilized from the shell is transferred to the yolk for use after hatching (de Matos, 2008).

1.1.6. **Role of 1,25(OH)$_2$D$_3$ in embryonic development**

The role of vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ in chick embryonic development was demonstrated in studies which have shown that when hens were fed only 1,25(OH)$_2$D$_3$ as a dietary source of vitamin D$_3$, a normal embryonic growth did not occur in the fertile eggs of laying hens (Ameenuddin, et al., 1987; Ameenuddin et al., 1983; Hart et al., 1986; Henry & Norman, 1978; Sunde et al., 1978) and chick embryos exhibited short upper mandible and usually die at 18 to 19 day of embryonic life.
Improvement in embryonic survival was observed after injection of vitamin D₃, 25-hydroxyvitamin D₃, or 1,25(OH)₂D₃ in vitamin D deficient eggs before incubation (Ameenuddin, et al., 1983; Sunde, et al., 1978). Therefore, it appears that vitamin D₃ or its metabolites, or both, are necessary for normal chick embryonic skeletal development.

The in ovo administration of 1,25(OH)₂D₃ to vitamin D deficient embryos showed increase hatchability, body and bone weight; improved bone resorption and mineralisation of trabeculae (Narbaitz & Tsang, 1989) and restored calcium and phosphate levels (Narbaitz, et al., 1987) confirming that vitamin D₃ metabolite 1,25(OH)₂D₃ is not only required for calcium and phosphate metabolism but plays an important role during chick embryonic development.

In non-vitamin D deficient chick embryos, in ovo administration of 1,25(OH)₂D₃ produced osteoid matrix at the peripheral part of the tibial diaphysis and also induced hypercalcemia and hypophosphatemia (Narbaitz & Fragiskos, 1984) and increased bone γ-carboxyglutamic acid containing protein suggesting a role of 1,25(OH)₂D₃ in bone formation (Tsutsumi et al., 1985).

1.1.7. **Role of 1,25(OH)₂D₃ in preventing bone disorders**

In the past, extensive research has been conducted to evaluate the efficacy of vitamin D₃ metabolite 1,25(OH)₂D₃ to improve leg disorders and enhance bone development. Poultry diet supplemented with various levels of 1,25(OH)₂D₃ was found to alleviate the incidence and severity of bone disorders in growing birds, however the effects of 1,25(OH)₂D₃ in foetal and neonatal bone development is poorly studied.
In 3 to 5 week old broilers, studies were conducted to evaluate the dietary level of 1,25(OH)_2D_3 for prevention of tibial dyschondroplasia (TD) under practical conditions. The results of study showed that 1,25,(OH)_2D_3 decreased the incidence and severity of TD and increased bone ash at 6μg/kg both at week 3 and 5 of age (Roberson & Edwards Jr, 1996).

The findings of earlier study demonstrated that addition of 1,25(OH)_2D_3 at 5 or 10μg/kg in an imbalanced diet (low calcium, phosphorous and cholecalciferol) dramatically reduced the incidence and severity of TD and increased tibia bone ash (Edwards Jr, 1989, 1990; Rennie, et al., 1993). The reduction in development of rickets and TD was observed in broiler cockerels after 10μg/kg supplementation of 1,25(OH)_2D_3 in diet with adequate levels of Ca and cholecalciferol (Elliot & Edwards, 1997). Also, in turkey poult s addition of 1,25(OH)_2D_3 at 10μg/kg in diet decreased the incidence of rickets and increased the bone ash (Sanders & Edwards Jr, 1991). The effect of 1,25,(OH)_2D_3 on incidence of TD was examined in two (high and low line TD) lines of chickens selected for TD and it was observed that dietary supplementation of 1,25(OH)_2D_3 at 5 and 10μg/kg reduced the incidence of TD to 21% and 0% respectively in the high line TD chickens. These findings suggest that 1,25(OH)_2D_3 reduces the incidence of TD not only induced by dietary imbalances but also in lines that are genetically selected for TD (Mitchell et al., 1997; Thorp et al., 1993). Increasing concentrations of 1,25(OH)_2D_3 from 0 to 1μg/kg in the feed of 75 week old laying hens markedly improved the tibia breaking strength, weight and density (Frost et al., 1990).

Previous studies in Australia showed that broiler flocks show a high incidence of endochondral ossification defects at an early age with
significant reduction in bone ash and serum vitamin D₃ metabolite, 1,25(OH)₂D₃ concentrations (Vaiano et al., 1994). The results of this study suggests that either failure of maternal transfer of this metabolite or higher circulating levels of 1,25(OH)₂D₃ are required to enhance early bone maturation in young broiler chickens.

1.1.8. Vitamin D₃ requirement of poultry

According to the recommendation of National Research Council (NRC, 1994), the vitamin D₃ requirement for broiler from 0 to 8 weeks of age is 200ICU/ kg of feed. Studies conducted by eliminating UV light in chick rooms revealed that the requirement of vitamin D₃ in young broiler birds is much greater than the recommended level of NRC (Elliot & Edwards Jr, 1997; Kasim & Edwards Jr, 2000). In broilers up to 2 weeks of age with sufficient calcium and phosphorous concentrations the dietary vitamin D₃ requirement may be in the range of 35 -50μg/kg (1400-2000IU) of feed for cortical bone quality. However, after 14 days the vitamin D₃ requirement decreases to less than 20μg/kg (400IU) of feed (Whitehead et al., 2004). The quantitative requirement of vitamin D₃ for broilers is variable and based on the criteria under evaluation. The birds required 275ICU of vitamin D₃/kg of feed for growth, 552ICU for blood plasma calcium, 503ICU for bone ash, and 904ICU for prevention of rickets (Edwards Jr, 1999). The diversity in the requirement of vitamin D₃ is based on parameter evaluated or largely related to the assessment for optimum performance whereas the amount suggested by the NRC is sufficient to prevent the signs of vitamin D₃ deficiency. The NRC recommends daily 300IU of vitamin D₃ at 100grams of feed per hen for white egg breeders whereas for brown-egg layers at
110 grams of feed per hen daily vitamin D₃ requirement is 30IU (NRC, 1994).

1.1.9. **Maternal supplementation of vitamin D₃ and its effects on progeny**

The nutritional status of the hen is important for the optimum production and transfer of essential nutrients into the egg from dam. Vitamin D₃ concentration in the yolk of chicks is crucial for embryonic and post-natal growth and bone development and thus maternal nutrition influences the bone development and leg problems in progeny (Oviedo-Rondon 2006). There are reports of positive correlation between vitamin D₃ in hen diet and amount of vitamin D₃ content in the egg. In three different experiments, vitamin D₃ was fed to laying hen at a level of 26.6, 62.4 and 216 µg/kg and after 6 weeks the vitamin D₃ content in the yolk were 1.4, 3.4 and 23 µg/100 grams of yolk respectively. The level of 25-hydroxycholecalciferol (25-OHD₃) in the yolk per 100 grams were 0.5, 1.0 and 1.5 µg (Mattila et al., 1999). These results indicate that in the egg yolk vitamin D₃ and 25-OHD₃ increases as the vitamin D₃ content increases in the poultry feed. The increase in body weight and tibia ash was observed in the chicks that were hatched after feeding high levels (2000 or 4000 IU) of vitamin D₃ in the maternal diet. In addition, maternal supplementation of 3200 IU of vitamin D₃ significantly increased the body weight, tibia ash and lowered the incidence of TD and rickets (Atencio et al., 2005). The results of this study suggests that to produce healthy and heavy chicks, the requirement of vitamin D₃ in broiler breeder is considerably higher than NRC recommendations, particularly when birds are maintained in UV light free environment.
1.1.10. Role of the extracellular matrix in bone development

Bone is made up of a mixture of different cell types embedded in mineralised extracellular matrix (ECM). ECM provides structural integrity to the skeleton. ECM is responsible for maintaining the microenvironment of cells embedded in ECM and consists of both an inorganic and organic component (Heng et al., 2004). The inorganic component of bone ECM is mineralised calcium salts in the form of hydroxyapatite and carbonate is the most abundant (Hellmich & Ulm, 2003; Rey et al., 1991). In addition, other inorganic salts like manganese, potassium fluoride, phosphate and citrate are also present in significant quantities (Posner, 1985; Rey et al., 1995). The organic component of ECM is primarily composed of type I collagen along with other isoforms (Bjorn Reino, 1996), non-collagenous proteins (osteocalcin, osteonectin, osteopontin, osteoadherin, fibronectin, bone sialoproteins (BSP), thrombospondin and proteoglycans) (Young et al., 1992). Collagen type I promotes cell adhesion and cell maturation of primary periosteal osteoblast in vitro. The ECM consists of a variety of proteins which are regulated by 1,25(OH)_{2}D_{3} and influence mineralisation. These proteins include osteocalcin (Ducy et al., 1996; Lian et al., 1989), osteopontin (Reinholt et al., 1990), BSP (Gordon et al., 2007), and collagens (Landis, 1999).

1.1.11. Difference between avian and mammalian growth plate

In certain aspects the avian growth plate differs from the mammalian growth plate. The understanding of these differences is useful to understand the pathophysiology of skeletal disorders in poultry. Though the biochemical and cellular events both in avian and mammals are similar during bone
development, there are certain variations in the longitudinal growth. In mammals’ blood vessel invasion and bone resorption takes place simultaneously throughout the growth plate in a horizontal manner. Because cartilage proliferation and resorption takes place in parallel, the growth plate thickness in mammals remain relatively constant whereas the overall thickness of growth plates increases markedly in avian growth regions (Galotto et al., 1994; Riddell, 1981; Roach, 1997).

In avian species, the developing long bone contains a cartilaginous growth plate without a secondary centre of ossification until hatching. The only secondary ossification centres are found in the proximal and distal end of tibia and distal end of metatarsus. Compared to the mammalian growth plate, there are more cells in each zone of avian growth plate and metaphyseal blood vessels penetrate deeply into hypertrophic cartilage of avian growth plate (Pines, 2007; Riddell, 1981).

1.1.12. Medullary bone in hen

The bone biology of the hen changes dramatically at the onset of sexual maturity. Osteoblasts switch their function from forming lamellar cortical bone producing a woven bone called medullary bone (MB). The MB present in the bone marrow of long bones and a unique feature of mature birds and crocodiles, begins 2 weeks before laying initiation (Whitehead, 2004). The formation and maintenance of MB is associated with combined effects of sex hormones such as estrogen and androgen (Bloom, et al., 1942). During insufficient dietary calcium supply MB serves as a mobile source of calcium for egg shell formation. The reduction in estrogen levels in hens at lay reverse the osteoblastic activity to form a lamellar cortical bone. The MB
gradually disappears with the beginning of structural bone formation (Whitehead, 2004). The mineralisation in medullary bone matrix at oviposition is stimulated by 1,25(OH)\(_2\)D\(_3\) and adequate intestinal supply of Ca\(^{2+}\) (Dacke et al., 1993; Kaetzel Jr & Soares Jr, 1984; Newbrey et al., 1992).

1.1.13. Growth plate and 1,25(OH)\(_2\)D\(_3\)

The cartilaginous growth plate forms the basis of longitudinal bone growth. In the growth plate, chondrocytes proliferate then undergo hypertrophy and subsequently form a mineralised trabecular bone. Any anomalies in the chondrocyte developmental sequence lead to bone deformities or reduced bone growth (Nilsson et al., 2005). In animals and children the alterations in growth plate maturation are largely seen in rickets caused by vitamin D deficiency (Bouillon et al., 2008; Donohue & Demay, 2002). Vitamin D\(_3\) metabolite 1,25(OH)\(_2\)D\(_3\) plays a major role in chondrocyte development in the growth plate (Ornøy et al., 1978) through stimulating specific genes that promote chondrocyte differentiation into hypertrophic chondrocytes (Norman & Hurwitz, 1993). In avian and mammals cellular events in endochondral ossification are similar however there are differences in the longitudinal bone growth (Roach, 1997). It is well known that proliferation and differentiation of chondrocyte is under the influence of many factors. Vitamin D\(_3\) metabolites are found to play a major role in chondrocyte metabolism and hence are required for normal bone development (Farquharson & Jefferies, 2000).
1.2. **Mesenchymal stem cells (MSCs)**

Almost 40 years ago Friedenstein and Petrakova described adult MSCs as non-hematopoietic stem cells present in bone marrow and most connective tissues of the body (Friedenstein et al., 1966). In adult bone marrow there are two different populations of adult stem cells. The first fully characterised cells are the hematopoietic stem cells that maintain continuous production of blood cells. The second marrow resident population of stem cells are termed as bone marrow stromal cells/ mesenchymal stem cells (Short et al., 2003).

MSCs provide a source of multipotent stem cells and have the capacity to differentiate into many cell types of connective tissue origins like bone, fat, cartilage, muscle and other tissues (Gerson, 1999; Haynesworth et al., 1996; Majumdar et al., 1998; Minguell et al., 2001; Pittenger, et al., 1999; Pountos & Giannoudis, 2005; Prockop, 1997, 2002; Serafini & Verfaillie, 2006; Zuk et al., 2002). The bone marrow consists of a minimal fraction of nucleated cells (0.001-0.01%), however isolation and expansion of MSCs and further stimulation to differentiate into multiple lineages is achievable under appropriate *in vitro* conditions (Jiang et al., 2002; Kalervo Väänänen, 2005).

MSCs can be rather easily isolated from bone marrow and can also be expanded *in vitro*. Consequently, they have become a prime target for researchers for tissue regeneration and presently considered as a potential candidate for several clinical applications (Vater et al., 2011). In addition to bone marrow, MSCs can also be isolated from tissues including periosteum, trabecular bone, fat, synovium, skeletal muscle, scalp tissue, deciduous teeth, placenta and umbilical cord blood (Campagnoli et al., 2001; De Bari
et al., 2001; De Ugarte et al., 2003; Fukuchi et al., 2004; Fukumoto et al.,
2003; Jankowski et al., 2002; Miura et al., 2003; Nöth et al., 2002; Shih et
al., 2005).

MSCs has been isolated from numerous species including chicken (Khatri et
al., 2009), humans (Pittenger, et al., 1999), rat (Polisetti et al., 2010), mouse
(Pereira et al., 1995; Tropel, et al., 2004), cat (Martin et al., 2002), dog
(Kadiyala et al., 1997), calves (Bosnakovski et al., 2005) and giant panda
(Y. Liu et al., 2013).

1.2.1. Identification criteria for MSCs

To define the identity of MSCs following criteria is set by The International
Society for Cellular Therapy in their position statement (Dominici et al.,
2006).

1. Adherence to plastic - MSCs must attach to the plastic surface of tissue
culture flasks when cells maintained in culture medium.

2. More than 95% of MSCs population must express CD105, CD73 and
CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79α
or CD19 and HLA class II.

3. MSCs must differentiate into trilineage (osteogenic, adipogenic and
chondrogenic) in vitro.

1.2.2. Differentiation potential of MSCs

Differentiation of MSCs into multiple lineages has been studied in variety of
species in vitro.

The ability of MSCs to differentiate into many cell types (Figure 1.2.) make
them a suitable candidate for tissue engineering, especially for the repair
and regeneration of bone, cartilage and tendon (Baksh et al., 2004). The gold standard used to characterise the MSCs is their ability to differentiate in vitro into osteoblasts, adipocytes and chondrocytes.

Osteogenic differentiation of MSCs in cultures is induced by addition of supplements like dexamethasone, β-glycerol phosphate and ascorbic acid that are capable of inducing osteogenesis. Dexamethasone is a glucocorticoid hormone required for osteogenic differentiation and bone nodule formation in vitro (Malaval et al., 1994). β- glycerol phosphate provides a source of inorganic phosphate and is essential for mineralisation and mineralised development of bone (Bloom et al., 1942; Kaetzel Jr & Soares Jr, 1984). Ascorbic acid is necessary for synthesis of collagen and triggers the mineralisation of bone matrix (Khatri, et al., 2009; Malaval, et al., 1994).

Adipogenic differentiation of MSCs is achieved by media containing insulin, isobutylmethylxanthine (IBMX) and dexamethasone and this medium has shown to enhance the differentiation of chicken, human, rat and mouse MSCs into adipogenic lineage (Bai et al., 2013; da Silva Meirelles et al., 2006; Khatri, et al., 2009; Pittenger, et al., 1999). It is known that during adipogenic differentiation insulin and glucocorticoid are responsible to stimulate uptake of deoxyglucose, conversion of glucose to CO₂ and lipid accumulation and IBMX found to increase the number and specificity of insulin receptors.

Chondrogenic differentiation of MSCs has been achieved by using high density pellet or micro mass cultures by adding transforming growth factor-β to serum free medium (Petitte et al., 2004; Rashidi & Sottile, 2009).
Cellular differentiation of each lineage is characterised by histological staining, gene expression and morphological properties. Characterisation of osteogenic, adipogenic and chondrogenic differentiation by histological staining is confirmed by calcium deposition (Alizarin Red stain), intracellular lipid rich vacuoles (Oil Red O) and deposition of proteoglycan matrix (Alcian blue staining) respectively. The osteogenic specific genes, ALP, osteocalcin, osteopontin, are up regulated in differentiated cells. Expression of aP2 and PPAR-γ genes specific to adipogenic lineage are induced in fat cells during the adipogenic differentiation of cells whereas cells committed to chondrogenic lineage show the expression of collagen-II genes (Guilak et al., 2006; Halvorsen et al., 2001; Jaiswal et al., 1997; Khatri, et al., 2009; T. M. Liu et al., 2007; Pittenger, et al., 1999).

**Figure 1.2.** Stem cells commitment to various mesenchymal phenotypes. End stage commitment to specific cell type is co-ordinated by transcription factors. Runx2- lead the cells to osteoblastic phenotype, PPARγ2- activation give rise to adipocytes, Sox9- activates the cells towards chondrocytes, Myoblasts differentiation occurs by MyoD activation (Aubin, 2008).

### 1.2.3. Application of MSCs

For the last decade, MSCs have been used as a significant tool in regenerative medicine (Caplan & Bruder, 2001; He, 2005; Luo et al., 2005; Luu et al., 2007) and use of bone marrow derived MSCs transplantation is
emerging (Barry & Murphy, 2004; Mayhall et al., 2004; Weissman et al., 2001). Despite of this, potential of MSCs in clinical cases of bone and musculoskeletal diseases is well known. This is further demonstrated by allogenic MSCs transplantation without any gene defect into young osteogenesis imperfecta patients as a short successful treatment (Horwitz et al., 2002; Horwitz et al., 1999). In addition, MSCs are considered as potential tool in many orthopaedic conditions as bone marrow stromal cells were utilised to treat segmental bone defects and articular cartilage defects in clinical trials (Kang et al., 2004; Krampera et al., 2006; Luo, et al., 2005; Luu, et al., 2007; Quarto et al., 2001; Wakitani et al., 2002).

1.2.4. Commitment of MSCs to osteogenic lineage

There are two distinct pathways of osteoblast differentiation during embryonic life (Harada & Rodan, 2003; Kingsley, 2008; Luo, et al., 2005; Olsen et al., 2000; Ralston & de Crombrugghe, 2006). Differentiation of mesenchymal progenitor cells directly into osteoblast without any cartilaginous template is called as intramembranous ossification (flat bone of skull) whereas formation of cartilage scaffold by MSCs and its replacement by osteoblast to form bone is called as endochondral ossification (long bone) (Ducy et al., 2000; Karsenty, 1999; Olsen, et al., 2000; Reddi, 1997).

Bone is considered as a dynamic tissue that continuously undergoes remodelling and regeneration process throughout the life. The process of continuous breakdown and re-building of bone is called as bone remodelling. This dynamic process of bone remodelling is controlled by two key cells, hematopoietically derived osteoclast and mesenchymal derived
osteoblast (Harada & Rodan, 2003; Olsen, et al., 2000; Ralston & de Crombrugghe, 2006). The functions of osteoclast and osteoblast in bone mass or the equilibrium between osteoclast and osteoblast activities is maintained through various signalling molecules that include various transcription factors, matrix proteins, growth factors and hormones (He, 2005; Karsenty, 1999; X. Li & Cao, 2006; Luo, et al., 2005; Luu, et al., 2007; Reddi, 1997).

Committed preosteoblasts are present near the surface of bone and express ALP, an early marker of osteogenic differentiation (Karsenty, 1999; Luu, et al., 2007). Preosteoblasts are then differentiated into mature osteoblasts that undergo phenotypic changes. These changes includes, enlargement of the nucleus, golgi apparatus and extensive endoplasmic reticulum to support the secretion of extracellular matrix protein (collagen type I). Later on, the cell terminally differentiates to form an osteocyte. Mature osteocytes are responsible for the regulation of mineral deposition and provide mechanical support (Olsen, et al., 2000).

