FUNCTIONAL CHARACTERIZATION OF PEROXISOMES AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA IN THE CHICKEN RESPIRATORY SYSTEM

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OCTOBER 2015
DECLARATION

I, Mutua Patrick Mbuvi, do declare that this thesis is my original work and has not been presented for degree or other awards in any other University.

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DEDICATION

To my dear mother Grace Mbaika and my profound friend Louise Bowditch, the world would have been a lesser place without you.
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TABLE OF CONTENTS

CONTENT                                                                 PAGE

TITLE PAGE                                                          i
DECLARATION                                                        ii
DEDICATION                                                         iii
ACKNOWLEDGEMENT                                                   iv
TABLE OF CONTENTS                                                 v
LIST OF TABLES                                                    x
LIST OF FIGURES                                                   xi
ABBREVIATIONS AND ACRONYMS                                        xii
DEFINITION OF TERMS                                               xiv
ABSTRACT                                                          xvii

CHAPTER 1: INTRODUCTION                                            1
1.1 Background information                                         1
1.2 Statement of problem                                           8
1.3 Justification of the study                                     9
1.4 Hypotheses                                                    10
1.5 Objectives                                                    10
1.5.1 General objectives                                          10
1.5.2 Specific objectives                                         11
1.6 Significance of the study                                     11

CHAPTER 2: LITERATURE REVIEW                                       12
2.1 The avian parabronchial lung                                  12
2.2 Respiratory air flow in avian lung 14
2.3 The avian respiratory immune system 15
2.3.1 The nasal associated lymphoid tissue 15
2.3.2 The avian trachea 16
2.3.3 The bronchus associated lymphoid tissue 16
2.3.4 The avian IgA system 17
2.3.5 The avian surfactant 17
2.3.6 The immune system in the gas exchange area 19
2.3.7 Techniques used for studying phagocytosis 23
2.3.8 Respiratory burst in the avian 24
2.3.9 Nitric oxide production in avian 24
2.3.10 Inflammation in the avian respiratory system 26
2.4 Particle uptake in the avian respiratory system 28
2.5 Avian lung antioxidant system 29
2.5.1 Peroxisomes 31
2.5.2 Peroxisome biogenesis 33
2.5.3 Cytochemical techniques for investigation of peroxisomes 34
2.6 Peroxisome proliferator activated receptors 36
2.6.1 Molecular mechanism of PPAR transcription 37
2.7 Troglitazone 43
2.8 Chicken antioxidant defense system 44
2.9 Chicken peroxisomes and PPARs 45
2.9.1 Chicken peroxisomes 45
2.9.2 Chicken PPARs
2.9.3 Expression patterns of chicken PPAR mRNAs
2.9.4 Regulators of chicken PPAR mRNAs
2.10 The amino acid homology between chicken and mammalian PPARs

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site
3.2 Study subjects
3.3 Sample size and sampling
3.4 Experimental design
3.5 Anesthesia and euthanasia of chicken
3.6 Chicken respiratory tract fixation and tissue harvesting
3.7 Localization of peroxisomes
3.8 Morphometric analysis of peroxisomes
3.9 Localization of catalase
3.10 Recovery of FARM by lavage
3.11 Counting of FARM
3.12 FARM in vitro viability tests
3.13 Morphology of FARM
3.14 Phagocytosis assays and morphometric analysis
3.15 Determination of phagocytic capacity of FARM
3.16 Measurement of TNF α production by FARM
3.17 Data analysis
### CHAPTER 4: RESULTS