Osteoblast differentiation is divided into three distinct stages namely; proliferation, matrix maturation and mineralisation. The sequence of osteoblast differentiation and associated markers are shown in Figure 1.3. During each stages of differentiation osteoblasts express phenotype markers of stage specific differentiation. Up regulation of cell cycle associated genes demonstrates the proliferation phase, expression of early osteogenic markers (ALP) demonstrates the maturation phase. Finally mineralisation phase is demonstrated by the expression of late markers of osteogenic differentiation such as osteoclacin and osteopontin (Olsen, et al., 2000).
1.2.5. Effects of 1,25(OH)$_2$D$_3$ during osteogenic differentiation

The active form of vitamin D$_3$ is 1,25(OH)$_2$D$_3$ and is involved in mineral metabolism and bone development, however the role of 1,25(OH)$_2$D$_3$ during osteogenic differentiation of MSCs is not well defined. The effect of 1,25(OH)$_2$D$_3$ on osteoblasts is biphasic. 1,25(OH)$_2$D$_3$ either inhibits or stimulates the osteoblasts development or gene expression depending upon the proliferation or differentiation stage of osteoblasts (Gurlek et al., 2002). In rat marrow derived osteoprogenitor cells, 1,25(OH)$_2$D$_3$ has been shown to inhibit the proliferation of cells (Rickard et al., 1995). Treatment of rat calvaria cultures with 1,25(OH)$_2$D$_3$ during the proliferative phase inhibited cell proliferation and reduced collagen synthesis and ALP activity. In addition, 1,25(OH)$_2$D$_3$ inhibited osteocalcin expression and blocked mineralised nodule formation. In contrast, 1,25(OH)$_2$D$_3$ treatment to more mature osteoblasts stimulates osteoblast marker genes such as osteocalcin and osteopontin (Ishida et al., 1993; Owen et al., 1991; Sodek et al., 1995).

In human marrow stromal cells and the human osteoblast cell line (SV-HFO), 1,25(OH)$_2$D$_3$ was found to promote osteogenic differentiation by enhancing the expression of osteogenic markers such as ALP, osteocalcin
and increasing mineralisation (Beresford et al., 1994; van Driel et al., 2006), whereas 1,25(OH)₂D₃ inhibited cell proliferation and osteoblastic differentiation in rat marrow stromal cells (Atmani et al., 2002; Atmani et al., 2003). Also, in murine primary osteoblast cultures 1,25(OH)₂D₃ down regulated the expression of *Phex*, a marker of differentiated osteoblast and inhibited matrix mineralisation (Ecarot & Desbarats, 1999). The effect of 1,25(OH)₂D₃ on two different osteoblast populations isolated from mouse calvaria and cortical bone showed significant differences in effects of 1,25(OH)₂D₃ on mineral deposition and gene expression which were largely dependent on maturational stages of osteoblast and skeletal origin (Yang et al., 2013). A marked difference in the effects of 1,25(OH)₂D₃ on osteoblastic cells from human, rat and mouse were shown during bone formation suggesting species difference exists in response to 1,25(OH)₂D₃ (Y. Li et al., 2008).

Together, results of previous studies suggest that effects of 1,25(OH)₂D₃ are depend on culture conditions, maturational stages of osteoblast and cells from different skeletal origin responds differently to 1,25(OH)₂D₃ (Chen et al., 2013). The effects of 1,25(OH)₂D₃ during osteogenic differentiation of chicken MSCs is not well defined and there is paucity of literature on how 1,25(OH)₂D₃ modulates osteogenesis and therefore how it is affecting bone growth in avian species.
1.3. **Research aim and hypotheses**

The overall aim of the project was to examine the effect of vitamin D₃ metabolite 1,25(OH)₂D₃ at various concentrations during embryonic bone development.

- It was hypothesized that *in ovo* injection of 1,25(OH)₂D₃ would affect the proliferation of chondrocytes in the different zones of growth plate during chicken embryonic bone development.

- It was also hypothesized that osteogenic differentiation of MSCs in response to 1,25(OH)₂D₃ differ between mammalian and avian species.

1.4. **Objectives of study**

1. To examine the effects of active vitamin D₃ metabolite 1,25(OH)₂D₃ at low (0.12μM/5ng) and high (1.2μM/50ng) concentration through *in ovo* administration on embryonic survival, growth and bone development in broilers.

2. To study the effects of vitamin D₃ metabolite 1,25(OH)₂D₃ at low (2.4 nM) and high (24 nM) concentrations on osteoblast proliferation, differentiation and mineralisation during osteogenic differentiation of MSCs *in vitro* with special emphasis on two important aspects.

   **A.** Species difference- To compare the response of 1,25(OH)₂D₃ between avian and rodent MSCs during *in vitro* osteogenic differentiation.

   **B.** Strain difference- To examine the effects of 1,25(OH)₂D₃ on osteoblasts from three (commercial broiler, layer and SPF layer)
different strains of chicken MSCs during *in vitro* osteogenic differentiation.
Chapter 2

Effects of *in ovo* administration of 1,25(OH)$_2$D$_3$ on chicken embryonic growth and bone development

2.1. Introduction

The biologically most active form of vitamin D$_3$ is 1, 25 dihydroxycholecalciferol (1,25(OH)$_2$D$_3$) involved in absorption of calcium and phosphorous in intestine and mineralisation of bone (DeLuca, 2004). It is well documented that dietary supplementation of 1,25(OH)$_2$D$_3$ reduces the incidence and severity of bone disorders in broilers (Edwards Jr, 1989, 1990; Rennie & Whitehead, 1996; Rennie, et al., 1993; Roberson & Edwards Jr, 1996). However, there is paucity of literature on the role of vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ during bone cell growth and differentiation during chicken embryonic development.

Previous studies have shown that *in ovo* administration of 1,25(OH)$_2$D$_3$ to vitamin D deficient embryos resulted in an increase in hatchability, body weight and bone weight. It also resulted in improved bone resorption and mineralisation of trabeculae (Narbaitz & Tsang, 1989) and restored calcium and phosphate levels (Narbaitz, et al., 1987) confirming that vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ is not only required for calcium and phosphate metabolism but plays an important role during chick embryonic development.

In non-vitamin D deficient chick embryos, *in ovo* administration of 1,25(OH)$_2$D$_3$ produced osteoid matrix at the peripheral part of the tibial diaphysis, hypercalcemia and hypophosphatemia (Narbaitz & Fragiskos,
1984) and increased bone $\gamma$-carboxyglutamic acid containing protein which is important for bone formation (Tsutsumi, et al., 1985).

Normal bone growth, particularly of the weight bearing long bones is critical for good health and well-being in both broiler and layer flocks. Improving bone development in both production systems can prevent early production losses due to bone breakages in juvenile and young adults and mitigate welfare concerns. The level of vitamin D$_3$ stored in the yolk sac of chicks is crucial for further growth and development of bone (Oviedo-Rondon et al., 2006). In newly hatched chickens the level of 1,25(OH)$_2$D$_3$ is found to be higher from hatching to day 7 of age (Abbas et al., 1985).

Early age is an important predisposing factor for the development of leg disorders in young broiler chicks due to their higher growth rate and inability to adequately metabolise vitamin D$_3$ (Bradshaw et al., 2002). Previous study in Australia showed that in broiler flocks there was a high incidence of endochondral ossification defects at an early age (day 14) with significant reduction in bone ash and serum 1,25(OH)$_2$D$_3$ concentrations (Vaiano, et al., 1994). From this study it could be hypothesized that serum concentration of 1,25(OH)$_2$D$_3$ is likely to influence the incidence and severity of leg disorders in young broiler chickens.

In longitudinal bone, chondrocytes proliferate, undergo hypertrophy and subsequently form a mineralised trabecular bone. Any anomalies in the chondrocyte developmental sequence lead to bone deformities or reduced bone growth (Nilsson et al., 2005). Vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ plays a major role in chondrocyte development in the growth plate (Ornoy et al., 1978) through stimulating specific genes that promote chondrocyte
differentiation into hypertrophic chondrocytes (Norman & Hurwitz, 1993). It is well known that proliferation and differentiation of chondrocyte is under the influence of Vitamin D$_3$ hence it is required for normal bone development (Farquharson & Jefferies, 2000). It was hypothesized that the biologically most active form of vitamin D$_3$ 1,25(OH)$_2$D$_3$ would affect the proliferation of chondrocytes in the different zones of growth plate during chicken embryonic bone development.

To date, little work has been carried out to determine the critical levels of vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ for optimal bone health in chickens and how this metabolite modulates the bone growth during foetal and neonatal development. The objective of the present study was to examine the effect of 1,25(OH)$_2$D$_3$ through *in ovo* administration on embryonic survival, growth and bone development in broilers.
2.2. Materials and Methods

All procedures involving embryos were approved by the Animal Care and Ethics Committee of Charles Sturt University (Protocol number 12/002). The doses and time of vitamin D injections were selected on the basis of results of previous studies (Elaroussi et al., 1993; Narbaitz and Fragiskos 1984).

Fertile eggs (Breed: Ross 308) for this experiment were obtained from the Baiada hatchery (Farm 1311, Snaidero Road, Griffith, NSW 2680, Australia). The broiler breeder hen age from which eggs were obtained for this study were 37, 31 and 44 weeks for run 1, 2 and 3 respectively.

A total of 120 eggs (Ross 308) were purchased from the Baiada hatchery and incubated. The 120 eggs were equally allocated to two experimental groups, i) day 8 (n=60) and ii) day 11 (n=60).

The eggs in the day 8 group and the day 11 group were allocated to one of the following four treatments as described below (n=15 each).

- Control (no treatment) n=15
- Sham control- in ovo injection with Phosphate Buffer Saline (PBS) n=15
- Low vitamin D- in ovo injection with [0.12μM (5ng)/egg] n=15
- High vitamin D- in ovo injection with [1.2μM (50ng)/egg] n=15 (Appendix I)

The eggs from the day 8 group received in ovo injection on day 8 of embryonic development (incubation) and the eggs in day 11 group received in ovo injection on day 11 of embryonic development (incubation).
Both the groups of eggs injected on day 8 or day11, were placed in an automatic rolling incubator (GQF 1502 "SPORTSMAN” GQF Inc.) at 37°C with 45-60% relative humidity. At day 18 incubator rolling was stopped and embryos were dissected from their shells on day 19. These experiments were conducted in triplicates.

2.2.1. Vitamin D₃ solution preparation

Vitamin D₃ metabolite, 1,25(OH)₂D₃ (D1530, Sigma Aldrich) was dissolved in ethanol to make a stock solution. The stock solution then was diluted in PBS (10010, Gibco, Life Technologies) to give a final concentration of 0.12μM (5ng) and 1.2μM (50ng) for low and high vitamin D treatments respectively (Appendix I). The final concentration of ethanol (v/v) injected /egg for both low and high vitamin D group was 0.0001 and 0.001% of the final injection volume.

2.2.2. Vitamin D₃ injection

Eggs were injected with vitamin D₃ metabolite 1,25(OH)₂D₃ as previously described with some modifications (Elaroussi et al., 1993; Narbaitz et al., 1987). Briefly at day 8 or day 11 of embryonic development eggs were removed from incubator, candled to outline the air cell with pencil for injections. The injection site was sterlised with 70% ethanol before injection. 100μl of 5ng or 50ng of 1,25(OH)₂D₃ was administered into the yolk of each egg using a 23 gauge needle. A silicon sealant was used to seal the injection site and eggs were returned to the incubator (Appendix VI).
2.2.3. **Analysis of viability percentage, gross examination, body weight and bone length of chicken embryo**

The percentage viability was determined on day 19 using the following formula,

\[ \text{Viability (\%) = \frac{\text{Number of viable embryos}}{\text{Number of fertile eggs injected}}} \times 100. \]

At day 19, the embryos were sacrificed and the number of non-viable embryos were recorded. Gross examination of viable embryos was performed and any abnormalities were recorded. The weight of embryos were recorded using an electronic weighing balance (Mettler Toledo®). Femur and tibial bones were separated from the embryo and the length of femur and tibia was measured using a ruler and recorded. Both bone samples were fixed in 4% paraformaldehyde (PFA) and stored at 4°C for later histological analysis (Appendix II).

2.2.4. **Histological analysis**

The bone samples were fixed in 4% PFA for 48 hours before removal to 70% ethanol (Appendix II). Six left tibiae from each group were decalcified for two hours in 5% formic acid solution (Appendix II) for histological and morphometric analysis (King et al., 1991; D. Liu et al., 2003) (Appendix V).

Hematoxylin and Eosin (H & E) stained sagittal sections of tibia were examined using standard light microscopy to measure the length (μm) of three different zones of growth plate i.e proximal and distal tibia: resting zone (Small, dense chondrocytes above the proliferating chondrocytes), proliferative zone (proliferative chondrocytes are arranged into columns...
with flattened and oblate sheproid shape) and hypertrophic zone (Swollen, mature and hypertrophic chondrocytes below proliferating zone) at three different intervals (Figure 2.1.). The mean of three measurements was considered as the zone length (King, et al., 1991). The length of each zone was compared between treatment groups to determine the effect of 1,25(OH)₂D₃ administration in ovo. Diaphysis width of tibia was also measured at the centre of bone section and compared among different treatment groups.

![Figure 2.1](image)

**Figure 2.1.** Measurement of each zone length of tibia growth plate at three different intervals (1, 2 and 3). The mean of three measurements of each zone was considered as the zone length (H & E; scale bar 500μm).

### 2.2.5. Statistical analysis

Statistical analysis was done by SPSS for Windows (version 20, IBM Chicago, IL, USA). A significance level of \( p < 0.05 \) was used to identify differences that were statistically significant and \( p < 0.10 \) are included to
show differences approaching significance. The data for all variables measured were analysed using generalised linear models with breeder age as a covariate, as the breeder age differed between the runs and treatment, run and their interaction as fixed effects. Factors included in the model were run, treatment and the interaction of treatment with run. When the analysis of variance suggested a significant treatment, treatment*run effect for a variable, the means were compared with Fischer’s least significance difference test. There was no treatment*run interaction effect for any of the variables measured.
2.3. Results

2.3.1. *Embryo viability is not affected by in ovo injection of 1,25(OH)$_2$D$_3$*

*In ovo* injection of, 1,25(OH)$_2$D$_3$ on either day 8 or day 11 of embryonic growth, did not reveal any significant difference in embryo viability % at day 19 between treatment groups (Table 2.1). However injection of 1,25(OH)$_2$D$_3$ on day 11 showed a trend towards decrease in viability % in high vitamin D compared to control ($p=0.057$) and low vitamin D group ($p=0.067$).

Table 2.1. Embryo viability % at day 19 of experiment after administration of vitamin D$_3$ metabolite, 1,25(OH)$_2$D$_3$ in ovo on day 8 or day 11 of embryonic development.

<table>
<thead>
<tr>
<th>Day of injection</th>
<th>Treatment group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sham control</td>
</tr>
<tr>
<td>Day 8 – embryo viability %</td>
<td>(45/45) 100±0.00</td>
<td>(40/44) 90.79±4.6</td>
</tr>
<tr>
<td>Day 11-embryo viability %</td>
<td>(36/39) 91.85±4.1$^A$</td>
<td>(36/42) 85.00±5.35</td>
</tr>
</tbody>
</table>

Numbers in bracket indicate total number of viable embryos/number of fertile eggs injected. Values are Mean ± SEM (average of three runs). $^A$ trend for significant difference – $p=0.057$ between Control & High vitamin D group; $p=0.067$ between Low vitamin D and High vitamin D group.

2.3.2. *No gross abnormalities were evident in the chick embryo by in ovo injection of 1,25(OH)$_2$D$_3$*

On terminal sacrifice (Day 19), chick embryos from all treatment groups were examined for any gross abnormality. There were no spontaneous or
treatment related gross abnormalities seen externally after *in ovo* injection of 1,25(OH)$_2$D$_3$ on day 8 or day 11 of embryonic development.

### 2.3.3. Body weight of chicken embryo was increased on day 19 in day 8 injection group whereas body weight was decreased on day 19 in day 11 injection group

Body weight of embryos for groups, day 8 and day 11 were taken on day 19 and the results are shown in Figure 2.2. The $p$ values of the ANOVA for day 8 and day 11 group of *in ovo* injections are 0.215 and 0.030 respectively. Mean body weights of chicken embryo for the day 8 group were significantly increased in sham control ($p=0.048$) and low vitamin D ($p=0.048$) compared to control group. For the day 11 group, the body weights of embryos in the low vitamin D group were significantly lower than sham control ($p=0.008$) and high vitamin D group ($p=0.017$; Figure 2.2.).

**Figure 2.2.** Overall effect of 1,25(OH)$_2$D$_3$ treatment on body weights of chicken embryo after *in ovo* injection of 1,25(OH)$_2$D$_3$ on day 8 or day 11. Values are Mean ± SEM (average of three runs). Comparison between treatment marked with an asterisk (*) are significantly different at $p<0.05$ based on linearly independent pairwise comparisons among estimated marginal means. Control (no injection), Sham control (only PBS), Low vitamin D [0.12μM (5ng)/egg] and High vitamin D [1.2μM (50ng)/egg].
2.3.4. **Femur length of chick embryo was reduced on day 19 by low doses of** 1,25(OH)$_2$D$_3$ **on day 11 of incubation**

To determine the effect of 1,25(OH)$_2$D$_3$ on bone length, fertile eggs were injected with or without 1,25(OH)$_2$D$_3$ on day 8 or day 11 of embryonic development and the length of femur and tibia was measured on day 19 of the experiment.

The results of femur length are shown in Figure 2.3. No significant difference was observed in femur length between treatment groups injected with 1,25(OH)$_2$D$_3$ on day 8 (ANOVA $p$ value =0.459). Femur length was significantly decreased in the low vitamin D ($p$=0.014; Figure 2.3) compared to sham control group after injecting 1,25(OH)$_2$D$_3$ on day 11 (ANOVA $p$ value =0.072 ). There was a trend towards increase in femur length in sham control ($p$=0.052) compared to control group of the day 11 group of *in ovo* injection.

![Figure 2.3](image)

**Figure 2.3.** Effect of vitamin D$_3$ metabolite,1,25(OH)$_2$D$_3$ treatment on femur length of chicken embryo after *in ovo* injection of 1,25(OH)$_2$D$_3$ on day 8 or day 11 of embryonic development. Values are Mean ± SEM (average from three runs). Comparison between treatment marked with an asterisk (*) are significantly different at $p$<0.05 based on linearly independent pair wise comparisons among estimated marginal means. Control (no
injection), Sham control (only PBS), Low vitamin D \([0.12 \mu M (5ng)/egg]\) and High vitamin D \([1.2 \mu M (50ng)/egg]\).

2.3.5. **Low dose of 1,25(OH)\(_2\)D\(_3\) injection decreased tibia length on day 19 in chick embryos injected on day 11 of incubation whereas tibia length was increased in sham control and low vitamin D group injected on day 8 of incubation**

Length of tibia was measured on day 19 of the experiment and results are shown in Figure 2.4. The ANOVA \(p\)-values for day 8 and day 11 injection group are 0.059 and 0.170 respectively. Tibia length of chicken embryo for the day 8 group was significantly increased in sham control \((p=0.038)\) and low vitamin D \((p=0.012)\) compared to control group. Length of tibia was significantly lower in low vitamin D as compared to sham control group chicken embryos injected on day 11 \((p=0.033;\) Figure 2.4.\).

**Figure 2.4.** Tibia length of chicken embryos on day 19 of experiment injected with vitamin D\(_3\) metabolite, 1,25(OH)\(_2\)D\(_3\) *in ovo* on day 8 or 11 of embryonic development. Values are Mean ± SEM (average of three runs). Comparison between treatment marked with an asterisk (*) are significantly different at \(p<0.05\) based on linearly independent pairwise comparisons among estimated marginal means. Control (no injection), Sham control (only PBS), Low vitamin D \([0.12 \mu M (5ng)/egg]\) and High vitamin D \([1.2 \mu M (50ng)/egg]\).
2.3.6. **Treatment with exogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} did not have an effect on proximal tibia resting zone, proliferative zone and hypertrophic zone length of embryonic chicken tibia**

The effect of vitamin D\textsubscript{3} metabolite, 1,25(OH)\textsubscript{2}D\textsubscript{3} on length of three different zones of proximal and distal end of tibia was analysed on day 19 of experiment after *in ovo* injection of 1,25(OH)\textsubscript{2}D\textsubscript{3} on day 8 or day 11 of embryonic development. The proximal and distal tibia was examined and the length of three zones was determined: 1) resting, 2) proliferative and 3) hypertrophic zone (Figure 2.5. and 2.6.). Each zone is described below in detail:

1) The resting zone is situated just below the epiphysis. Chondrocytes progenitors in this zone are spherical in shape with limited or no proliferation potential.

2) The proliferative zone contains flattened, differentiated chondrocytes with oblate spheroid shape arranged in columns. In this zone, the chondrocyte divides and amount of chondrocyte production determines the tubular bone length.

3) The hypertrophic zone contains large, spherical chondrocytes which produces extracellular matrix for calcification (Aziz et al., 2005; Farquharson & Jefferies, 2000; D. Liu, et al., 2003).
The length of proximal tibia resting zone was analysed on day 19 of experiment. There were no significant differences observed in the length between treatment groups after injection of 1,25(OH)₂D₃ on day 8 or day 11 of embryonic development and the results are shown in Table 2.2. However, for the day 8 group of in ovo injection, a trend towards increase in proximal tibia resting zone length was observed in control compared to low vitamin D group (p=0.083).

Chick embryos injected with various doses of 1,25(OH)₂D₃ on day 8 or day 11 of embryonic development did not reveal significant difference in

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**Figure 2.5.** Photomicrograph of a longitudinal section of tibia from a 19 day old chicken embryo showing the three different zones of growth plate measured in this study (H & E; scale bar 500μm).

**Figure 2.6.** Chondrocytes within three different zones of growth plate of embryonic chick tibia (H & E; scale bar 200μm). Abbreviations: A, resting zone; B, proliferative zone; C, hypertrophic zone.
proximal tibia proliferative zone length of 19 day old chicken embryonic tibia (Table 2.2.).

Tibia of 19 day old chicken embryo did not reveal significant difference in the hypertrophic zone length of proximal tibia after *in ovo* injection on day 8 or day 11 of embryonic development (Table 2.2.).
Table 2.2. The length (µm) of different zones of proximal tibia (x5) from 19 day old chicken embryo injected with or without 1,25(OH)\textsubscript{2}D\textsubscript{3} on day 8 or day 11 of embryonic development.

<table>
<thead>
<tr>
<th>Day 8 of in ovo injection-Proximal tibia zone length-µm</th>
<th>Treatment groups</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sham control</td>
</tr>
<tr>
<td>Resting zone</td>
<td>1035.62 ±27.03\textsuperscript{A}</td>
<td>991.82 ±24.26</td>
</tr>
<tr>
<td>Proliferating zone</td>
<td>1180.44 ±22.20</td>
<td>1144.80 ±26.67</td>
</tr>
<tr>
<td>Hypertrophic zone</td>
<td>5949.05 ±200.99</td>
<td>5676.38 ±162.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 11 of in ovo injection-Proximal tibia zone length-µm</th>
<th>Treatment groups</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sham control</td>
</tr>
<tr>
<td>Resting zone</td>
<td>966.30 ±36.90</td>
<td>994.08 ±36.08</td>
</tr>
<tr>
<td>Proliferating zone</td>
<td>1148.42 ±28.23</td>
<td>1122.21 ±28.21</td>
</tr>
<tr>
<td>Hypertrophic zone</td>
<td>6211.62 ±180.17</td>
<td>6236.24 ±186.97</td>
</tr>
</tbody>
</table>

\textsuperscript{A} trend for significant difference for the day 8 in ovo injection group in proximal tibia resting zone length- p=0.083 between Control & Low vitamin D group. n=18 in each group. Values are Mean ± SEM (average of three runs). Control (no injection), Sham control (only PBS), Low vitamin D [0.12µM (5ng)/egg] and High vitamin D [1.2µM (50ng)/egg].
2.3.7. Length of distal tibia resting zone, proliferative zone and hypertrophic zone was not altered by in ovo injection of $1,25(OH)_2D_3$

Growth plate morphology of distal tibia was evaluated in 19 day old chicken embryos and the results are shown in Table 2.3. Injection of $1,25(OH)_2D_3$ on day 8 or day 11 of embryonic development did not have significant effect on distal tibia resting zone length. However, in the day 8 injected group a trend towards increase in proximal tibia resting zone length was observed in sham control ($p=0.085$) and high vitamin D ($p=0.072$) compared to control group (Table 2.3.). Similarly, in the day 11 injected group a trend towards increase in resting zone length was observed in high vitamin D compared to control group ($p=0.097$; Table 2.3.).

There were no significant differences revealed in the proliferative zone length of 19 day old chicken embryonic tibia after in ovo administration of $1,25(OH)_2D_3$ on day 8 or day 11 of embryonic development (Table 2.3.).

Tibia of 19 day old chicken embryo did not reveal significant difference in the hypertrophic zone length after in ovo injection of vitamin D$_3$ metabolite $1,25(OH)_2D_3$ both on day 8 or day 11 (Table 2.3.). However, in the day 11 group a trend towards increase in distal tibia hypertrophic zone length was observed in sham control ($p=0.097$) and low vitamin D ($p=0.086$; Table 2.3.) compared to high vitamin D group.
Table 2.3. Length of different zones (µm) of distal tibia growth plate (x5) from 19 day old chicken embryo injected with or without 1,25(OH)₂D₃ on day 8 or day 11 of embryonic development.

<table>
<thead>
<tr>
<th>Day 8 of <em>in ovo</em> injection- Distal tibia zone length- µm</th>
<th>Treatment groups</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sham control</td>
<td>Low vitamin D</td>
<td>High vitamin D</td>
<td><em>p</em>-value</td>
<td></td>
</tr>
<tr>
<td>Resting zone</td>
<td>1282.22 ±26.22^A</td>
<td>1361.37 ±34.04^A</td>
<td>1354.76 ±33.96</td>
<td>1364.97 ±32.68^A</td>
<td>0.220</td>
<td></td>
</tr>
<tr>
<td>Proliferating zone</td>
<td>1119.11 ±26.79</td>
<td>1105.02 ±29.25</td>
<td>1083.79 ±26.30</td>
<td>1108.38 ±32.81</td>
<td>0.796</td>
<td></td>
</tr>
<tr>
<td>Hypertrophic zone</td>
<td>5319.06 ±242.24</td>
<td>5244.79 ±133.50</td>
<td>5430.08 ±141.69</td>
<td>5375.66 ±156.43</td>
<td>0.893</td>
<td></td>
</tr>
</tbody>
</table>

| Day 11 of *in ovo* injection- Distal tibia zone length- µm | Treatment groups |  |  |  |  |  |
|----------------------------------------------------------|------------------|------------------|------------------|------------------|------------------|
|                                                         | Control         | Sham control     | Low vitamin D    | High vitamin D   | *p*-value        |
| Resting zone                                            | 1338.47 ±25.00^A| 1398.64 ±24.73   | 1363.03 ±35.13   | 1415.10 ±40.06^A| 0.333            |
| Proliferating zone                                       | 1085.93 ±40.65  | 1063.13 ±22.67   | 1065.51 ±38.57   | 1102.55 ±27.26   | 0.770            |
| Hypertrophic zone                                        | 5421.65 ±158.05 | 5492.93 ±160.44^A| 5502.12 ±216.64^A| 5106.51 ±121.73^A| 0.267            |

^A trend for significant difference for the day 8 *in ovo* injection group, Distal tibia resting zone length- Control and Sham control (*p*=0.085), Control and High vitamin D (*p*=0.072); for the day 11 *in ovo* injection group, Distal tibia resting zone length: Control and High vitamin D (*p*=0.097), Distal tibia Hypertrophic zone length- High vitamin D and Sham control (*p*=0.097), High vitamin D and Low vitamin D (*p*=0.086). Values are Mean ± SEM (average of three runs). *n*=18 in each group. Control (no injection), Sham control (only PBS), Low vitamin D [0.12µM (5ng)/egg] and High vitamin D [1.2µM (50ng)/egg].
2.3.8. \textit{1,25(OH)\textsubscript{2}D\textsubscript{3} injection on day 11 showed a trend towards increase in tibia diaphysis width of chick embryos}

Tibia diaphysis width was measured in the centre of longitudinal section of 19 day old chick embryonic tibia after \textit{in ovo} injection of vitamin D\textsubscript{3} metabolite 1,25(OH)\textsubscript{2}D\textsubscript{3} on day 8 or day 11 of embryonic development. There was no significant difference observed in the width of tibia diaphysis in all treatment groups on day 8 of \textit{in ovo} injection. However, in day 11 group of \textit{in ovo} injection the diaphysis width was significantly increased in sham control compared to control group ($p=0.010$) and trend towards increase in tibia diaphysis width was observed in high vitamin D compared to control group ($p=0.080$; Table 2.4.).

\textbf{Table 2.4.} Effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} on tibia diaphysis width (\textmu m) evaluated by morphometric analysis (x5). Chicken embryos injected with or without 1,25(OH)\textsubscript{2}D\textsubscript{3} on day 8 or 11 of embryonic development and diaphysis width was measured on day 19 of experiment.

<table>
<thead>
<tr>
<th>Tibia diaphysis width- \textmu m</th>
<th>Treatment groups</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sham control</td>
</tr>
<tr>
<td>Day 8 of \textit{in ovo} injection</td>
<td>1375.85 ±32.94</td>
<td>1440.40 ±29.11</td>
</tr>
<tr>
<td>Day 11 of \textit{in ovo} injection</td>
<td>1335.24 ±47.79\textsuperscript{AB}</td>
<td>1468.20 ±32.34\textsuperscript{B}</td>
</tr>
</tbody>
</table>

\textsuperscript{A} trend for significant difference in tibia diaphysis width of day 11 group ($p=0.074$) between Control and High vitamin D ($p=0.080$).

\textsuperscript{B} significant difference in tibia diaphysis width between Control and Sham control ($p=0.010$) group of day 11 \textit{in ovo} injection. n=18. Values are Mean ± SEM (average of three runs). Control (no injection), Sham control (only PBS), Low vitamin D [0.12\textmu M (5ng)/egg] and High vitamin D [1.2\textmu M (50ng)/egg].
2.4. Discussion

The key aspect of this study was to examine the in ovo effects of 1,25(OH)$_2$D$_3$ at 0.12µM (5ng) and 1.2µM (50ng) either on day 8 or day 11 of embryonic development on viability, body weight and bone development in commercial broiler strain of chicken. The results of the study shows that administration of 1,25(OH)$_2$D$_3$ had a significant influence on the above parameters measured.

2.4.1. Viability of chicken embryo was not affected by 1,25(OH)$_2$D$_3$

The results of present study demonstrated that in ovo injection of 1,25(OH)$_2$D$_3$ on day 11 of embryonic development showed a trend towards decrease in viability of chicken embryos injected with high doses [1.2µM (50ng)] of 1,25(OH)$_2$D$_3$ suggesting the higher concentration of 1,25(OH)$_2$D$_3$ could be lethal and do not meet the requirements for embryo survival. These results are in agreement with (Moriuchi & Deluca, 1974) who observed that 32 pmoles of 1,25(OH)$_2$D$_3$ were well tolerated by chicken embryos whereas 70% mortality was observed when 9 day old embryos were injected with 325 pmoles of 1,25(OH)$_2$D$_3$. In a preliminary study for dose selection, a high embryo mortality was observed in chicken embryos injected with 750 pmoles of 1,25(OH)$_2$D$_3$ on 15$^{th}$ day of incubation but in a confirmatory study 70, 150 and 300 pmoles of 1,25(OH)$_2$D$_3$ showed survival rates similar to control group embryos (Narbaitz & Tolnai, 1978). The low and high doses of 1,25(OH)$_2$D$_3$ used in the present study were 120,000 (0.12µM) and 120,000 pmoles (1.2µM) respectively which are almost 400 to 4000 times higher than Narbaitz & Tolnai’s study. Though the doses used in this study appear to be higher than doses used by Narbaitz & Tolnai’s study, 43%
mortality of chick embryo was reported by 100ng doses of 1,25(OH)2D3 when injected on day 9 and 10 of incubation (R. Narbaitz and B. Fragiskos 1984). Altogether the findings of previous studies suggest that doses of 1,25(OH)2D3 below 300pmoles are well tolerated by chicken embryos and increasing concentration does not support embryonic survival. Therefore, it is likely that the high doses of 1,25(OH)2D3 will reduce the survival rate of embryos but interestingly the low dose in the present study did not affect the viability percentage of chicken embryos. The probable explanation for this may be the low doses of 1,25(OH)2D3 are rapidly metabolised and inactivated whereas high doses has resulted into hypercalcemia due to mobilisation of shell calcium into circulation and thereby reduction in embryo survival.

In this study vitamin D3 metabolite, 1,25(OH)2D3 was dissolved in ethanol and the final concentration of ethanol (v/v) injected for both low and high vitamin D group/egg was 0.0001 and 0.001%. Also, in previous studies 1,25(OH)2D3 have been dissolved in absolute ethanol for in ovo injection and showed no adverse effects of ethanol (Narbaitz and Fragiskos 1984., Narbaitz and Tsang 1989). Therefore, the effects observed in response to vitamin D treatment could not be attributed to ethanol.

In the previous studies, ethanol, dimethysulphoxide (DMSO) and phosphate buffer saline (PBS) has been used as a diluent for injection during in vivo studies (Elaroussi et al., 1993; Kai et al., 2009; Roberto Narbaitz & Fragiskos, 1984; R Narbaitz & Tsang, 1989; Rochford et al., 2013). In the present study, vitamin D3 metabolite 1,25(OH)2D3 was dissolved in ethanol with a very minimal quantity to make final stock solutions. The stock solution was further diluted with sterile PBS for in ovo injection (Refer
appendix I). Therefore, in this study PBS was used as a vehicle to inject the sham control group and PBS is well accepted diluent for in vivo studies (Kai et al., 2009; Rochford et al., 2013).

Data of present study indicate that the time window selected for in ovo injection was appropriate and only higher doses of 1,25(OH)₂D₃ could be unfavourable for embryo survival.

2.4.2. In ovo injection of 1,25(OH)₂D₃ did not produce developmental anomalies in chicken embryo

In the present study there were no gross abnormalities detected on terminal sacrifice (Day 19) in the embryos injected with various concentrations of 1,25(OH)₂D₃ which is in agreement with the previous findings (Narbaitz, et al., 1987). But earlier studies have also shown that replacement of 1,25(OH)₂D₃ for vitamin D₃ in the diet of hens significantly impaired the hatchability of eggs (Henry & Norman, 1978; Sunde, et al., 1978) and caused abnormalities of upper mandible of the embryos hatched from such hens (Sunde, et al., 1978).

It is well known that metabolism of vitamin D₃ is important during embryonic development. The enzyme 1-α hydroxylase is required for the conversion of 25-OHD₃ to active metabolite 1,25(OH)₂D₃. This enzyme is present as early as day 12 of embryonic life and its concentration increases as growth progress (Turner et al., 1987). In vitamin D deficient embryos due to lack of 1-α hydroxylase enzyme formation of 1,25(OH)₂D₃ is not adequate. As a result calcium is not mobilised from shell and transported across chorioallantoic membrane and calcium deficiency leads to reduced

The exact cause of malformations in mandible development is unclear however; poor calcium transport or defect in collagen synthesis may be involved (Sunde, et al., 1978). Therefore it seems that the eggs utilized in present study had adequate vitamin D₃ and or vitamin D₃ metabolites from maternal sources and calcium in the circulation of chick embryo to support normal survival and proper skeletal development.

2.4.3. In ovo injection of 1,25(OH)2D3 reduced body weight of embryos injected with low dose of vitamin D on day 8 of incubation whereas body weight of embryos was increased in sham control and low vitamin D group injected on day 11 of incubation.

The results of present study showed that injection of 1,25(OH)₂D₃ when administered on day 11 of embryonic development at 0.12μM (5ng) dose retarded the body weight of chicken embryo.

In day 8 injected groups, body weight was significantly increased in sham control and low vitamin D compared to control group. However, there were no significant differences observed in body weights of low and high vitamin D compared to sham control group. Hence, increase in the body weights observed in the sham control and low vitamin D group do not corroborate the treatment effect. The exact cause of increased body weight in sham control and low vitamin D group could not be ascertained.

Previous studies reported an improvement in body weight of embryos after injecting 10ng of calcitriol (1,25(OH)₂D₃) on day 14 of incubation to
vitamin D deficient embryos whereas 100ng of calcitriol caused a reduction
in the body weight (Narbaitz & Tsang, 1989). The lower dose of calcitriol
positively improved the body weights in vitamin D deficient embryos which
could be a result of stimulation of bone resorption and increased
mineralisation of bone. Whereas a high dose of calcitriol has failed to
improve bone, muscle weight and bone resorption which could have
resulted in lower body weights of chicken embryo.

In the present study, the eggs were sourced from vitamin D fed parent flock
for experiment and would have adequate levels of vitamin D₃ metabolite
sufficient for normal embryonic growth and development. However,
additional in ovo injection on day 11 at 5ng of 1,25(OH)₂D₃ reduced the
body weight which could be due to hypercalcemia as a result of increase
calcium mobilisation from shell, reduction in plasma phosphorus levels due
to increase absorption of calcium. The findings of present study cannot be
compared with previous literature.

In vitamin D deficient embryos the cause of retarded growth is not fully
understood but is influenced by Somatomedin C, a main factor responsible
for regulating bone and muscle growth. However, other factors such as
thyroid hormones, corticoids and vitamin D and or calcium could contribute
to the embryo growth control (Scanes et al., 1984).

In the present study, the test system did not use embryos that were vitamin
D deficient and it was found that additional doses of 1,25(OH)₂D₃ used did
not improve the body weights of chicken embryo.

Injection of vitamin D₃ or its metabolite during the developmental stages of
vitamin D deficient embryos corrects the hatchability and growth rate. This
confirms the role of vitamin D₃ or its metabolite that are primarily required if eggs are vitamin D deficient. On the other hand the results of present study suggests that if the eggs are not vitamin D deficient or adequate in vitamin D₃ or its metabolite, additional doses of 1,25(OH)₂D₃ do not influence the survival and body weight of chicken embryo. But further studies using lower as well as higher doses of 1,25(OH)₂D₃ than the doses used in the current study are required to ascertain whether the different doses of vitamin D₃ metabolite 1,25(OH)₂D₃ are capable of improving the viability and growth rate of chicken embryo or producing signs of toxicity.

2.4.4. **On day 19 bone length was reduced by low dose of 1,25(OH)₂D₃ injected on day 11 of incubation**

The results of the present study showed that *in ovo* injection of 1,25(OH)₂D₃ on day 11 of embryonic development at 0.12μM (5ng) dose reduced femur as well as tibia length of chicken embryo and this finding is well correlated with decrease in body weights of low vitamin D group chicken embryo. The improvement in tibia length was observed after *in ovo* injection of 1,25(OH)₂D₃ on day 8 of embryonic development in sham control and low vitamin D [0.12μM (5ng)] compared to control group. This increase in tibia length is also concurring with corresponding increase in body weight of these groups. Moreover, in day 8 group of *in ovo* injection there were no significant differences observed in the tibia length between sham control, low and high vitamin D group.

Previous study in 17 day old white leghorn embryos injected on day 15 with 300pmoles of 1,25(OH)₂D₃ reduced tibia length and weight (Narbaitz & Tolnai, 1978). Though the net effect of 1,25(OH)₂D₃ on tibia length in both
studies was similar, the dose in the present study that has reduced the tibia length was 120,000 pmoles (0.12µM) which is 400 times higher and time of in ovo injection was different from the earlier reported study. The data of bone length in the present study suggests that in ovo injection of 1,25(OH)₂D₃ during embryonic development negatively affect the bone growth in chicken embryo.

2.4.5. Length of growth plate zones and diaphysis width of tibia was not affected by in ovo injection of 1,25(OH)₂D₃

Assessment of growth plate morphology is useful to determine bone cell growth (King, et al., 1991). In this study the effect of 1,25(OH)₂D₃ on bone cell growth was determined after in ovo injection of 1,25(OH)₂D₃ on day 8 or day 11 of embryonic development.

Histological examination of tibia from day 19 old chicken embryos revealed three distinctive zones namely resting, proliferating and hypertrophic zone with characteristics morphology of chondrocytes in each zone. Cartilage within the physis forms a basis of longitudinal bone growth as a result of chondrocyte proliferation, hypertrophy and synthesis of extracellular matrix. Once the cartilage is formed, it undergoes calcification, degradation and finally replaced by osseous tissue. These generative and degenerative processes occur at both the ends in the growth plate epiphysis. The growth of cartilage cells in the growth plate is regulated by many factors. Unlike mammals, the avian growth plate contains longer chondrocyte columns with random orientation and more cells in each zone. Blood vessels penetrate deeply into growth plate and in hypertrophic zone the cells are not apparent in columns (Pines & Hurwitz, 1991). Disturbances in cellular responses and
metabolism of chondrocytes can lead to the dysplasias of growth plate in poultry (Lian et al., 2003).