4.1 Morphological characteristics of peroxisomes in the chicken respiratory system 61

#### 4.1.1 Light microscopy 61

#### 4.1.2 Electron microscopy 61

#### 4.1.3 Peroxisome morphometry 64

4.2 Localization of catalase in the chicken respiratory system 65

4.3 Lavage and numerical characteristics of FARM 68

#### 4.3.1 FARM in vitro Viability characteristics 71

#### 4.3.2 Morphological characteristics of the chicken FARM 72

#### 4.3.3 Effect of troglitazone on phagocytic potential of FARM 72

#### 4.4 Effect of troglitazone on TNF α production by FARM 76

### CHAPTER 5: DISCUSSION AND HYPOTHESES TESTING

5.1 Distribution of peroxisomes in the chicken respiratory system 77

5.2 Distribution of catalase in the chicken respiratory system 79

5.3 Effect of troglitazone on phagocytic capacity of FARM 80

5.4 Effect of troglitazone on TNF α production by FARM 82

5.5 Hypotheses testing 83

### CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions 84

6.2 Recommendations 85

6.3 Suggestions for further research 86

### REFERENCES 87
APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix I</td>
<td>Protocol for tissue fixation and detection of peroxisomes</td>
<td>103</td>
</tr>
<tr>
<td>Appendix II</td>
<td>Protocol for preparation of diaminobenzidine</td>
<td>104</td>
</tr>
<tr>
<td>Appendix III</td>
<td>Protocol for processing cells for transmission electron microscope</td>
<td>105</td>
</tr>
<tr>
<td>Appendix IV</td>
<td>Stains and staining methods</td>
<td>108</td>
</tr>
<tr>
<td>Appendix V</td>
<td>Publications from this research</td>
<td>110</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

**Table 2.1:** Peroxisomal enzymes producing ROS/RNS 32

**Table 2.2:** Peroxisomal antioxidant enzymes 32

**Table 4.1:** Summary of mean diameter of peroxisomes 64

**Table 4.2:** *In vitro* viability tests of troglitazone treated and untreated FARM 69

**Table 4.3:** Comparison of instilled and aspirated lavage fluids in chicken lungs 70

**Table 4.4:** Number of FARM recovered per lavage and per chicken 71

**Table 4.5:** Mean diameter of troglitazone treated and untreated FARM 73
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Light micrographs of chicken and rabbit lung portions</td>
<td>13</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>A diagram of the avian lung air sac system</td>
<td>14</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Air flow through avian respiratory system</td>
<td>15</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Exogenous and endogenous sources of oxidants</td>
<td>30</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>A composite model of peroxisome biogenesis</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>General structure of PPAR</td>
<td>37</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Molecular mechanism of PPAR activation</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Monocyte differentiation into M1 and M2 macrophage phenotypes</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Lavage set up in the chicken respiratory system</td>
<td>55</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Light micrographs of peroxisomes</td>
<td>62</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Electron micrographs of peroxisomes</td>
<td>63</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Mean volume density of peroxisomes trachea, lungs, and bronchi</td>
<td>64</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Micrographs showing catalase in chicken trachea, bronchi and lung</td>
<td>65</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Negative controls for catalase in chicken trachea, bronchi and lung</td>
<td>67</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Micrograph of cells recovered by lavage of chicken lung air sac system</td>
<td>68</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Mean number of FARM recovered in the five progressive lavages</td>
<td>71</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td>Micrograph showing morphology of chicken FARM</td>
<td>72</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Volume density of internalized particles in treated and untreated FARM</td>
<td>74</td>
</tr>
<tr>
<td>Figure 4.10</td>
<td>Light micrographs of particles in treated and untreated FARM</td>
<td>74</td>
</tr>
<tr>
<td>Figure 4.11</td>
<td>Electron micrographs of internalized particles in untreated FARM</td>
<td>75</td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>Electron micrographs of particles in troglitazone treated FARM</td>
<td>75</td>
</tr>
<tr>
<td>Figure 4.13</td>
<td>TNF α production by FARM treated with varying doses of troglitazone</td>
<td>76</td>
</tr>
</tbody>
</table>
ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine phosphodiester guanine</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>FAO</td>
<td>Food and agriculture organization</td>
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<tr>
<td>FARM</td>
<td>Free avian respiratory macrophages</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>KIPPRA</td>
<td>Kenya institute for public policy research and analysis</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial anti-viral signaling protein</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor κ-light-chain enhancer of activated B cells</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
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<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromoles</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>P (c)</td>
<td>Particles phagocytized in an entire cell</td>
</tr>
<tr>
<td>P (p)</td>
<td>Points falling onto profiles of phagocytized particles</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
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<td>PPAR</td>
<td>Peroxisome proliferator activated receptors</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TNF α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>USAID</td>
<td>United states agency for international development</td>
</tr>
<tr>
<td>Vv (p, c)</td>
<td>Volume density of the phagocytized particles in a FARM</td>
</tr>
<tr>
<td>Vv (p, t)</td>
<td>Volume density of peroxisomes in the tissues</td>
</tr>
</tbody>
</table>
### DEFINITION OF TERMS