In the present study, *in ovo* administration of vitamin D₃ metabolite 1,25(OH)₂D₃ on day 8 or day 11 of embryonic development did not affect the length of any zone of tibia growth plate at both the ends. However, for the day 8 or day 11 group of *in ovo* injection the resting zone length was approaching towards significance (increase or decrease) either in proximal or distal tibia in control group. In contrast, there was no significant difference in the length of resting zone length between sham control, low and high vitamin D at both ends of tibia growth plate for day 8 or day 11 group of *in ovo* injection. Also, a trend towards decrease in hypertrophic zone length was observed in high vitamin D compared to sham control and control group in the distal tibia of the day 11 group of *in ovo* injection. However, there were no differences observed in the length of hypertrophic zone length of proximal tibia in any of the treatment group for the day 11 group of *in ovo* injection suggesting the variations occurred in hypertrophic zone length of distal tibia than proximal tibia of high vitamin D in day 11 group of *in ovo* injection. It is unlikely that such variations could occur only either at the proximal or distal ends within the same long bone in avian species because the rate of longitudinal bone growth is related to the thickness of avian growth plate (Roach, 1997). Such variations in the growth rate of hypertrophic chondrocytes at both ends of same long bone are reported in mammalian species (Farquharson & Jefferies, 2000). The data of growth plate morphology in this study did not show the evidence of positive effect on length of different zones of both proximal and distal tibia...
after administration of $1,25\text{(OH)}_2\text{D}_3$ on day 8 or day 11 of embryonic development.

The tibia diaphysis width was increased in sham control group on day 11 of \textit{in ovo} injection and this finding is well correlated with the observed increase in body weight and tibia length of this group.

In high vitamin D group a trend towards increase in tibia diaphysis width was observed compared to control group however, no significant differences were observed in the tibia diaphysis width between sham control, low vitamin and high vitamin D group on day 11 of \textit{in ovo} injection. The exact mechanism by which diaphysis growth occurs is unclear. However, it is reported that in long bones the diaphysis grows through new formation of trabecular rows at the periphery of cortex and this is accompanied by resorption of old trabeculae at the endosteal side by osteoclasts (Fell, 1925).

It has been observed in 4 week old rat tibia that sub chronic (1μg/kg) and intermittent (3μg/kg) administration of $1,25\text{(OH)}_2\text{D}_3$ caused narrowing of growth plates affecting proliferation, maturation and apoptosis of chondrocytes suggesting that $1,25\text{(OH)}_2\text{D}_3$ impede endochondral ossification and bone development (Idelevich et al., 2011). This study has tested the effects of $1,25\text{(OH)}_2\text{D}_3$ on growth plate development during postnatal growth and development of rat and largely differs from our study which looked at the effect of $1,25\text{(OH)}_2\text{D}_3$ during chick embryonic development. To our knowledge there is a dearth of published data that describes the relationship between \textit{in ovo} injection of $1,25\text{(OH)}_2\text{D}_3$ during embryonic development and growth plate morphology of chicken embryonic bone.
In this study at many places the differences between treatment groups of individual runs were observed in the various parameter studied and these differences could be attributed to the eggs sourced from different donor flock age, health and nutritional status of flock and environmental conditions (Edwards Jr et al., 2002).

Over the decades improved genetic selection has resulted in rapid growth of poultry, bone weight, length, bone strength and composition. The present study looked at bone length and different zones of growth plate to determine the chick embryonic bone development in response to vitamin D$_3$ metabolite treatment \textit{in ovo}. However, there are numerous indicators to determine the bone status of chicken such as bone ash, mineral content, bone density and bone breaking strength (Shim et al., 2012) which in future studies would further elucidate the effect of \textit{in ovo} administration of 1,25(OH)$_3$D$_3$ on embryonic bone development.

In summary, the ameliorative effect of vitamin D$_3$ and its metabolite in correcting bone disorders are well established using vitamin D deficient chicken model and in growing birds, however limited studies have been conducted to study the effects of \textit{in ovo} administration of vitamin D supplements in non-vitamin D deficient flock. The results of present study showed that 1,25(OH)$_2$D$_3$ at the doses tested did not improve the growth and early embryonic bone development suggesting additional doses of vitamin D$_3$ metabolite 1,25(OH)$_2$D$_3$ are not essential provided the eggs sourced from vitamin D fed parent flock.

This study has also demonstrated that in ovo administration of exogenous high levels of vitamin D3 metabolite 1,25(OH)$_2$D$_3$ did not have profound
impact on the growth of chondrocytes of proximal and distal end of longitudinal bone during incubation. Since cell proliferation, differentiation and mineralisation of bone matrix are the important aspects of osteogenesis further *in vitro* studies examining the role of 1,25,(OH)$_2$D$_3$ during osteogenic differentiation of MSCs are required to mimic the effects caused by high levels of vitamin D3 metabolite *in ovo*. 
CHAPTER 3

Effects of $1,25(OH)_2D_3$ during in vitro osteogenic differentiation of chicken mesenchymal stem cells

3.1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with both self-renewal capacity and the ability to differentiate into osteogenic, adipogenic and chondrogenic lineages (Pittenger, et al., 1999). MSCs cultured in the presence of ascorbic acid, beta-glycerol phosphate and dexamethasone will differentiate into osteocytes and form mineralised bone-like structures in vitro (Malaval et al., 1999; Maniotopoulos, et al., 1988). Osteogenic differentiation of bone marrow-derived MSCs is well characterised in the rat (Maniotopoulos, et al., 1988), mouse (Song, et al., 2003; Tropel, et al., 2004) human (Frank, et al., 2002) and chicken (Khatri, et al., 2009).

The vitamin D$_3$ metabolite, $1,25(OH)_2D_3$ is involved in calcium and phosphorous homeostasis and bone development (DeLuca, 2004). Treatment of rat osteoblasts, human marrow stromal cells and the human osteoblastic cell line SV-HFO with $1,25(OH)_2D_3$ was found to promote osteogenic differentiation (Beresford, et al., 1994; Gurlek & Kumar, 2001; van Driel, et al., 2006). In contrast, $1,25(OH)_2D_3$ inhibited osteoblastic differentiation in rat bone marrow stromal cells and murine primary osteoblast cultures (Atmani, et al., 2002; Atmani, et al., 2003; Ecarot & Desbarats, 1999; Yang, et al., 2013). Together, results of previous studies suggest that effects of $1,25(OH)_2D_3$ largely rely on the maturational stages of osteoblasts.
The effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} during osteogenic differentiation of chicken MSCs are not well characterised. Moreover, there is little information available on how 1,25(OH)\textsubscript{2}D\textsubscript{3} modulates osteogenesis in avian species. It is hypothesized that osteogenic differentiation of MSCs in response to 1,25(OH)\textsubscript{2}D\textsubscript{3} differ between mammalian and avian species. To explore this, I have examined the effects of low (2.4nM) and high (24nM) concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} on mineralisation comparing avian and rodent MSCs. Effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on calcium deposition, osteoblast differentiation and cell proliferation from three different strains of chicken MSCs (commercial broiler, commercial layer and SPF layer) were studied during \textit{in vitro} osteogenic differentiation. The knowledge gained from this study could help to determine the critical levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} for optimal bone integrity during foetal and neonatal development in avian species and poultry in particular.
3.2. Materials and Methods

All procedures involving animals and birds were approved by the Charles Sturt University Animal Care and Ethics Committee (Protocol number: 12/002).

3.2.1. Isolation of rat and chicken MSCs

MSCs were derived from femur of 21 day old juvenile Wistar rats and 19 day old chicken embryos from three different strains of poultry; commercial broiler (Ross 308), layer (Hy-Line) and SPF layer (Lohmann LSL) as previously described (Khatri, et al., 2009; Kocamaz et al., 2012) (Appendix VI).

Briefly, the femur from the hind limb was excised under sterile conditions and placed in a petri dish containing phosphate buffered saline (PBS) (10010, Gibco, Life Technologies). Both proximal and distal ends of the femur were removed and a 23 gauge needle was inserted to flush out the bone marrow with complete culture medium (Appendix III). The bone marrow cell suspension was filtered through a 70μm cell strainer (352350, BD Falcon™) and centrifuged at 1000rpm (Heraeus Biofuge Primo R Centrifuge, Thermo scientific) for five minutes to pellet the stromal cells. The cell pellet was then resuspended in 10mls complete culture medium (Appendix III and VI) and cultured in T25 or T75 flask (3123-075, Iwaki® cell biology) until MSCs colonies were clearly identifiable.

Non adherent cell populations were eliminated by daily washing of the culture flask for 3 days with complete culture medium and cells were grown until 80% confluence at 37°C in a humidified environment containing 5%
CO₂. The cells were then passaged at a split ratio of 1:2 or 1:3 and propagated at the density of 1.5x10⁶ cells per T75 flask for further growth.

Cells from all strains of chicken were routinely passaged to passage 2 and then frozen for long term storage and subsequent use in differentiation experiments.

3.2.2. Routine cell passage

Once the cells reached to 80% confluency they were passaged by dissociation using TrypLE™ (1x) (12604, Gibco, Life Technologies) as per standard protocols (Appendix VI).

3.2.3. Confirmation of multilineage potential of chicken MSCs

Chicken MSCs were isolated by virtue of their adherent qualities as described previously (Polisetti, et al., 2010). Adherent cells were observed to form small colonies after a few days in culture (Figure 3.1A) and colonies were allowed to grow until 80% confluency. Cells were then passaged for expansion.

MSCs were validated for their ability to differentiate into adipogenic and osteogenic lineage. To demonstrate the differentiation capacity of broiler MSCs into adipogenic and osteogenic lineage, cells were incubated in adipogenic and osteogenic induction media respectively (see section 3.2.4. and 3.2.5.). Differentiation of MSCs into adipogenic and osteogenic lineage was confirmed by staining with Oil Red O and Alizarin Red respectively (Figure, 3.1B and C). The multilineage differentiation (osteogenic and adipogenic) of chicken MSCs was observed however, differentiation into chondrogenic lineage was not confirmed in this study.
Figure 3.1. (A-C) Lineage differentiation of chicken MSCs. A. Isolated MSCs revealed spindle shaped fibroblastic morphology and formed small colonies. B. Broiler MSCs in adipogenic stimulating media for 21 days showing lipid droplets by Oil Red O stain. C. Osteogenic differentiation of chicken MSCs maintained in osteogenic induction media. Calcium particles stained positive by Alizarin Red stain on day 3 of culture period. Scale bar 200μm.

3.2.4. Adipogenic differentiation of MSCs

Differentiation of broiler MSCs into an adipogenic lineage was assessed as previously described (Polisetti, et al., 2010). Briefly, broiler MSCs were grown in complete culture media to 100% confluency. Cells were then dissociated and cultured in 24 well plates for 21 days in adipogenic induction medium containing 0.5μM dexamethasone (D4902, Sigma-Aldrich), 0.5mM isobutyl methylxanthine (I7018, Sigma-Aldrich), and 10ng/ml insulin (I2643, Sigma-Aldrich) (Appendix III). Adipogenic media was changed every three days. After 21 days of culture adipogenic differentiation was identified by formation of lipid rich vacuoles by Oil Red O staining (O0625, Sigma-Aldrich) (Appendix II and VI) (Figure 3.1B).

3.2.5. Osteogenic differentiation of MSCs

Rat or chicken MSCs were seeded in 4 or 24-well plates (144444, Thermo Scientific; 662160, CELLSTAR®, Greiner bio-one) at a density of 2.5 x 10^4 cells/cm² and cultured in complete media until 100% confluent. Osteogenic differentiation was induced by supplementing the cells with osteogenic induction media containing 10mM β-glycerol (50020, Sigma-Aldrich),
0.05mM Ascorbic acid (A4544, Sigma-Aldrich) and 100nM dexamethasone (D4902, Sigma-Aldrich) (Appendix III). Media was changed every 3 days alternating between induction and maintenance media containing low (2.4nM-LVD) or high (24nM-HVD) concentrations of vitamin D₃ metabolite, 1,25(OH)₂D₃ for the desired culture period (Appendix I). Negative control cultures (NC) of chicken or rat MSCs were cultured only in complete media whereas no treatment control cultures (NT) received induction/maintenance media containing no exogenous 1,25(OH)₂D₃. Chicken and rat cells were cultured for 7 and 21 days post induction respectively (Figure 3.2.).

![Figure 3.2. A schematic representation of osteogenic differentiation protocol of rat and chicken MSCs. Rat and chicken MSCs were seeded at 2.5 x 10⁵ cells/cm² per well, after 100% confluence (Day 0) medium was changed to only complete media, induction media with or without low (2.4nM-LVD) or high (24nM-HVD) concentration of 1,25(OH)₂D₃ and medium was again changed to maintenance media after 3 days. Media was alternated between induction and maintenance for 7 and 21 days for chicken and rat cells respectively.](image)

**3.2.6. Analysis of calcium deposition**

Chicken MSCs were examined during *in vitro* osteogenic differentiation for calcium deposition by Alizarin Red staining on day 3, 5 and 7 of culture. Rat cultures were stained only on day 21 of culture. To achieve visualisation of mineral deposition, cells were fixed in cold 4% paraformaldehyde (PFA) for five minute, washed with PBS three times and stained with Alizarin Red stain (106278, Merck) (2% aqueous solution) for five minutes (Appendix II
and VI). Stained cultures were photographed using a Leica DMLB inverted light microscope at x10 magnification. Six images were obtained from each well in both colour and black and white for image analysis (Appendix VI).

Calcium deposition was quantified histomorphometrically using Image J™ (http://rsb.info.nih.gov/ij/). Briefly; a threshold in the range of 0 to 255 was applied to define the extent of mineralisation from each black and white image. The area fraction of dark regions that corresponds to calcium deposition was obtained by thresholding each image from different treatment groups for analysis (Appendix VII).

3.2.7. **Histochemical staining for alkaline phosphatase**

Alkaline Phosphatase (ALP) is an early marker of an osteoblastic cell phenotype (Marom et al., 2005; Yamamoto et al., 2002). Chicken MSCs were examined for ALP expression using an enzymatic stain on day 3, 5 or 7 of *in vitro* osteogenic differentiation. Cells were fixed in cold 4% PFA for two minutes and staining was carried out using ALP staining kit as per manufacturer’s instructions (SCR 004, Millipore) (Appendix VI). Stained cultures were photographed using standard light microscopy at x10 magnification.

3.2.8. **Analysis of cell proliferation**

Cell proliferation was determined by measuring incorporation of the thymidine analogue Bromodeoxyuridine (BrDU) into host DNA (Taupin, 2007). Briefly, chicken MSCs were plated in complete culture medium at a density of 2.5x10⁴ cells/cm² in 24 well plates. Cells from negative control (without osteogenic medium-NC), no treatment control (osteogenic
differentiation media without exogenous vitamin D$_3$-NT) and 1,25(OH)$_2$D$_3$ treated wells were exposed to BrDU (10mg/ml) for two hours on day 0 of induction. On either day three or five of osteogenic differentiation cells were fixed with cold 4% PFA and cells visualised for the presence of BrDU incorporation by immunocytochemistry (Appendix IV and VI).

To quantitate the number of BrDU positive cells, cultures were counterstained with Harris’ Haematoxylin and cells photographed using standard light microscopy at x20 magnification. Six randomly selected images were acquired for each treatment well for image analysis (Appendix VII).

3.2.9. **Quantitation of BrDU positive cells and cell density**

A standard area (1.38cm$^2$) from each well per treatment group was counted using Adobe Photoshop (Adobe photoshop CS6) (Appendix VII). The proportion of BrDU positive cells was determined by calculating the number of BrDU labelled cells as a proportion of the total cell number.

The cells were counted within a standard area in each BrDU stained image. The cell density was determined by calculating the total number of cells relative to surface area in each well and the density of cell was expressed as cells/cm$^2$.

3.2.10. **Statistical Analysis**

All results were analysed for statistical significance using student’s t test (Microsoft Excel 2010). Where data was not normally distributed the non-parametric test such as Mann Whitney U test or Wilcoxon rank sum test was
applied using Graph Pad Software, Inc. USA. Data are expressed as means ± SEM and values of $p<0.05$ were considered as significant.
3.3. Results

In this study, the effects of exogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} on mineralisation, osteogenic differentiation and cell proliferation were examined between different species (rat and chicken) and strains of chicken (broiler and layer) MSCs during *in vitro* osteogenic differentiation.

### 3.3.1. Mineral deposition was inhibited by exogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} in both mammalian and avian mesenchymal stem cells

Rat MSCs were induced to an osteogenic lineage in the presence or absence of low (2.4nM) and high (24nM) concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} and deposition of calcium was confirmed by Alizarin Red staining on day 21 of osteogenic differentiation. It was observed that addition of exogenous vitamin D\textsubscript{3} metabolite, 1,25(OH)\textsubscript{2}D\textsubscript{3} at both 2.4 and 24nM inhibited calcium deposition in rat MSCs (Figure 3.3).

Calcium deposition in the extracellular matrix on day 21 of osteogenic differentiation of rat cells was confirmed by Alizarin Red staining and analysed using Image J (see section 3.2.6.). Alizarin Red staining showed many mineralized nodules after 21 days of osteogenic induction (Figure 3.3A). Quantitative analysis of calcium deposition showed that treatment of rat MSCs with high doses (24nM) of 1,25(OH)\textsubscript{2}D\textsubscript{3} significantly inhibited calcium deposition (NT mean- 20.10±6.23, HVD mean- 2.92±0.82, *p*=0.026, *n*=3, Figure 3.3C and D). There was also a trend towards a significant decrease in calcium deposition between the low vitamin D and no treatment control cultures (LVD mean- 7.09±1.86, NT mean- 20.10±6.23, *p*=0.058, *n*=3, Figure 3.3B and D) and between low vitamin D and high vitamin D cultures (LVD mean- 7.09±1.86, HVD mean-
2.92±0.82, p=0.055, n=3, Figure 3.3C and D) although in both cases the trend did not quite reach significance.

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**Figure 3.3.** (A-C) Alizarin Red staining of rat MSCs treated with 1,25(OH)$_2$D$_3$ for 21 days during osteogenic differentiation. Calcium deposition was reduced after treatment of rat MSCs with 24nM of 1,25(OH)$_2$D$_3$ (HVD, C) when compared to no treatment control cultures (NT, A). Scale bar 200μm. D). Quantitative analysis of calcium deposition analysed by Image J and expressed as area fraction (% of pixels). Values are Mean ± SEM (n= 3). Comparisons marked with an asterisk (*) are significantly different (p<0.05, student’s t- test). Abbreviations: NT, no treatment with vitamin D; LVD, low vitamin D (2.4nM); HVD, high vitamin D (24nM).

To determine whether avian cells responds in the same way as mammalian cells to exogenous vitamin D$_3$, MSCs were isolated from three strains of chicken embryos; commercial broiler (Ross 308), SPF layer (Lohmann LSL) and commercial layer (Hy-Line) chickens (Appendix VI). The effect of 1,25(OH)$_2$D$_3$ during osteogenic differentiation in three different strains of chicken MSCs was then examined.
All three strains of chicken MSCs (commercial broiler, commercial layer and SPF layer) were examined at 3, 5 or 7 days of osteogenic differentiation and calcium deposition was confirmed using Alizarin Red staining. In contrast to rat, calcium deposition was evident as early as day three (D3) of osteogenic induction, compared to rat where the first morphologically detectable bone nodules have only been first reported on day 10 of the culture period (Malaval, et al., 1994). This finding identifies a species specific difference in onset and rapidity of in vitro osteogenic differentiation between avian and mammalian species.

The results of calcium deposition in the three strains of chicken MSCs at different intervals of incubation are presented in Figures 3.4.-3.6. and Table 3.1. Calcium deposition in the NT cultures of all three strains increased linearly over the culture period however, the extent and magnitude of calcium deposition was higher in broiler MSCs cultures compared to SPF and Hy-line layer MSCs (Figures 3.4., 3.5. and 3.6.)

Vitamin D₃ metabolite, 1,25(OH)₂D₃ at low (2.4nM) or high (24nM) concentrations was found to inhibit calcium deposition in all chicken MSCs at all days examined during in vitro osteogenic differentiation (Figures 3.4.-3.6.). However, differences in calcium deposition were observed between strains in response to 1,25(OH)₂D₃ treatment.

3.3.1.1. Strain dependent differences

High doses (24nM) of 1,25(OH)₂D₃ inhibited mineralisation of broiler MSCs. On day 3 of osteogenic induction, high doses of 1,25(OH)₂D₃ significantly inhibited mineralisation compared to no treatment control
cultures (NT mean- 40.64±7.17, HVD mean- 10.94±4.83, \(p=0.001\), n=11, Figures 3.4D and M).