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adjuvant:</strong></td>
<td>Is a pharmacological or immunological agent that modifies the effect of other agents. Adjutants enhance the body immune responses against antigens.</td>
</tr>
<tr>
<td><strong>Antioxidant:</strong></td>
<td>An antioxidant is any of various substances that inhibit oxidation or reactions promoted by oxygen and peroxides and that include many held to protect the living body from deleterious effects of free radicals.</td>
</tr>
<tr>
<td><strong>Catalase:</strong></td>
<td>A common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is very important enzyme in protecting the cell from oxidative damage by reactive oxygen species.</td>
</tr>
<tr>
<td><strong>Cytokines:</strong></td>
<td>These are a broad category of small proteins (5 – 20 Daltons) that are important in cell signaling. Their release has an effect on the cells around them.</td>
</tr>
<tr>
<td><strong>Diaminobenzidine:</strong></td>
<td>Is an organic compound that is both chemically and thermodynamically stable. It is a derivative of benzidine and is used in staining cellular or tissue components that degrade hydrogen peroxide. Hydrogen peroxide oxidizes diaminobenzidine into a brown precipitate.</td>
</tr>
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<td><strong>Free avian respiratory macrophages:</strong></td>
<td>These are respiratory macrophages resident in the lungs and air sacs of birds and participate both in innate and adaptive immune protection mechanisms of the avian respiratory</td>
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</tbody>
</table>
system. Free avian respiratory macrophages are harvestable by lavage of the avian lung air sac system and are also referred to as avian respiratory macrophages.

**Immunoglobulin:** This refers to any class of proteins present in the serum and cells of the immune system that function as antibodies.

**Inflammation:** Is a localized physical condition in which part of the body becomes reddened, swollen, hot, and often painful, especially as a reaction to injury or infection.

**Lipopolysaccharide:** These are also known as lipoglycans and endotoxins. They are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. They are found in the outer membrane of gram negative bacteria and elicit strong immune responses in animals.

**Peroxisome biogenesis:** Is the process through which new peroxisomes arise from pre-existing peroxisomes. The process involves peroxisome membrane formation, import of peroxisome matrix proteins and fission of the newly formed peroxisome into daughter peroxisomes.

**Peroxisome proliferator activated receptor ligand:** It is a substance which is either natural or synthetic and binds with peroxisome proliferator activated receptor protein ligand binding domain with subsequent conformational change of the receptor leading to transcription of genes by the peroxisome proliferator activated receptor.
Peroxisome proliferator activated receptors: These are a group of nuclear receptor proteins that function as transcription factors regulating expression of genes. They play essential roles of cellular differentiation, development and metabolism.

Peroxisome targeting signal: This is a protein present in a peroxisome matrix protein recognized by a receptor (Pex 5 or Pex 7) for translocation and docking of the peroxisome matrix protein into peroxisome lumen.

Peroxisomes: These are small, membrane-enclosed organelles that contain enzymes involved in a variety of metabolic reactions, including antioxidant enzymes that degrade reactive oxygen species.

Polystyrene particles: These are synthetic aromatic polymer based plastic solid beads.

Reactive oxygen species: These are chemically reactive chemical species containing oxygen. Examples include peroxides, super oxides, hydroxyl radical, and singlet oxygen.

Surfactant protein: These are compounds composed of a mixture of proteins and lipids that function to lower the surface tension between a surface and a solid. In the lungs, surfactant proteins are secreted by alveolar type II cells and reduce collapse of alveoli during gaseous exchange.

Troglitazone: Is an anti-diabetic and anti-inflammatory drug and a member of the thiazolidinediones.