On day 5, a significant decrease in mineral deposition was observed between high vitamin D and both the no treatment control (NT mean-
47.18±8.82, HVD mean- 5.39±5.03, \(p=0.004\), n= 6) and low vitamin D cultures (LVD mean- 38.73±11.33, HVD mean- 5.39±5.03, \(p=0.015\), n=6, Figure 3.4H and M).

There was also significant reduction in mineral deposition between high vitamin D and no treatment control cultures on day 7 of osteogenic induction (NT mean- 57.36±5.97, HVD mean- 6.16±4.34, \(p=0.001\), n= 7, Figure 3.4J, L and M). Together, this data suggests that calcium deposition in broiler MSCs is inhibited in dose dependent manner by 1,25(OH)\(_{2}\)D\(_{3}\) on all days examined.
Figure 3.4. (A-L) Inhibition of calcium deposition at high doses (24nM) of 1,25(OH)₂D₃ in broiler MSCs in vitro (D, H and L). Broiler MSCs were grown in complete, osteogenic induction media with or without 2.4nM (low vitamin D) and 24nM (high vitamin D) concentrations of 1,25(OH)₂D₃. Cultures were observed for calcium deposition with Alizarin Red stain on day 3, 5 or 7 of osteogenic differentiation. Scale bar 200μm. M). Calcium deposition was quantified by Image J and expressed as area fraction (% of pixels). Values are Mean ± SEM. Comparisons marked with an asterisk (*) are significantly different (p<0.05, Mann Whitney U test). Day 3 n=11; day 5 n=6 and day 7 n=7. Abbreviations: D, day; NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, high vitamin D.

SPF layer MSCs showed a marked sensitivity to low and high doses of 1,25(OH)₂D₃ compared to broiler cells. On day 3 and 5 of osteogenic differentiation, both low (2.4nM) and high (24nM) concentrations of 1,25(OH)₂D₃ completely inhibited mineralisation in cultures of SPF layer MSCs (Figures 3.5C, D, G and H).
On day 7 of osteogenic differentiation calcium deposition was observed in SPF layer cultures but this was significantly reduced in the low vitamin D and high vitamin D cultures compared to both no treatment control cultures ($p=0.029$ and $p=0.000$ respectively, NT mean- $53.46 \pm 8.28$, LVD mean- $8.46 \pm 8.26$, HVD mean- $1.0 \pm 0.00$, n=4, Figures 3.5J, K, L and M) and compared to broiler MSCs cultures (Figure 3.4.). Calcium deposition was also significantly reduced in high vitamin D compared to low vitamin D cultures on day 7 of osteogenic induction in SPF layer cultures (LVD mean- $8.46 \pm 8.26$, HVD mean- $1.0 \pm 0.00$, $p=0.001$, n=4, Figure 3.5L and M).

Together, these data suggest that SPF layer derived MSCs are more sensitive to increasing levels of exogenous vitamin D$_3$ in our culture system than their broiler counterparts.
Figure 3.5. (A-L) In SPF layer culture, 1,25(OH)2D3 inhibited calcium deposition in both low vitamin D (2.4nM) and high vitamin D cultures (24nM) on all days of osteogenic differentiation (C, D, G, H, K, L). Though the evidence of mineralisation was observed on day 3 in no treatment control cultures of SPF layer cells, the extent of mineralisation was less on day 3 and 5 compared to no treatment control cultures of broiler MSCs. Scale bar 200μm. M). Calcium deposition in each treatment culture was analysed by Image J and values represent area fraction (% of pixels). Values are Mean ± SEM. Comparisons marked with an asterisk (*) are significantly different (p<0.05, Mann Whitney test and Wilcoxon signed rank test). In all cases n=4.

Abbreviations: D, day; NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, high vitamin D.

Consistent with observations in SPF layer MSCs, evidence of early mineralisation was also observed on day 3 of osteogenic differentiation of Hy-Line layer MSCs. However, the amount of calcium deposition in no treatment control cultures of Hy-Line layer was very much less than no treatment control cultures of broiler and SPF layer MSCs under the same
experimental conditions on all days examined (Figure 3.6B, F and J). Results of quantitative analysis of calcium deposition in these cultures are shown in Table 3.1 and reflect the differences observed visually. Due to a low number of experimental replicates statistical significance could not be established for this strain.

$1,25(\text{OH})_2\text{D}_3$ at high concentrations (24nM) was observed to completely inhibit mineral deposition on all days examined (Figure 3.6D, H and L). Low doses (2.4nM) of $1,25(\text{OH})_2\text{D}_3$ also dramatically reduced mineral deposition (Figure 3.6C, G and K). Together, this data suggests that Hy-Line layer MSC cells showed the highest sensitivity to both low and high concentrations of $1,25(\text{OH})_2\text{D}_3$ compared to all other poultry strains examined.
Figure 3.6. (A–L) Calcium deposition in the Hy-Line layer MSCs undergoing osteogenic differentiation in the presence of increasing doses of vitamin D₃ metabolite 1,25(OH)₂D₃. Complete absence of calcium deposition in Hy-Line layer cultures by 1,25(OH)₂D₃ in high vitamin D cultures (24 nM) at all days of culture period (D, H and L) whereas in low vitamin D cultures (2.4 nM) the extent of calcium deposition was reduced (C, G and K) compared to no treatment control cultures. Scale bar 200μm. In all cases n=1. Abbreviations: D, day; NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, high vitamin D.

Table 3.1. In vitro calcium deposition by Hy-Line layer MSCs cultured in the presence or absence of low (2.4nM) and high (24nM) concentrations of 1,25(OH)₂D₃ during osteogenic differentiation. Calcium deposition in each culture was analysed by Image J and values represent area fraction (% of pixels). Statistical significance could not be achieved in this strain due to inadequate number of independent culture runs (n=1).

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Hy-Line layer mean area fraction (% of pixels)</th>
</tr>
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</table>
3.3.2. Early osteogenic differentiation of chicken MSCs was reduced by exogenous 1,25(OH)$_2$D$_3$

In an attempt to elucidate whether the reduction in calcium deposition in the presence of exogenous 1,25(OH)$_2$D$_3$ treatment was as a result of reduction in cells of an osteoblastic phenotype, cultures of chicken MSCs were examined for ALP staining on day 3, 5 or 7 of osteogenic differentiation. Alkaline phosphatase (ALP) is used as marker of early osteoblast phenotype and is essential for bone matrix formation (Atmani, et al., 2003; Beck et al., 1998; Halvorsen, et al., 2001; Hughes & Aubin, 1997; Malaval, et al., 1994; zur Nieden et al., 2003). ALP is also expressed in undifferentiated stem cells including embryonic stem cells (Horiuchi et al., 2004; Thomson et al., 1998; zur Nieden, et al., 2003).

Cells from negative control cultures (NC), those cultured in the absence of osteogenic induction factors expressed ALP. Similar results were observed in all strains of chicken MSCs (Figures 3.7.) This staining was highly focal in its distribution and the staining intensity was higher than vitamin D$_3$ treated cultures. This staining pattern suggests that an undifferentiated MSCs population is still present in these cultures.

In no treatment control cultures (NT), ALP staining was intense and widespread whereas in vitamin D$_3$ treated cultures (LVD and HVD) reduced intensity and more focal accumulation of ALP staining was observed (Figure 3.7.).

The change in localisation and intensity of ALP staining observed in these cultures suggests that fewer osteoprogenitor cells are maturing to become ALP expressing osteoblasts in the presence of 1,25(OH)$_2$D$_3$. 

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Overall, the results of ALP staining suggest that treatment of chicken MSCs of all strains with exogenous $1,25(\text{OH})_2\text{D}_3$ dose dependently reduces the progression of osteoprogenitor cells to cells of an osteoblastic phenotype.
Figure 3.7. (A-jj) Histochemical detection of ALP in broiler (A-L), SPF layer (M-X) and Hy-Line layer (Y-jj) MSCs cultured in complete media and osteogenic induction media in the presence or absence of 2.4nM (Low vitamin D) and 24nM (High vitamin D) concentrations of 1,25(OH)\(_2\)D\(_3\) for 3, 5 or 7 day of culture. Broiler MSCs: day 3 n=6, day 5 n=5, day 7 n=4. In SPF layer MSCs, there was dose dependent reduction in positive staining of ALP in vitamin D cultures (O, P, S, T, W and X). At all-time points examined n=6 in all cases. 1,25(OH)\(_2\)D\(_3\) dose dependently reduced the ALP staining in Hy-Line cells during osteogenic differentiation. ALP staining was diffuse in NT cultures (Z, dd and hh) whereas in LVD and HVD cultures pattern of staining is reduced and focal in nature indicating inhibition of osteoblastic development by 1,25(OH)\(_2\)D\(_3\) (aa, bb, ee, ff, ii, and jj). For day 3 and 5 n = 2, day 7 n = 3. Scale bar 200μm.

Abbreviations: D, day; NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, high vitamin D.
3.3.3. **Exogenous 1,25(OH)_{2}D_{3} reduces the proportion of BrDU positive cells in chicken MSCs during osteogenic differentiation**

Proliferation of osteoprogenitor cells is a vital step during normal osteogenesis (Driel et al., 2004; Neve et al., 2011). To examine whether exogenous vitamin D$_{3}$ was influencing cell proliferation in our cultures the effects of exogenous 1,25(OH)$_{2}$D$_{3}$ on cell proliferation were investigated by Bromodeoxyuridine (BrDU) incorporation into cultured cells during osteogenic induction. Chicken MSCs were acutely pulsed with BrDU on day 0 of osteogenic induction and BrDU positive cells quantified on day 3 or 5. A schematic representation of BrDU labelling and cell analysis is shown in Figure 3.8A and B.
Figure 3.8. A). BrDU incorporation for analysis of cell proliferation in chicken MSCs. Cells were acutely pulsed with BrDU for 2 hours on day 0 of osteogenic induction. BrDU positive cells were quantified on day 3 or 5 of osteogenic differentiation. This method allows identification of BrDU labelled population that continued to proliferate over the period during osteogenic differentiation.

B). Diagram showing the labelling of cell populations after BrDU exposure. On an acute pulse of BrDU cells in S-phase of the cell cycle incorporate BrDU. The cells that failed to undergo mitosis will be heavily stained whereas cells that proceed through mitosis for one division or more post-labelling will show a reduced staining intensity after immunohistochemistry. Thus cells will be either ‘heavily’ labelled or ‘lightly’ labelled according to this experimental paradigm.
Representative images of BrDU immunostaining of chicken MSCs undergoing osteogenic differentiation in the presence or absence of 1,25(OH)$_3$D$_3$ are shown in Figure 3.9. The quantitative analysis of proportions of BrDU labelled cells from each treatment culture for all strains of chicken MSCs are shown in Figure 3.10.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
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<td>U</td>
<td>V</td>
<td>W</td>
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</tr>
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</table>

**Figure 3.9.** Photomicrograph of BrDU immunostaining of broiler (A-H), SPF (I-P) and Hy-Line layer (Q-X) MSCs after day 3 or 5 of incubation in complete media (negative control) and osteogenic induction media with or without 1,25(OH)$_3$D$_3$ (2.4nM- LVD and 24nM - HVD). Cells were pulsed with BrDU (10mg/ml) for 2 hours and the effect of 1,25(OH)$_3$D$_3$ on cell proliferation was determined by quantitating proportions of BrDU labelled cells on day 3 or 5 of osteogenic differentiation. For broiler MSCs in all cases n=4, SPF layer n=3 and Hy-Line layer n=2. Scale bar 200μm.

Abbreviations: D, day; NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, high vitamin D.
Figure 3.10. (A-C) Proportions of BrDU positive cells in 3 strains of chicken MSCs (A-broiler, B- SPF layer, C- Hy-Line layer). Cells were cultured in the complete media, presence or absence of osteogenic media and increasing concentrations of 1,25(OH)₂D₃. Cells were pulsed with BrDU for 2 hours on day 0 and percentage of cells stained positive for BrDU was quantitated on day 3 or 5 of osteogenic differentiation. Compared to no treatment controls, cell proliferation was reduced in both low and high vitamin D cultures on day 3 or 5 for all strains examined. A marked decrease in BrDU positive cells was noted in negative control cultures compared to all other cultures. Values are Mean ± SEM. For broiler n=4, SPF layer n= 3, Hy-Line layer n=2. Comparisons marked with an asterisk (*) are significantly different (p<0.05, student’s t-test).
These results indicate that exposure of chicken MSCs undergoing osteogenic differentiation exposed to increasing concentrations of exogenous 1,25(OH)\(_2\)D\(_3\) reduced the total proportions of BrDU positive cells suggesting a decrease in the number of proliferating cells over time in all cultures analysed (Figure 3.10.)

Both high and low doses of vitamin D\(_3\) metabolite 1,25(OH)\(_2\)D\(_3\) decreased cell proliferation at all time points and in all strains examined. No induction cultures had the lowest proportion of BrDU labelled cells for all strains at all days examined.

3.3.4. Treatment with exogenous 1,25(OH)\(_2\)D\(_3\) significantly reduce cell proliferation in broiler and layer MSCs cultures undergoing osteogenic differentiation

In broiler cultures, on day 3 of osteogenic differentiation proportions of BrDU labelled cells were significantly reduced in low vitamin D and high vitamin D compared to no treatment control cultures ($p=0.045$ and $p=0.000$ respectively, NT mean- 93.37±0.84, LVD mean- 91.00±0.81, HVD mean-84.74±1.20, n=4, Figures 3.9B, C, D and 3.10A). Also, there was significant reduction in the proportion of BrDU positive cells in high vitamin D compared to low vitamin D cultures (LVD mean- 91.00±0.81, HVD mean-84.74±1.20, $p=0.002$, n=4; Figures 3.9C, D and 3.10A).

On day 3 of osteogenic induction, negative control cultures which received only complete media and not osteogenic induction factors showed significant reduction in BrDU positive cells compared to no treatment controls, low vitamin D and high vitamin D cultures (NC mean- 68.45±4.21, NT mean- 93.37±0.84, $p=0.000$, LVD mean- 91.00±0.81, $p=0.001$, HVD
mean- 84.74±1.20, p=0.005, n=4 in all cases ; Figures 3.9A, B, C, D and 3.10A).

In broiler cultures, the proportions of BrDU labelled cells on day 5 of osteogenic induction were significantly reduced at high concentration (24nM) of 1,25(OH)_{2}D_{3} compared to no treatment control culture (NT mean- 95.46±1.09, p=0.000, n=4, HVD mean- 89.54±0.28) and low vitamin D cultures ( LVD mean- 93.54±0.35, p=0.000, n=4; Figures. 3.9F, G, H and 3.10A.) whereas proportions of BrDU labelled cells in negative controls was significantly reduced compared to no treatment control, low vitamin D ( NC mean- 81.72±2.85, NT mean- 95.46±1.09, p=0.002, n=4, LVD mean- 93.54±0.35, p=0.003, n=4) and high vitamin D cultures (HVD mean 89.54±0.28, p=0.018, n=4; Figures 3.9E, F, G, H and 3.10A.).

In SPF layer cultures, on day 3 of osteogenic differentiation the proportions of BrDU labelled cells were significantly lower in high vitamin D compared to no treatment control cultures (NT mean- 91.03±3.51, HVD mean- 82.28±1.28, p=0.040, n=3; Figures 3.9J, L and 3.10B).

On day 5 of osteogenic induction, proportions of BrDU labelled cells were significantly reduced in high vitamin D compared to no treatment control (NT mean- 94.09 ±1.50, HVD mean- 88.13±1.61, p=0.028, n=3) and low vitamin D cultures (LVD mean- 92.35±0.89, p=0.042, n=3; Figures 3.9N, O, P and 3.10B.). In negative control cultures, on day 5 of osteogenic differentiation, there was significant decrease in proportions of BrDU labelled cells compared to no treatment control (NT mean- 94.09±1.50, p=0.006 n=3) low vitamin D (LVD mean- 92.35±0.89, p=0.007, n=3) and
high vitamin D cultures (HVD mean- 88.13±1.61, \( p=0.032, n=3 \); Figures 3.9M, N, O, P and 3.10B).

The effect of 1,25(OH)\(_2\)D\(_3\) treatment on cell proliferation in Hy-Line layer MSCs could not be examined statistically due to insufficient independent culture runs (n=2). However, there is marked reduction in proportion of BrDU labelled cells in negative control and vitamin D cultures on both day 3 and day 5 of osteogenic differentiation (Figure 3.9Q, T, U and X.).

Together, these data suggest that increasing concentration of 1,25(OH)\(_2\)D\(_3\) exerts anti-proliferative action in all strains examined: Broiler, SPF layer and Hy-Line layer MSCs during \textit{in vitro} osteogenic differentiation. In both broiler and SPF layer MSCs the proportion of BrDU positive cells was significantly decreased on both days of osteogenic differentiation with increasing concentration of 1,25(OH)\(_2\)D\(_3\) and a similar trend was evident in cultures of Hy-Line layer MSCs (Figure 3.10C). This data demonstrates that the anti-proliferative properties of 1,25(OH)\(_2\)D\(_3\) during \textit{in vitro} osteogenic differentiation are similar across all the strains of chicken MSCs.

3.3.5. \textit{Temporal effect of exogenous 1,25(OH)\(_2\)D\(_3\) on cell proliferation between 0-3 and 0-5 days of osteogenic induction}

In order to determine the effect of 1,25(OH)\(_2\)D\(_3\) on cell proliferation over time during osteogenic differentiation, the relative proportions of BrDU positive cells between day 3 and day 5 of each treatment group were compared, results are shown in Table 3.2.

In broiler cultures, proportions of BrDU positive cells were significantly increased between day 3 and day 5 of osteogenic induction in negative
control (Day 3 mean- 68.45± 4.21, Day 5 mean- 81.72±2.85, p=0.031, n=4), no treatment control (Day 3 mean- 93.37±0.84, Day 5 mean- 95.46±1.09, p=0.003, n=4) and high vitamin D cultures (Day 3 mean- 84.74±1.20, Day 5 mean- 89.54±0.28, p=0.023, n=4). Moreover, a trend towards increase in proportions of BrDU positive cells between day 3 and day 5 was also observed in low vitamin D cultures (Day 3 mean- 91.00±0.81, Day 5 mean- 93.54±0.35, p=0.053, n=4).

The comparative analysis of proportions of BrDU positive cells between day 3 and day 5 of each treatment group suggests that cell proliferation increases over the time period during the osteogenic differentiation of broiler MSCs with the greatest increase observed in high vitamin D treated cultures.

There was no significant difference observed in proportions of BrDU positive cells between day 3 and day 5 of negative control and no treatment control cultures of SPF layer MSCs. However, a significant increase in population of BrDU positive cells was observed between day 3 and day 5 of high vitamin D cultures (Day 3 mean- 82.28±1.28, Day 5 mean- 88.13±1.61, p=0.024, n=3). Similar to broiler MSCs, a trend towards an increase in proportions of BrDU positive cells in SPF layer MSCs was observed between day 3 and 5 of low vitamin D cultures (Day 3 mean- 85.95±2.36, Day 5 mean- 92.35±0.89, p=0.052, n=3). The data of proportions of BrDU positive cells in SPF layer between day 3 and day 5 suggests that cell proliferation increased in vitamin D₃ treated cultures over time during osteogenic differentiation.
In Hy-Line layer MSCs, the proportions of BrDU positive cells between day 3 and day 5 of each treatment group could not be compared statistically due to inadequate experimental replicates (n=2). However, in osteogenic induced cultures numerically slight increase in proportions of BrDU positive cells were observed between day 3 and day 5 suggesting fewer cell populations proliferated between day 3 and day 5 of osteogenic differentiation in Hy-Line layer cultures.
Table 3.2. Effect of 1,25(OH)₂D₃ on cell proliferation during \textit{in vitro} osteogenic differentiation of chicken MSCs. Comparison of % of BrDU positive cells between day 3 and day 5 of each treatment group from broiler, SPF layer and Hy-Line layer MSCs. Values are Mean ± SEM. \( p<0.05 \) considered statistically significant (student’s t-test).

\textit{Abbreviation: NA- Not applicable}

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<tr>
<th>Treatment</th>
<th>Broiler MSCs</th>
<th>SPF layer MSCs</th>
<th>Hy-Line layer MSCs</th>
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<td>Day 3</td>
<td>Day 5</td>
<td>( p )-value</td>
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<td>Negative control</td>
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</tbody>
</table>
3.3.6. **Cell number was reduced over time in presence of exogenous 1,25(OH)\(_2\)D\(_3\) treatment**

To determine if the reduction in proportion of BrDU positive cells observed in treated cultures resulted in an absolute reduction in cell number, cell density was calculated in all cultures examined.

Increasing concentrations of vitamin D\(_3\) metabolite 1,25(OH\(_2\))D\(_3\) decreases the cell number over time. This effect was similar in all strains examined; a trend in Hy-Line layer MSCs was same as broilers and SPF layer where overall cell density is significantly reduced. Osteogenic induction (no treatment control) increased the cell number in all strains examined.
Figure 3.11. Total cell density was analysed as described in materials and methods section and compared between day 3 and day 5 of osteogenic differentiation. Overall, numbers of cells from negative control cultures were decreased compared to all other treatment cultures in all strains of chicken MSCs. Osteogenic induction cultures shows a significant increase in cell density compared to negative control whereas a trend towards a reduction in cell density with increasing doses of exogenous 1,25(OH)_{2}D_3. For broiler day 3 and 5 n= 4; SPF layer n= 3; Hy-Line layer n=2. Values are Mean ± SEM. p<0.05 considered statistically significant (student’s t-test). Due to insufficient experimental replicates statistical significance could not be achieved for Hy-Line layer MSCs.

Abbreviations: NC, negative control; NT, no vitamin D; LVD, low vitamin D; HVD, High vitamin D.
In order to determine if the observed increase in proportion of BrDU labelled cells has resulted in an increase in cell density over time, cell density (cells/cm$^2$) was examined and data is shown in Table 3.3.

There was no significant difference observed in cell density of broiler MSCs between day 3 and day 5 for all treatments. In SPF layer MSCs the cell density was significantly increased in high vitamin D cultures between day 3 and day 5 (Day 3 mean- 826.71±63.07, Day 5 mean- 994.46 ±28.52, $p=0.036$, n=3; Table 3.3.). This increase in cell density coincides with increased proportions of BrDU positive cells between day 3 and day 5 of osteogenic induction in high vitamin D cultures.

Cell density between day 3 and day 5 of each treatment culture of Hy-line layer MSCs could not be compared statistically due to inadequate numbers of experimental replicates. However the trend is similar to that observed in both broiler and SPF layer cultures.

Overall cell density analysis suggests that very little cell proliferation occurred between day 3 and day 5 of osteogenic induction except in the presence of high level of vitamin D.
### Table 3.3. Analysis of cell density (cells/cm²) between day 3 and 5 for each strain and treatment group during osteogenic differentiation. Values are Mean ± SEM. *p*<0.05 considered statistically significant (student’s t-test).

*Abbreviation: NA- not applicable.*

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<th>Treatment</th>
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<th>Hy-Line layer MSCs (n=2)</th>
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<td><strong>High vitamin D</strong></td>
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</table>
3.4. Discussion

The aim of this study was to examine the effect of exogenous 1,25(OH)$_2$D$_3$ at increasing concentrations during *in vitro* osteogenic differentiation of chicken MSCs *in vitro*. Species specific differences in osteogenic differences of MSCs were observed in this study. Early onset of mineralisation was evident in avian species compared to mammalian species. Vitamin D$_3$ metabolite, 1,25(OH)$_2$D$_3$ significantly inhibited calcium deposition in all strains and species examined, in a dose dependent manner. Strain differences between broiler and layer strains of poultry were also investigated. In all strains of chicken MSCs examined, layer strains were highly sensitive to increasing concentrations of exogenous 1,25(OH)$_2$D$_3$. Early osteogenic differentiation and cell proliferation was significantly reduced in all strains of chicken MSCs with exogenous 1,25(OH)$_2$D$_3$ at all days examined.

3.4.1. Early onset of mineralisation was evident in all strains of chicken MSCs

In this study, evidence of an early onset of mineralisation was apparent in all strains of chicken MSCs. This is a novel finding, as onset of mineralisation during osteogenic induction of MSCs has not been examined previously in poultry, particularly in relation to different poultry strains.

One possible explanation for early onset of mineralisation in poultry MSCs compared to mammalian MSCs is suggested by previous studies in chick. Osteoblasts express osteocalcin during post proliferative phase and its expression is maximal during mineralisation, hence osteocalcin is considered as maker of matured osteoblasts (Lian, et al., 1989; Owen et al.,
1990). The level of osteocalcin in chick osteoblasts isolated from calvaria was found to be almost five times that found in 21 day rat calvaria osteoblasts (Hauschka et al., 1983; Lian et al., 1985; Lian et al., 1982). Therefore, it may be that avian bone contains developmentally more matured osteoblasts and fewer pre-osteoblast cells than cells isolated from mammalian species. Thus, avian cells may require less time to form matured osteoblasts, initiate extra cellular matrix maturation and form bone nodules than their mammalian counterparts (Aronow et al; 1990). In the present study, cell populations from both poultry and rat were isolated from femur bone marrow rather than calvaria and analysis of osteocalcin levels was not compared, however, this finding could suggest a mechanism for the early mineralisation observed in avian species and similar studies could be carried out in the future to determine the mechanism behind this mineralisation.

3.4.2. Calcium deposition was inhibited by 1,25(OH)\(_2\)D\(_3\) in rat and chicken MSCs in vitro

High doses (24nM) of 1,25(OH)\(_2\)D\(_3\) have been shown to have a potent inhibitory action on mineralisation in many species in vitro (Broess et al., 1995; Ecarot & Desbarats, 1999; Fromigué et al., 1997; Yang, et al., 2013). However, to date no strain specific differences have been identified in response to 1,25(OH)\(_2\)D\(_3\) treatment during osteogenic differentiation.

Inhibition of mineralisation has been reported by (Yamaguchi & Weitzmann, 2012) where 1,25(OH)\(_2\)D\(_3\) at 10nM and 100nM potently inhibited mineralisation in the pre osteoblastic cell line \(MC3T3-E1\) and primary mouse bone marrow stromal cells. Acute and chronic treatment of osteoblasts derived from chicken calvaria with 10nM of 1,25(OH)\(_2\)D\(_3\)
markedly decreased mineralisation of the extracellular matrix (Broess, et al., 1995).

Overall, *in vitro* studies conducted in different species are in accordance with the observations of the present study suggesting that 1,25(OH)\(_2\)D\(_3\) inhibits mineral deposition during osteogenic differentiation.

Mineralisation of extracellular matrix in osteoblastic culture is a function of mature osteoblasts and a final step in osteoblast differentiation (Quarles et al., 1992). It is well known that osteocalcin, osteopontin and *phex* are markers of differentiated osteoblasts and their expression is associated with matrix mineralisation (Beck, et al., 1998; Ecarot & Desbarats, 1999). 1,25(OH)\(_2\)D\(_3\) treatment of rat, chicken and mouse osteoblast cultures inhibited collagen type I, osteocalcin, osteopontin production and *phex* expression and thus inhibition of these osteoblastic markers is suggested to induce inhibition of mineral deposition (Broess, et al., 1995; Ecarot & Desbarats, 1999; Owen, et al., 1991).

The data from the present study shows that increasing concentrations of 1,25(OH)\(_2\)D\(_3\) inhibited calcium deposition during osteogenic induction of MSCs in mammals and avian species in a dose dependent manner.

### 3.4.3. Osteogenic differentiation was inhibited by 1,25(OH)\(_2\)D\(_3\)

The process of osteogenic differentiation occurs in three different phases; proliferation, matrix maturation and mineralisation and during each phase distinct markers are expressed by cells of osteoblastic lineage (Olsen, et al., 2000).
Alkaline phosphatase (ALP) is a marker of early osteoblast differentiation (Fromigué, et al., 1997; Malaval, et al., 1994). 1,25(OH)₂D₃ has been shown to have both an inhibitory (Broess, et al., 1995; Yang, et al., 2013) and stimulatory (Fromigué, et al., 1997; Manolagas et al., 1981; Matsumoto, et al., 1991) action on ALP expression.

The results of this study identified a dose dependent reduction in ALP-positive cells in all chicken strains examined after 1,25(OH)₂D₃ treatment (Figure 3.7.). Together, this data suggests that avian MSCs were unable to differentiate into mature mineralising osteoblasts in the presence of increasing concentrations of 1,25(OH)₂D₃. This finding is consistent with previous studies which have shown a reduction in ALP staining of mouse bone marrow stromal cells at 1nM concentrations of 1,25(OH)₂D₃ with a complete disappearance at 10nM concentrations (Y. Li, et al., 2008).

Treatment of primary chicken osteoblasts isolated from embryonic calvaria exposed to 0.1 and 10nM doses of 1,25(OH)₂D₃ for 30 days also showed down regulation of ALP enzyme activity (Broess, et al., 1995). These findings are consistent with data presented in this study.

In the present study, the evidence of dose dependent reduction in ALP staining in all strain of chicken MSCs supports a hypothesis that 1,25(OH)₂D₃ inhibits the differentiation of osteoprogenitor cells into more mature osteoblasts, which would result in the inhibition of mineralisation observed during *in vitro* osteogenic differentiation.
3.4.4. \textit{1,25(OH)}_{2}\textit{D}_3 \textit{exerts an anti-proliferative effect on chick MSCs undergoing osteogenic differentiation}

Data from this study showed that increasing concentrations of \textit{1,25(OH)}_{2}\textit{D}_3 exerted a potent anti-proliferative effect during \textit{in vitro} osteogenic differentiation of chicken MSCs (Figures 3.9.-3.10.). This finding is consistent with earlier reports that in human bone marrow stromal cells (Fromigué, et al., 1997; Geng et al., 2011), mouse C3H10T1/2 mesenchymal multipotent cells (Artaza et al., 2010), mouse bone marrow stromal cells (Y. Li, et al., 2008) and human keratinocytes (Takahashi et al., 2003) \textit{1,25(OH)}_{2}\textit{D}_3 showed reduced cell proliferation. An anti-proliferative effect of \textit{1,25(OH)}_{2}\textit{D}_3 is also well documented in many tumour cell lines, for example, addition of \textit{1,25(OH)}_{2}\textit{D}_3 to neuroblastoma cell lines and the human breast cancer cell line MCF-7 resulted in a decrease in the number of proliferating cells (Gumireddy et al., 2003; Jensen et al., 2001).

Previous work has suggested that the inhibitory effect of \textit{1,25(OH)}_{2}\textit{D}_3 on cell proliferation is dependent on the maturational state of the osteoblasts cells used for the study. Histone gene expressions are strictly confined to proliferating cells and associated with DNA replication. Thus cell growth related genes H4 histone reflects DNA synthesis (Lian & Stein, 1992). A biphasic effect of \textit{1,25(OH)}_{2}\textit{D}_3 has been demonstrated in rat osteoblast cultures where \textit{1,25(OH)}_{2}\textit{D}_3 down regulated the cell growth controlling histone gene expression during the proliferative period (days 8-15) whereas no significant effect was observed in histone mRNA levels in later periods (days 15-30) of culture (Owen, et al., 1991). This results suggests that the stage of osteoblast differentiation might be important to exert the anti-proliferative effect of \textit{1,25(OH)}_{2}\textit{D}_3.
In the present study, chicken MSCs were truly undifferentiated pre-osteoblastic cells (MSCs) labelled with BrDU on day 0 of osteogenic differentiation when cells were still undergoing significant cell division. It was hypothesised that this early stage of the culture protocol was a critical time window for 1,25(OH)₂D₃ to exert an anti-proliferative action.

Data from the present study showed that treatment of undifferentiated chicken MSCs with increasing concentrations of 1,25(OH)₂D₃ decreased the rate of cell proliferation (proportion of BrDU positive cells). These results support the hypothesis that the anti-proliferative effect of 1,25(OH)₂D₃ occurs during the earliest stages of differentiation into cells of osteogenic lineage. This hypothesis is also supported by analysis of cell density as cell proliferation is completed by day 3 and very less cell proliferation occurred between day 3 and day 5 of osteogenic induction.
CHAPTER 4

General discussion

The biologically active form of vitamin D₃ is, 1,25(OH)₂D₃ involved in various physiological processes such as calcium, phosphorus metabolism and bone mineralisation (DeLuca, 2004). This thesis has investigated the role of 1,25(OH)₂D₃ on embryonic bone development by in ovo injection and during osteogenic differentiation of chicken mesenchymal stem cells (MSCs) in vitro. The effects of exogenous 1,25(OH)₂D₃ were examined during osteogenic induction between avian and mammalian species. This thesis, for the first time has investigated the response of exogenous 1,25(OH)₂D₃ treatment between both broiler and layers strains during osteogenic differentiation. The key findings of both in ovo and in vitro experiments examining the effects of 1,25(OH)₂D₃ during bone development are discussed below and where appropriate, the directions for future studies are proposed. At the end, the limitations observed during the conduct of experiments are briefly discussed.

4.1. Effects of 1,25(OH)₂D₃ during in ovo osteogenesis

As discussed in the literature review, most of the earlier studies examined the effect of 1,25(OH)₂D₃ either in vitamin D deficient embryos or the embryos hatched from hens fed 1,25(OH)₂D₃ as only source in their diet and demonstrated the efficacy of 1,25(OH)₂D₃ in improving embryo survival and growth rate. These studies suggest vitamin D₃ deficiency during egg formation may have a significant impact on embryo development and that adequate transfer of nutrients from the maternal source is important for
successful embryonic development (Moran, 2007; Oviedo-Rondón et al., 2006).

The first experiment examined the effect of 1,25(OH)$_2$D$_3$ in ovo injection on either day 8 or day 11 of embryonic development on various parameters such as embryo viability, gross abnormalities, bone length and proliferation of cells from different zones of tibial growth plate at day 19.

4.1.1. Viability of chicken embryo was not affected by in ovo injection of 1,25(OH)$_2$D$_3$

In this study, there was no significant difference observed in viability percentage. However, at high (1.2µM/12,00000pmoles/50ng) concentration of 1,25(OH)$_2$D$_3$ a trend towards a decrease ($p=0.057$) in viability was observed, suggesting high concentrations of 1,25(OH)$_2$D$_3$ could be lethal for embryonic survival. Similarly Narbaitz & Fragiiskos (1984) found that the higher doses (100ng) of 1,25,(OH)$_2$D$_3$ resulted into higher mortality of chick embryos injected on day 9 and 10 of incubation. On the other hand, earlier studies (Moriuchi & Deluca, 1974; Narbaitz & Tolnai, 1978) reported significant reduction in embryo viability after in ovo injections of 1,25(OH)$_2$D$_3$ above 300pmoles whereas doses below 300pmoles were well tolerated by embryo and did not impact on survival rate.

The doses of vitamin D$_3$, both 0.12µM/12,00000pmoles/5ng and 1.2µM/12,00000pmoles/50ng used in the present study suggest that the higher concentration of vitamin D$_3$ is detrimental to the survival of chick embryo and the doses below 300pmoles could be well tolerated by chick embryo. It is well known that 1,25(OH)$_2$D$_3$ is responsible for mobilisation of calcium from the egg shell across the chorioallantoic membrane to the
developing embryo. It is postulated that the higher concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3} has resulted in a hypercalcemia and thereby causing the reduction in embryo viability.

Though the trend towards decrease in viability was observed in high vitamin D of day 11 injection group, further studies are required to deduce the significance of this finding. This hypothesis might not be complete until further experiments are conducted with other parameters such as systemic levels of calcium and phosphorous after exogenous administration of 1,25(OH)\textsubscript{2}D\textsubscript{3}.

4.1.2. **In ovo injection of 1,25(OH)\textsubscript{2}D\textsubscript{3} reduced body weight and bone length**

There was a significant reduction in body weight and bone length of both femur and tibia as a result of *in ovo* injection of 1,25(OH)\textsubscript{2}D\textsubscript{3} at a concentration of 0.12μM (5ng) when injected on day 11 of embryonic development. In an earlier study by (Narbaitz & Tsang, 1989) an improvement in body weight was observed in vitamin D deficient embryos injected with 10ng of calcitriol, however higher level of calcitriol (100ng) caused reduction in body weight of chick embryos. They concluded that calcitriol had increased the bone resorption and mineralisation of bone and thereby improved the body weights when lower dose of calcitriol (5ng) was injected to vitamin D deficient embryos. Conversely, a higher concentration of calcitriol (100ng) had reduced the muscle, bone weight and bone resorption as a result lowering the body weight in chicken embryo.

In the present study, administration of 5ng of 1,25(OH)\textsubscript{2}D\textsubscript{3} on day 11 reduced body weight of embryos. This could be a result of an increase in
calcium mobilisation from shell and reduction in circulating levels of phosphorous due to increased calcium absorption.

The exact mechanism of reduced body weight and bone length is still unclear. The embryonic bone and muscle growth is mainly under the influence of Somatomedin C produced in the liver. In addition, there are other factors known to contribute embryonic growth such as corticoids and thyroid hormones that indirectly influence the production of Somatomedin C (Scanes, et al., 1984). In this study the Somatomedin production was not analysed however, this finding could suggest a mechanism behind the reduction in body weight and bone length of chicken embryo. Further studies examining involvement of Somatomedin and other growth hormones could answer the mechanism of growth reduction in chicken embryo.

In the present study, the test system did not use embryos that were vitamin D deficient and these findings suggest that additional doses of 1,25(OH)$_2$D$_3$ do not influence the survival and body weight of chicken embryo provided the eggs are adequate in vitamin D$_3$ or its metabolite from maternal circulation. Future studies, to explore the role of 1,25(OH)$_2$D$_3$ on embryo viability and body weight or to produce toxicity signs are required at the higher doses than doses examined in the present study.

4.1.3. Growth plate morphology was not influenced by in ovo injection of 1,25(OH)$_2$D$_3$

Earlier studies in growing birds demonstrated the preventive role of 1,25(OH)$_2$D$_3$ to combat incidence and severity of bone disorders such as rickets and tibial dyschondroplasia which are the result of failure of chondrocyte proliferation (Edwards Jr, 1989, 1990; Elliot & Edwards Jr,
It is well known that vitamin D₃ metabolites regulate chondrocyte metabolism and bone formation. The biological effects of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR) in the target tissues.

Previous studies in developing chicken embryo and rat identified the receptors for 1,25(OH)₂D₃ in proliferating and hypertrophic chondrocytes, suggesting a role of vitamin D₃ metabolite in chondrocyte metabolism (Klaus et al., 1991; S. Suda et al., 1985).

In chickens, longitudinal growth occurs by proliferation and hypertrophy of chondrocytes forming a cartilage in the growth plate of the long bones. Subsequent mineralisation of cartilage forms the osseous tissue by the actions of osteoblasts and osteoclasts (Farquharson & Jefferies, 2000; D. Liu, et al., 2003). There is little information available on the role of 1,25(OH)₂D₃ in chondrocyte proliferation and differentiation during chicken embryonic development; it was hypothesized that administration of 1,25(OH)₂D₃ would enhance the bone growth during embryonic development.

In the first experiment, chondrocyte proliferation in three different zones of the proximal and distal end of the embryonic tibia growth plate was examined after in ovo injections of 1,25(OH)₂D₃. Histomorphometric analysis of the growth plates showed no difference in length of different zones of proximal and distal tibia both at day 8 or day 11 of in ovo injection group suggesting no effect of 1,25(OH)₂D₃ on chondrocyte proliferation.

In vitamin D deficient embryo, hypocalcemia and hypophosphatemia inhibits the mineralisation of extracellular matrix. As a result, chondrocyte
in proliferative and hypertrophic zone fail to mature thereby making the growth plate zones wider, a characteristic of vitamin D deficiency. Previous studies showed that *in ovo* injection of vitamin D₃ on day 8 of embryonic development at 300ng was able to correct rickets and the height of growth plate zones usually seen in vitamin D deficient chicks (King, et al., 1991; Lacey & Huffer, 1982) however, the present study examined the effect of 1,25(OH)₂D₃ in normal avian embryos and did not find the ameliorative effect on cell proliferation of different zones of growth plate.

There are reports of a positive correlation between vitamin D₃ in the hens diet and the amount of vitamin D₃ content in the egg (Atencio, et al., 2005; Mattila, et al., 1999). The findings of our study suggests that, from breeder level the adequate carryover of cholecacliferol and vitamin D₃ metabolites to the developing chick embryo may be sufficient and additional *in ovo* administration of 1,25(OH)₂D₃ does not stimulate chondrocyte proliferation in the growth plate of the long bones.

In avian and mammals, cellular events in endochondral ossification are similar however there are differences in the longitudinal bone growth (Roach, 1997). It is well known that proliferation and differentiation of bone cells is under the influence of many factors. Vitamin D₃ metabolites are found to play a major role in bone cell metabolism and are required for normal bone development (Farquharson & Jefferies, 2000). Therefore, future *in vitro* and *in vivo* studies examining the pathways involved in the cellular proliferation, and differentiation of osteoprogenitor cells would aid in understanding the precise mode of action of 1,25(OH)₂D₃ in skeletal disorders or bone diseases in poultry.
In addition, there are other sensitive indicators used to determine bone quality such as bone ash, bone breaking strength and bone mineral density measurement by quantitative computed tomography which in future studies would provide an in depth knowledge of embryonic bone development in response to vitamin D₃ administration *in ovo*.

4.2. *Effects of 1,25(OH)₂D₃ during in vitro osteogenic differentiation of mesenchymal stem cells*

The role of 1,25(OH)₂D₃ during *in vitro* osteogenesis is well characterised in rat (Atmani, et al., 2003; Owen, et al., 1991), mouse (Ecarot & Desbarats, 1999) and human (Fromigué, et al., 1997; P. Liu et al., 1999) however its role in avian species is poorly understood. Use of MSCs as a model aids to elucidate the process of bone development and key proteins involved during *in vitro* osteogenesis. Therefore, second experiment was carried out to examine the effects of exogenous 1,25(OH)₂D₃ on both rat and chicken MSCs at low (2.4nM) and high (24nM) concentrations during osteogenic differentiation. In addition, the second experiment also explored the effects of 1,25(OH)₂D₃ on mineralisation, early osteogenic differentiation and cell proliferation in three different strains of poultry (commercial broiler, commercial layer and SPF layer). Till date, there are no published reports that has examined the strain specific differences in response to exogenous 1,25(OH)₂D₃ during *in vitro* bone formation. The following section describes key findings of the *in vitro* experiment and where appropriate directions of future studies are suggested.
4.2.1. **Difference in osteogenic differentiation between avian and mammalian species**

*In vitro* osteogenesis is characterised by mineralisation of the extracellular matrix and provides critical information of osteoblast differentiation and maturation. The novel finding of this experiment was early evidence of calcium deposition as early as day three (D3) of osteogenic induction in all strains of chicken MSCs examined, whereas in rat cultures the appearance of bone nodules were reported on day 10 of osteogenic differentiation (Malaval, et al., 1994). Such early and rapid onset of calcium deposition during *in vitro* osteogenic differentiation has not been reported yet in poultry and this is the first study to identify species specific difference during osteogenic differentiation between avian and mammalian species.

The mechanism behind the early onset of mineralisation in avian species is unclear. Osteocalcin is an abundant non-collagenous protein in bone produced by osteoblasts. Osteocalcin is considered a marker of matured osteoblast (Driel, et al., 2004; Owen, et al., 1990). The results of previous studies have shown that the level of osteocalcin in chick osteoblasts was almost five times higher than found in 21 day rat calvaria osteoblast (Hauschka, et al., 1983; Lian, et al., 1985; Lian, et al., 1982). Therefore, avian bone may contain more mature osteoblasts and less pre-osteoblast populations than mammalian species and consequently the time required to form an extracellular matrix and subsequent mineralisation may be much less compared to mammalian cell population. In the present study, the cell populations used for the osteogenic differentiation from both poultry and rat were isolated from the femur bone and an expression of osteocalcin was not examined. However, future studies investigating the expression of late stage
markers such as osteocalcin and osteopontin (Olsen, et al., 2000) could help to understand the mechanism of early mineralisation in avian species.

4.2.2. **Exogenous 1,25(OH)\(_2\)D\(_3\) suppresses mineralisation during in vitro osteogenic differentiation of rat and chicken MSCs**

In this study, exogenous 1,25(OH)\(_2\)D\(_3\) inhibited calcium deposition in a dose dependent manner both in avian and mammalian species and the effect was maximal at higher concentration (24nM). To my knowledge, this is the first study that has explored strain specific differences in response to exogenous 1,25(OH)\(_2\)D\(_3\) treatment during *in vitro* osteogenic differentiation. Compared to broilers, layer strains were found to be most sensitive to increasing doses of exogenous 1,25(OH)\(_2\)D\(_3\). Inhibition of extracellular matrix mineralisation by 1,25(OH)\(_2\)D\(_3\) at 10 or 100nM has been reported in mouse bone marrow stromal cells, MC3T3-E1 pre osteoblastic cells and chick osteoblasts derived from calvaria (Broess, et al., 1995; Yamaguchi & Weitzmann, 2012). The findings of this study shows the importance of critical doses of 1,25(OH)\(_2\)D\(_3\) between the species and strains examined and this needs to be balanced to prevent the inhibitory action of 1,25(OH)\(_2\)D\(_3\) on bone forming cells. Further *in vivo* studies under practical conditions would be appropriate to understand the precise mechanism of 1,25(OH)\(_2\)D\(_3\) action in bone formation between different commercial strains of poultry.

There are various markers that are expressed at different stages of osteogenic differentiation and involved in matrix mineralisation. The results of previous studies (Broess, et al., 1995; Ecarot & Desbarats, 1999; Owen, et al., 1991) demonstrated that treatment of rat, chicken and mouse osteoblast cells with 1,25(OH)\(_2\)D\(_3\) inhibited collagen type I, osteocalcin,
osteopontin and phex expression and inhibition of these osteoblastic markers could contribute to the decrease in mineralisation of the extracellular matrix.

TGF-β and BMP2 are well known inducers of osteoblast differentiation. However, TGF-β and BMP2 induced-activation of the Smad pathway was strongly inhibited by 1,25(OH)₂D₃ in the MC3T3 osteoblastic cell line, which may account for the inhibition of mineralisation in vitro (Yamaguchi & Weitzmann, 2012). Therefore, future studies directed towards investigation of the TGF-β and BMP2 induced-activation of Smad pathway in the chicken MSCs could answer the question of the mechanism behind suppression of calcium deposition observed in the present study.

It is known that 1,25(OH)₂D₃ at multiple level of osteoblastic growth regulates the transcriptional and post transcriptional control of gene expression involved in bone development (Y. Li, et al., 2008). Thus, regulation of molecular processes in response to 1,25(OH)₂D₃ between species must be complex. Previous work has shown the differences between rat and mouse osteoblasts nucleotide sequence in the distal portion of osteocalcin vitamin D receptor elements (VDRE). These variations were associated with different effects of 1,25(OH)₂D₃ (Lian et al., 1997). Based on the data of the present study further investigations are needed to determine the differences between rat and chicken nucleotide sequence of VDRE that are controlling the osteoblast growth and differentiation and thus contributing to different response to 1,25(OH)₂D₃ treatment during osteogenic differentiation.
4.2.3. **Cellular differentiation of chicken MSCs is reduced by exogenous $1,25(\text{OH})_2\text{D}_3$**

During osteogenic induction, cells need to differentiate into bone forming cells. There are markers which are well correlated with the differentiation of the osteoblast phenotype. One of the markers of early osteogenic differentiation is ALP and its induction is associated with matrix mineralisation and bone formation (Atmani, et al., 2003; Declercq et al., 2005; zur Nieden, et al., 2003).

In the second experiment of this thesis, chicken MSCs from all strains were examined for ALP by histochemical staining at different time points to explore the possibility that the observed inhibition of calcium deposition in cultures by exogenous $1,25(\text{OH})_2\text{D}_3$ treatment is a consequence of reduction in osteoblastic phenotype. The results of ALP staining showed that exogenous $1,25(\text{OH})_2\text{D}_3$ at dose dependent concentration decreased ALP staining in all strains and at all days examined. These results are compatible with reduction in ALP observed in mouse bone marrow stromal cells and chick calvarial osteoblasts by exogenous $1,25(\text{OH})_2\text{D}_3$ treatment (Broess, et al., 1995; Y. Li, et al., 2008).

The findings of the present study suggest that $1,25(\text{OH})_2\text{D}_3$ acts on osteoprogenitor cells and prevent their progression towards matured osteoblasts. A brief hypothesis offered in this experiment states that the less number of functional osteoblasts in the culture could be responsible for decrease in matrix maturation and mineralisation.

Based on the results of this study, chicken MSCs are identified as a very sensitive model to study the effect of vitamin D$_3$ metabolite $1,25(\text{OH})_2\text{D}_3$
during bone development. The findings of this study has given new insight to further study of the underlying developmental differences and regulatory processes involved during osteogenic differentiation of chicken MSCs in vitro.

It is known that osteoblast differentiation is divided into three different developmental stages; proliferation, matrix maturation and mineralisation. During each stage of differentiation osteoblasts express phenotype markers of stage specific differentiation (Olsen, et al., 2000). Up regulation of cell cycle associated genes demonstrates the proliferation phase (Collagen I) while expression of early osteogenic markers (alkaline phosphatase) demonstrates the maturation phase. Finally mineralisation of extracellular matrix is demonstrated by the expression of late markers of osteogenic differentiation such as osteoclastin, osteopontin and bone sialoprotein (Olsen, et al., 2000; Owen, et al., 1991; zur Nieden, et al., 2003). In addition, differentiation of MSCs to an osteogenic lineage occurs through activation of specific transcription factors. Runt related transcription factor 2 (Runx2) is a primary or master regulator of osteoblast differentiation and chondrogenesis (Coffman, 2003; Ducy et al., 1999; Gerard, 2000; Komori, 2006; Komori et al., 1997). Therefore, to understand the influence of 1,25(OH)2D3 on osteoblast growth and differentiation further studies investigating the molecular mechanism at specific stages of maturation and involvement of key transcription factors that governs osteoblast differentiation are warranted.
4.2.4. **Exogenous 1,25(OH)\(_2\)D\(_3\) exerts anti-proliferative action on chicken MSCs in vitro**

Osteoprogenitor cells originate from MSCs and through proliferation and differentiation develop into pre-osteoblasts, mature osteoblasts and terminally differentiate into either osteocytes or bone lining cells (Blair et al., 2002). Therefore, understanding cell proliferation in response to exogenous 1,25(OH)\(_2\)D\(_3\) treatment is fundamental during bone formation.

As seen in the second experiment, the proportion of BrDU positive cells was reduced by exogenous 1,25(OH)\(_2\)D\(_3\) in a dose dependent manner both at day 3 and day 5 of osteogenic induction in all strains of chicken MSCs (Figures 3.9-3.10.). This finding suggest a potent anti-proliferative action of 1,25(OH)\(_2\)D\(_3\) during *in vitro* osteogenic differentiation in all strains of chicken MSCs examined. Several studies have reported similar anti-proliferative action of 1,25(OH)\(_2\)D\(_3\) in many cell types including tumour cell lines (Artaza, et al., 2010; Geng, et al., 2011; Gumireddy, et al., 2003; Y. Li, et al., 2008).

In this thesis, BrDU content in cells were detected by immunocytochemical methods to assess cell proliferation. This is a method that aims to identify the cell proliferation over time in culture; however this study has looked at only proportions of BrDU positive cells. Further investigations examining the alterations that occurred in cell cycle progression after exogenous 1,25(OH)\(_2\)D\(_3\) would be useful to determine cell cycle kinetics in response to 1,25(OH)\(_2\)D\(_3\) treatment.

Cell proliferation is under the regulation of extracellular signals that control the cell cycle by monitoring many proteins. These include cyclins, cyclin
kinases and cyclin dependent kinase inhibitors (Song et al., 2011). It has been suggested that the inhibitory effects of 1,25(OH)$_2$D$_3$ on cell proliferation are mediated via several mechanisms namely: stimulation of p21$^{Waf1/Cip1}$ and reduction in the expression of cyclinD1 (Geng et al., 2011).

From the results of the present study, it could be postulated that the possible involvement of cell cycle signalling pathways or key cell cycle regulators are influenced by 1,25(OH)$_2$D$_3$. Further in depth research in avian MSCs is necessary to delineate the involvement of cell cycle related proteins controlling the cell proliferation.

**Limitations of study**

In this project, there were certain limitations observed in the first experiment which has caused variations between runs on different parameters. However, these variations did not impact the overall results of parameters studied and has been proved by statistical analysis.

In future, to overcome such differences in between experiments, homogeneity in the experimental conditions is essential. The factors that should be taken care of for future experiments are, use of fertile eggs from similar broiler breeder hen age and minimising the duration of pre incubation to incubation. Also, additional treatment group for 1,25(OH)$_2$D$_3$ that could elicit the toxic response would help to understand the actual mode of action and the effect of vitamin D$_3$ metabolite, 1,25(OH)$_2$D$_3$ in chicken embryonic bone development.
Concluding remarks

The aim of this thesis was to study the effects of exogenous $1,25(\text{OH})_2\text{D}_3$ at varying concentrations during *in ovo* and *in vitro* osteogenesis in chickens. The effects of exogenous $1,25(\text{OH})_2\text{D}_3$ were examined on different parameters in both *in ovo* and *in vitro* studies. Based on the results of these studies the following conclusions can be drawn:

1. *In ovo* injection of exogenous $1,25(\text{OH})_2\text{D}_3$ on day 8 or day 11 of embryonic development did not affect embryo viability, reduced body weight and bone length and no significant effect was observed on growth plate morphology of chicken embryo.

2. This thesis has explored a novel finding for the first time; the differences in osteogenic differentiation between avian and mammalian species. The occurrence of mineralisation was early and rapid in avian compared to mammalian species during *in vitro* osteogenic differentiation.

3. Mineralisation was inhibited both in mammalian and avian species in dose dependent manner however, the direct effects of exogenous $1,25(\text{OH})_2\text{D}_3$ on osteoblasts was strain dependent. In all strains of chicken MSCs examined, layer strain were highly sensitive to increasing concentrations of exogenous $1,25(\text{OH})_2\text{D}_3$.

4. Exogenous $1,25(\text{OH})_2\text{D}_3$ inhibits cell proliferation and early osteogenic differentiation in a dose dependent manner in all strains of chicken MSCs examined.

5. Moreover, critical doses of vitamin $\text{D}_3$ are different between avian and mammalian species and between different strains of poultry that has strong impact on regulation of skeletal integrity and bone development.
6. Therefore, optimal species-specific concentration needs to be established by means of this economic and reproducible *in vitro* MSCs model system, to examine the critical levels and role of vitamin D$_3$ for optimal bone health and welfare of poultry and other production species.
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gene expression are related to the proliferative and differentiated state of the bone cell phenotype: Dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. *Endocrinology, 128*(3), 1496-1504. doi: 10.1210/endo-128-3-1496


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Appendices

I. Vitamin D₃ metabolite, 1,25(OH)₂D₃ stock and working solution preparation

II. Fixatives, decalcification solution and histological stains

III. Cell culture chemicals, stock solutions and media preparation

IV. Buffers and immunocytochemistry solutions

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Appendix I

Vitamin D₃ metabolite, 1,25(OH)₂D₃ stock and working solution preparation

Reagents used:

1,25(OH)₂D₃  
Sigma Aldrich (D 1530)

Mass - 10μg

Formula weight - 416.64

Dissolve 10μg of 1,25(OH)₂D₃ in 20μl of ethanol first then add 80μl of PBS = 100μl = 240.0154μM.

Dissolve this 100μl in 9900μl of PBS to give final stock 2.4μM.

Prepare 10, 100 and 500μl aliquots and store at -20°C.

Low vitamin D group treatment is 0.120μM/5 ng per egg. To prepare this solution take 100μl aliquot of 2.4μM stock and dissolve in 1900μl of PBS prior to injection.

High vitamin D group treatment is 1.2μM/50 ng per egg. To prepare this solution take 1ml aliquot of 2.4μM stock and dissolve in 1ml of PBS prior to injection.
**Reagents used:**

1,25(OH)$_2$D$_3$ Santa Cruz Biotechnology Inc (SCZSC-202877A)

Mass- 1mg

Formula weight- 416.64

Dissolve 1mg of 1,25(OH)$_2$D$_3$ in 10mls of ethanol to give a final concentration of 240.0384μM.

Aliquot 100μl of 240.0384μM solution and dissolve into 9900μl of complete media, this solution is now 2.4μM which can be further used for *in vitro* experiments.

For low vitamin D media add 10μl of 2.4μM 1,25(OH)$_2$D$_3$ into 9990μl of complete media to give a final concentration of 2.4nM.

High vitamin D media was prepared by adding 100μl of 2.4μM 1,25(OH)$_2$D$_3$ to 9900μl of complete media to give a final concentration of 24 nM.
Appendix II

Fixatives, decalcification solution and histological stains

Reagents used:

Ethanol
Scharlau (ET-00072500)

Alizarin red S
Merck (106278)

Paraformaldehyde (PFA)
Merck (1.04005)

Formic acid
Sigma Aldrich (33015)

Oil Red O stain
Sigma Aldrich (O0625)

Isopropanol
Fisher Scientific (P/7507/17)

70% ethanol – 1 litre

Ethanol
700mls

Distilled water (dH₂O)
300mls

4% PFA- 1 litre

PFA
40g

PBS
800mls

Heat to a 65°C using water bath or hot plate. Add 1-2mls of 1M NaOH to dissolve PFA, continue heating until PFA is fully dissolved. Make up final volume up to 1 litre. Filter PFA solution through Whatmann filter paper or 0.22μm filter. Prepare 50mls aliquots and store at -20°C.
5% formic acid solution- 1 litre

Formic acid 50mls

dH₂O 950mls

Alizarin red stain

Alizarin Red S 2g

dH₂O 100mls

Mix well. Adjust the pH to 4.1-4.3 with 10% ammonium hydroxide. Once solution is prepared it is stable for 3 months. Keep the stain in dark.

Oil Red O Stain stock solution

Oil Red O 0.5g

Isopropanol 100mls

Incubate stock solution at room temperature for 1 hour, filter through a 0.2μm filter. Stock solution can be kept at room temperature up to 1 year.

Oil Red O working solution

Oil Red O stock solution 6mls

dH₂O 4mls

Incubate at room temperature for 1 hour, filter through a 0.2μm filter. The working solution is stable for approximately 3 hours at room temperature.
Appendix III

Cell culture chemicals, stock solutions and media preparation

Reagents used:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ascorbic acid</td>
<td>Sigma Aldrich (A4544)</td>
</tr>
<tr>
<td>B-glycerol phosphate disodium salt pentahydrate</td>
<td>Sigma Aldrich (50020)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Sigma Aldrich (D4902)</td>
</tr>
<tr>
<td>Heat inactivated foetal bovine serum (HIFBS)</td>
<td>Gibco, Life Technologies (10082)</td>
</tr>
<tr>
<td>Embryonic stem cell foetal bovine serum (ES cell FBS)</td>
<td>Gibco, Life Technologies (10439)</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>Gibco, Life Technologies (11360)</td>
</tr>
<tr>
<td>PenStrep (Penicillin &amp; Streptomycin)</td>
<td>Gibco, Life Technologies (15140)</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Gibco, Life Technologies (10010)</td>
</tr>
<tr>
<td>Glasgow Minimum Essential Medium 1x (GMEM)</td>
<td>Gibco, Life Technologies (11710)</td>
</tr>
<tr>
<td>TrypLE™ express (1x)</td>
<td>Gibco, Life Technologies (12604)</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Aldrich (D4540)</td>
</tr>
<tr>
<td>Ammonium hydroxide</td>
<td>Sigma Aldrich (320145)</td>
</tr>
<tr>
<td>Trypan blue stain 0.4%</td>
<td>Gibco, Life Technologies (15250)</td>
</tr>
<tr>
<td>Normal goat serum</td>
<td>Fitzgerald (88-NG22S)</td>
</tr>
</tbody>
</table>
### Reagents used:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier and Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell strainer-70μm</td>
<td>BD Falcon™ (352350)</td>
</tr>
<tr>
<td>Isobutylmethyaxanthine</td>
<td>Sigma-Aldrich (I7018)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Sigma-Aldrich (I2643)</td>
</tr>
<tr>
<td>T75 flask</td>
<td>3123-075, Iwaki® cell biology</td>
</tr>
<tr>
<td>Viraclean disinfectant</td>
<td>Whiteley Medical (R 69000)</td>
</tr>
</tbody>
</table>
Stock Solutions:

L-ascorbic acid stock solution (20mM stock)

L-ascorbic acid 176.12mg
dH₂O 50mls

Allow to dissolve, filter sterilise and store at 4°C.

β-glycerol phosphate disodium salt pentahydrate stock solution (200mM stock)

β-glycerol phosphate disodium salt pentahydrate 306.11mg
dH₂O 5mls

Allow to dissolve, filter sterilise and store at 4°C

Dexamethasone stock solution (25mM)

Dexamethasone 100mg
Ethanol 10.19mls

Aliquot once dissolved into 1.5μl eppendorf tube. Aliquots can be stored at -20°C for 6 months.

Dexamethasone working solution (1mM)

Dexamethasone stock solution (25mM) 40μl
Complete media 960μl

Store working stock solution at 4°C.
**Insulin from human pancreas (200μM) (1.16mg /ml) stock solution**

- Insulin  
- dH₂O

Add 10-15mls of dH₂O to insulin powder. If insulin fails to dissolve completely add 0.01M HCL up to 1ml then add remaining dH₂O up to 21.52mls. Then store at 4°C.

---

**Potassium hydroxide (KOH) (0.5N)**

- KOH 1.4028g
- Deionised water 50mls

Allow to dissolve, filter sterilize through 0.22μm syringe filter. Store at room temperature

---

**Isobutylmethylxanthine (IBMX) stock solution (25mM)**

- IBMX 100mg
- 0.5 N KOH 17.998mls

Mix well, filter sterilize through 0.22μm syringe filter before aliquoting into 1.5μl eppendorf. Store at -20°C and keep 2-3 eppendorf for regular use at 4°C.

---

**Preparation of complete media/ Maintenance media (50mls)**

- GMEM 44mls
- HI FBS (10%) 5mls
- Sodium pyruvate (1%) 500μl
- PenStrep (1%) 500μl
**Preparation of osteogenic differentiation / induction media**

Add to 10mls of complete media the following components:

- β-glycerol phosphate disodium salt pentahydrate (10mM) 500μl of stock solution
- L-ascorbic acid (0.05mM) 25μl of stock solution
- Dexamethasone (100nM) 1μl of working stock

**Preparation of freezing media (10mls)**

Complete media 6mls
HI FBS 2mls
Dimethyl sulfoxide 2mls

For freezing cell, mix 1:1 v/v of freezing media and required cell suspension.

**Preparation of adipogenic differentiation media**

Add to 10mls of complete media the following components:

- Dexamethasone (1mM working stock) 1μl
- Insulin (Stock solution 200μM) 8.62μl
- IBMX (25mM stock solution) 200μl
**Preparation of MSCs derivation media (50mls)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMEM</td>
<td>44mls</td>
</tr>
<tr>
<td>ES cell FBS (10%)</td>
<td>5mls</td>
</tr>
<tr>
<td>Sodium pyruvate (1%)</td>
<td>500μl</td>
</tr>
<tr>
<td>PenStrep (1%)</td>
<td>500μl</td>
</tr>
</tbody>
</table>
### Appendix IV

**Buffers and immunocytochemistry solutions**

<table>
<thead>
<tr>
<th>Reagents used:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Merck (6.10241)</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Merck (6.10198)</td>
</tr>
<tr>
<td>Di-sodium hydrogen phosphate (Na$_2$HPO$_4$)</td>
<td>Merck (6.10249)</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH$_2$PO$_4$)</td>
<td>Merck (6.10203)</td>
</tr>
<tr>
<td>Tris (hydroxymethyl)aminomethane</td>
<td>Sigma Aldrich (T-87602)</td>
</tr>
<tr>
<td>Tween® 20 (Polyoxyethylene sorbitan monolaurate)</td>
<td>Sigma Aldrich (T-87602)</td>
</tr>
<tr>
<td>Bromodeoxyuridine (BrDU)</td>
<td>Sigma Aldrich (B5002)</td>
</tr>
<tr>
<td>Triton™ X-100</td>
<td>Sigma Aldrich (X100)</td>
</tr>
<tr>
<td>Hydrochloric acid (HCL)</td>
<td>Merck (6.10307)</td>
</tr>
<tr>
<td>Anti–BrDU antibody</td>
<td>Becton Dickinson (347580)</td>
</tr>
<tr>
<td>Dako Real Envision Detection System, peroxidase/DAB, rabbit /Mouse</td>
<td>Dako (K007)</td>
</tr>
</tbody>
</table>
Preparation of BromoDeoxyUridine (10mg/ml stock solution)

Dissolve BrDU in PBS by heating to 60°C after raising the pH very slightly with 1-2 drops of NaOH. Store in 0.2mls aliquots at -20°C freezer until use.

1 M HCL

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>HCL</td>
<td>4.35mls</td>
</tr>
<tr>
<td>dH₂O</td>
<td>45.65mls</td>
</tr>
</tbody>
</table>

Rinse buffer- (TBST) 1x - 1 litre

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.8g</td>
</tr>
<tr>
<td>KCL</td>
<td>0.2g</td>
</tr>
<tr>
<td>Tris</td>
<td>3g</td>
</tr>
<tr>
<td>Tween 20</td>
<td>500μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1000mls</td>
</tr>
</tbody>
</table>

Adjust to pH 7.4. Sterilise by filtration or autoclaving before use.
PBS (10x)

Dissolve the following in 800mls distilled water:

- NaCl 80g
- KCl 2.0g
- Na₂HPO₄ 14.4g
- KH₂PO₄ 2.4g

Adjust to pH 7.4. Make up final volume to 1 litre with dH₂O. Sterilise by autoclaving prior to use. To make 1X PBS add 100mls of 10x PBS to 900mls of dH₂O.

PBSTX (0.1%)

- PBS 1x 9.9mls
- Triton X 0.1ml

Normal goat serum (10%)

- PBSTX (0.1%) 9mls
- Normal goat serum 1ml
Appendix V

Histology stains, tissue processing and H & E staining protocol

Reagents used:

- Suripath® Harris’ Haematoxylin
- Leica microsystems (3801560)
- Eosin Y
- Australian Biostain (ADEY-50G)

Tissue processing protocol

Instrument used: Shandon Excelsior ES, Thermo Electron Corporation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time (hours)</th>
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</thead>
<tbody>
<tr>
<td>1. Alcohol (75%)</td>
<td>0:45</td>
</tr>
<tr>
<td>2. Alcohol (85%)</td>
<td>0:45</td>
</tr>
<tr>
<td>3. Alcohol (95%)</td>
<td>0:45</td>
</tr>
<tr>
<td>4. Alcohol(100%)</td>
<td>0:50</td>
</tr>
<tr>
<td>5. Alcohol(100%)</td>
<td>0:50</td>
</tr>
<tr>
<td>6. Alcohol(100%)</td>
<td>1:00</td>
</tr>
<tr>
<td>7. Histopure</td>
<td>1:30</td>
</tr>
<tr>
<td>8. Histopure</td>
<td>1:30</td>
</tr>
<tr>
<td>9. Histopure</td>
<td>1:30</td>
</tr>
<tr>
<td>10. Paraffin wax</td>
<td>1:30</td>
</tr>
<tr>
<td>11. Paraffin wax</td>
<td>1:30</td>
</tr>
<tr>
<td>12. Paraffin wax</td>
<td>1:30</td>
</tr>
</tbody>
</table>
# H & E autostaining protocol

**Instrument used:** Shandon Gemini ES, Thermo Fischer Scientific.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dry storage</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>Histopure</td>
<td>4 minutes</td>
</tr>
<tr>
<td>3.</td>
<td>Histopure</td>
<td>3 minutes</td>
</tr>
<tr>
<td>4.</td>
<td>Histopure</td>
<td>2 minutes</td>
</tr>
<tr>
<td>5.</td>
<td>Ethanol 100%</td>
<td>2 minutes</td>
</tr>
<tr>
<td>6.</td>
<td>Ethanol 100%</td>
<td>2 minutes</td>
</tr>
<tr>
<td>7.</td>
<td>Ethanol 95%</td>
<td>2 minutes</td>
</tr>
<tr>
<td>8.</td>
<td>Running water</td>
<td>2 minutes</td>
</tr>
<tr>
<td>9.</td>
<td>Haematoxylin</td>
<td>6 minutes</td>
</tr>
<tr>
<td>10.</td>
<td>Running water</td>
<td>1 minutes</td>
</tr>
<tr>
<td>11.</td>
<td>Acid alcohol</td>
<td>8 seconds</td>
</tr>
<tr>
<td>12.</td>
<td>Running water</td>
<td>1 minutes</td>
</tr>
<tr>
<td>13.</td>
<td>Scott’s blueing water</td>
<td>1 minutes</td>
</tr>
<tr>
<td>14.</td>
<td>Running water</td>
<td>1 minutes</td>
</tr>
<tr>
<td>15.</td>
<td>Aqueous eosin</td>
<td>20 seconds</td>
</tr>
<tr>
<td>16.</td>
<td>Running water</td>
<td>30 seconds</td>
</tr>
<tr>
<td>17.</td>
<td>Ethanol 95%</td>
<td>10 seconds</td>
</tr>
<tr>
<td>18.</td>
<td>Ethanol 100%</td>
<td>1 minutes</td>
</tr>
<tr>
<td>19.</td>
<td>Ethanol 100%</td>
<td>1 minutes</td>
</tr>
<tr>
<td>20.</td>
<td>Histopure</td>
<td>2 minutes</td>
</tr>
<tr>
<td>21.</td>
<td>Histopure</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>
Appendix VI

In ovo injection of vitamin D

Procedure:

1. Wear lab coat and latex gloves.
2. Remove the tray of eggs from incubator.
3. Candle each egg to check for viability and mark the air shell with pencil. Also, mark with pencil a dot showing the position of the embryo.
4. Wipe the air shell area with a swab of 70% ethanol.
5. Make a hole in the centre of air shell with 19gauge needle.
6. Place a needle onto a petri dish containing a 70% ethanol soaked swab to keep it sterile.
7. Fill a 1ml tuberculin syringe fitted with a 25gauge needle with 100μl of the vitamin D solution or the vehicle (PBS).
8. Insert a needle into the hole of air shell. Angling needle diagonally away from embryo, lower the needle into the egg until it is inserted up to the hub of the syringe.
9. Slowly inject the 100μl of fluid into the yolk sac.
10. Remove the needle from the syringe and discard immediately into a sharps container.
11. Seal the hole in the air shell with silicone sealant.
12. Put the tray of eggs back into the incubator within 20 minutes of injections.
13. Dispose all needles into sharps container after injections.
14. Discard gloves and used syringes in biological waste bin and autoclave.
Poultry strains

The detailed information of eggs and their source used for derivation of mesenchymal stem cells (MSCs) is presented below.

<table>
<thead>
<tr>
<th>Strain name and total eggs for cell derivation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial broiler (Ross 308) n=48</td>
<td>The Báiada hatchery, Farm 1311, Snaidero Road, Griffith, NSW 2680, Australia.</td>
</tr>
<tr>
<td>SPF layer (Lohmann LSL) n=30</td>
<td>Hy-line Australia Pty Ltd 1555 Midland Hwy 3551 Bendigo, Victoria, Australia.</td>
</tr>
<tr>
<td>Commercial layer (Hy-Line) n=45</td>
<td>Australian SPF Services Pty. Ltd 14 James Road P.O. Box 641 Woodend, Victoria, Australia 3442</td>
</tr>
</tbody>
</table>
Isolation and culture of chicken bone marrow derived mesenchymal stem cells

Procedure:

1. Derivations must be performed in a biosafety cabinet.
2. Fertile eggs, commercial broiler (Ross 308) from the Baiada hatchery (Farm 1311, Snaidero Road, Griffith, NSW 2680, Australia); SPF layer (Lohmann LSL) Hy-line Australia Pty Ltd 1555 Midland Hwy 3551 Bendigo, Victoria, Australia; Commercial layer (Hy-Line) Australian SPF Services Pty. Ltd 14 James Road P.O. Box 641 Woodend, Victoria, Australia 3442 are used for derivation.
3. Place the eggs in an automatic rolling incubator (GQF 1502 SPORTSMAN"GQF Inc.) at 37°C with 45-60% relative humidity to day 19.
4. Dissect foetus from eggs and euthanize by cervical decapitation.
5. Place the chick foetus in a sterile petri dish. Harvest both hind limbs removing skin and muscle. Cut the limb above the hip and below the ankle joint; it is important to maintain the proximal and distal end of femur to ensure the sterility of the bone marrow.
6. Carefully dissect the knee joint and strip remaining connective tissue from the femur.
7. Collect all femurs, briefly wipe bones with gloved fingers dipped in 70% ethanol and place bones in dish of sterile 1x PBS (Pre-warmed to 37°C).
8. Wash bones by transferring through sterile pre-warmed PBS three times in three different petri dishes.
9. Remove the proximal and distal end of femur with scissors or a scalpel blade.

10. Attach 23 or 25gauge needle. Fill 10 or 20ml syringe with pre-warmed derivation media.

11. Use the syringe to force media through bone shaft using 23gauge needle to flush the marrow into 50mls conical tube. Repeat to ensure all marrow is removed. Bone should look white when finished. Continue until all bones are demarrowed.

12. Pipette cell mixture up and down a few to dissociate cells. You can also use syringe to pull large marrow pieces through needle to dissociate further.

13. Pass the cell suspension through a cell strainer (70μm) to remove any large cell clumps or bone particles.

14. Pool all cell suspensions into a 50mls conical centrifuge tube. Centrifuge at 400g for 10 minutes at RT.

15. After centrifugation, remove the supernatant and resuspend the cell pellet using complete media. Determine the number of viable cells using trypan blue.

**Trypan blue cell viability staining protocol.**

1. Remove 100μl of cell suspension and mix with 100μl of 0.4% trypan blue solution.

2. Count the viable cells from four corners of a standard haemocytometer. Calculate mean cell number multiply by dilution factor (2) and 10000 to give the viable cell count per ml.
16. Plate the cells at 1-2 x $10^6$/cm$^2$ in either a T25 or T75 flasks. Incubate cells at $37^\circ$C with 5% CO$_2$.

17. After 3 hours, remove the non-adherent cells by carefully removing the media and replacing with fresh derivation media.

18. After 8 hours of culture, replace media with fresh pre-warmed derivation media. Thereafter repeat this step every 8-12 hours up to 72 hours of initial culture.

19. Once cells are 80 to 90% confluent remove media and wash once with sterile PBS.

20. Passage the cells with Tryple Express$^\text{TM}$ 1x and split 1:3 or 1:5 v/v.

21. Up to passage one use, derivation media thereafter use maintenance media.

22. Expand cells to 70-90% confluent, changing media every 3-4 days. Cells can either passaged for experimental use or frozen for storage.
Cell passage

Procedure

1. Pre-warm all reagents to 37°C in water bath. Spay the bottles and laminar flow with 70% ethanol.

2. Aspirate culture media from the T75 or T25 cell culture flask in to beaker containing Viraclean disinfectant.

3. Wash the cells with 10mls of PBS. Remove the PBS and add 1ml per 25 cm² of Tryple™ express (1x) to the culture flask (3mls for a T75 culture flask and 1ml for T25 flask). Return the flask to the incubator for 5 minutes. After five minutes, examine the cells under a microscope. Fully trypsinized cells appear round and are separate from the surface of the flask.

4. Gently dislodge the cells by tapping the flask on solid surface. If the cells are not fully detached, return the flask to the incubator and leave to digest for a further 1-2 minutes.

5. Once all cells are detached, add complete media to a volume of 2-3x that of the Tryple™ express (1x) to the flask (7mls of complete media for a T75 culture flask and 3mls of complete media for a T25 culture flask).

6. Mix gently and remove cells to a 15mls centrifuge tube. Centrifuge for five minutes at 1100rpm.

7. Following centrifugation, remove supernatant and resuspend the cells in 2-5mls of maintenance media.

8. Remove 100μl of cell suspension and mix with 100μl of 0.4% trypan blue solution to determine cell viability as described previously.
9. Resuspend the cells in an appropriate volume of maintenance medium and dispense into sterile T75 flasks or 24 well plates. For T75 flask minimum cell seeding density is $1.2-1.5 \times 10^6$. For 24 well plates minimum cell seeding density is $5 \times 10^4$ cells per well.

10. Incubate at $37^\circ$C with 5% CO$_2$ and replacing media every 2-3 days.
Freezing cells

Procedure:

1. Allow cells to grow to 80-90% confluent. Dissociate and resuspend as described previously to a concentration of 2-3x10^6 cells/ml.

2. Prepare freezing medium immediately before use.

3. Remove 1ml of cell suspension to each freezing vial; gently add an equal volume of freezing medium to the cells.

4. Label the cryovials with date; passage number, cell type and cell density. Place cryovials at -20°C for one hour then transfer to -80°C freezer.

5. Cryovials can be then stored in liquid nitrogen for long-term storage.
Thawing of cells

Procedure:

1. Pre-warm prepared maintenance media to 37°C.

2. Remove the cryovial from deep freezer or from liquid nitrogen tank, thaw by immersing into 37°C water bath or place in 37°C incubator for 5-10 minutes.

3. Transfer the cells into a 15mls sterile conical tube. Slowly add 7mls maintenance media to the cells in a drop wise manner. Gently mix to re-suspend the cells then centrifuge the cells for 5 minutes at 1000rpm.

4. Aspirate the supernatant and resuspend cells in 2mls of maintenance media.

5. Add cell suspension to a T75 flask containing 10mls maintenance media.

6. Incubate cells at 37°C, 5% CO₂ and 95% humidity and allow the cells to adhere.

7. After 24 hours, replace the medium with an equal volume of fresh, pre-warmed maintenance media.

8. Change the medium every 2–3 days or until cell passage.
**Alizarin Red staining**

**Procedure:**

1. Gently remove the media from wells, wash once with 1x PBS.

2. Fix the cells with ice cold 4% PFA for five minutes. Wash wells twice with 1x PBS for five minutes.

3. Add 500μl of Alizarin Red stain to each well, leave to five minutes. Wash wells three times with dH₂O. Add 1ml of 1x PBS to each well to prevent from drying. Photograph each well under x10 magnification.

**Oil Red O Staining**

**Procedure:**

1. Aspirate media from culture wells.

2. Fix cells with 4% cold PFA for 5 minutes. Remove fixative and wash 3 times with 1x PBS.

3. Add 500μl of Oil Red O solution and leave for 1 hour at room temperature

4. Remove stain and wash with distilled water three times. Add 1ml of 1x PBS in each well to prevent from drying. Photograph at x10 magnification.
Alkaline phosphatase (ALP) staining

Alkaline Phosphatase Detection Kit

Catalogue Number: SCR004 Millipore

Procedure:

1. Gently remove the media from wells.

2. Fix the cells with 4% PFA for one to two minutes. Rinse the wells once with rinse buffer.

3. Prepare reagents for ALP staining from kit components as per manufacturer’s instructions- Fast red violet: Napthanol AS- BI phosphatase solution: dH₂O (2:1:1 ratio).

4. Add 250μl stain to each well and incubate in the dark at room temperature for 15 minutes.

5. Remove ALP stain from wells. Rinse the wells once with rinse buffer.

6. Add 1ml of 1x PBS to each well to prevent from drying.

7. Photograph each well under x10 magnification.
BrDU immunocytochemistry

Procedure:

1. Gently remove the media from wells. Wash with 1x PBS once.
2. Fix cells with ice cold 4% PFA for five minutes. After fixing, wash the wells with 1x PBS twice for five minutes each.
3. Add 500μl of 1M HCL to each well. Place dishes in oven at 60°C for 30 minutes.
4. Wash wells with 0.1% PBSTX twice for five minutes.
5. Block cells in 10% goat serum in PBSTX for 15 minutes - use 250μl in each well.
6. Add primary antibody-Mouse Anti BrDU at 1: 200 dilution. Dilute primary antibody in 0.1% PBSTX. Use 120μl of primary antibody for each well.
7. Place dishes at 4°C overnight. Cover the plates with parafilm to prevent from drying.
8. After incubation, remove primary antibody from wells by washing with 0.1% PBSTX twice for five minutes.
9. Add secondary antibody (Dako kit (K007)- Bottle A). Dilute at 1: 1 concentration in 0.1% PBSTX- for 30 minutes at room temperature.
10. Remove secondary antibody, wash the wells with 0.1% PBSTX three times.
11. Stain using DAB. Prepare reagent as per manufacturer’s instructions (K007) (20μl DAKO Real DAB chromogen, Bottle C + 1ml of DAKO Real substrate buffer, Bottle B).
12. Watch staining reaction carefully until desired colour intensity is achieved.
13. Wash the wells three times with 1x PBS.

14. To counterstain, add 250μl of Harris’ Haematoxylin to each well, leave 90 seconds. Remove Haematoxylin and wash the wells with 1x PBS three times.

15. Add 1ml 1x PBS to each well then photograph at x20 magnification.
Appendix VII

Image analysis

Procedure:

1. Go ‘file’ tab of image J software and click open.

2. Open black and white images from folder to be analysed. If image is not black and white then click ‘image- type- 8 bit’ to convert to black & white images.

3. Select on ‘image- adjust- threshold’.

4. Adjust the threshold in between 0 to 255 that covers all dark areas.

2. Then ‘apply’.


4. Results will appear on screen, save the summary results of each image for statistical analysis.
Cell counting method using Adobe Photoshop CS6

Procedure:

1. Open ‘Adobe Photoshop’ software.
2. Go to file – open the image.
3. Select the ‘count’ tool (I) on the left side of menu list
4. Make a 5x5 cm box using the rectangular marquee tool (M) in each corner of the image.
5. Create a new count group, type a count group name and assign count group colour.
6. Select cell, the total cell count will display on the top left side of software screen.
7. Enter the cell count in excel sheet manually for further analysis.