Tumor necrosis factor alpha: This is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction.
Poultry farming is important in mitigating food insecurity through provision of animal proteins. However, growth of poultry is constrained by high mortality of domestic birds occasioned by respiratory disease conditions. In the mammalian lungs, therapeutic agents improve the ability of peroxisomes to discharge antioxidant enzymes which resolve oxidant mediated inflammatory disease conditions by degrading reactive oxygen species. Despite incessant exposure of the avian respiratory system to exogenous and endogenous oxidants with subsequent risk of inflammatory injury, information regarding distribution and the antioxidant properties of peroxisomes in the chicken respiratory system remains scarce. Further, in the mammalian lungs, peroxisome proliferator activated receptor gamma (PPAR γ) ligands activate respiratory macrophages to restore alveolar architecture through clearance of inflammatory sites with diminished proinflammatory cytokine production. There is no empirical data to support assertion that PPAR γ ligands induce anti-inflammatory properties in the free avian respiratory macrophages (FARM). The aim of this study was to establish distribution of peroxisomes and catalase, the principal peroxisome antioxidant enzyme, in the chicken respiratory system, and to elucidate anti-inflammatory roles of PPAR γ ligands in the FARM. The study was conducted in the department of Zoological Sciences, Kenyatta University and electron microscopy done at the department of Veterinary Anatomy and Physiology, Chiromo Campus, University of Nairobi. A total of 44 indigenous chickens aged 8 months were used. Diaminobenzidine and immunohistochemical staining methods were used for identification of peroxisomes and catalase respectively. FARM were recovered by lavage of the chicken respiratory system and treated with troglitazone, a selective synthetic PPAR γ ligand, for one hour. To determine the phagocytic capacity of treated FARM, the cells were co-cultured with particles for three hours. Further, treated FARM were stimulated with LPS and TNF α secretion by the cells assessed using ELISA test. Peroxisomes formed fine electron dense granular matrix and were spherical in shape with an average diameter of 0.9 µm. Peroxisomes were equivalently ($P > 0.05$) distributed in the lung and bronchi tissues which had a mean volume density of $38 \pm 1.5 \%$ and $37 \pm 1.7 \%$ respectively. However, the trachea had significantly ($P < 0.05$) fewer peroxisomes compared to lung and bronchi. The mean volume density of peroxisomes in the trachea was $16 \pm 1.8 \%$. Catalase was abundant in the trachea ciliated epithelial cells and in the epithelia lining the bronchiolar junctions. In the lungs, catalase was abundant in the epithelia lining of air capillaries and the enzyme was expressed in FARM. Troglitazone treated FARM exhibited significantly ($P < 0.05$) higher phagocytic index than untreated FARM. The mean volume density of internalized particles per unit volume of a FARM was 41 % and 21 % in the troglitazone treated and untreated FARM respectively. Additionally, troglitazone treated FARM significantly ($P < 0.05$) decreased LPS-induced TNF α production in a dose dependent manner. In conclusion, peroxisomes and catalase are strategically located in the avian respiratory system. Further, PPAR γ ligands induce anti-inflammatory properties by enhancing phagocytic ability of the FARM and by attenuating pro-inflammatory cytokine production in activated FARM. Therefore, therapeutic agents targeting peroxisomes and PPAR γ ligands could be used to supplement the current regimen of vaccines and antibiotics in treatment and management of inflammatory disease conditions of the avian respiratory system.
CHAPTER 1: INTRODUCTION

1.1 Background information

Food insecurity, especially inadequate supply of animal proteins, is a major challenge facing people in the developing world (McKeever and Morrison, 1998). In 2007, global meat production was estimated to be 275 million tons, out of which 88 million tons of poultry meat, representing 33 % of the total global meat production, was reported (FAO, 2007). According to a report on the food outlook, in 2007 the relative contribution to the global poultry meat production from the chicken, turkey and ducks was 87 %, 6.7 % and 4 % respectively, while geese, pigeons, quails, pheasants, ostriches and emu combined accounted for 2.3 % (FAO, 2007). Even though demand for poultry meat is higher in developing countries than in the developed nations (FAO, 2007), poultry meat production is low in the least developed countries. For instance, in 2007, the relative contribution to global poultry meat production by the USA and Africa was 22 % and 4.2 % respectively (FAO, 2007). In 2050, global poultry meat production is estimated to rise to 180 million tons and will account for 38 % of the global meat production projected at 465 million tons (FAO, 2008).

In Kenya, poultry farming provides 22,000 tons of meat annually (Winrock international, 2010), an important source of animal protein and income generating activity mainly for rural smallholder families contributing to the livelihoods of an estimated 21 million people (KIPPRA, 2009). Kenyan poultry population stands at 32 million of which 6 million are commercial hybrids and the rest are indigenous (KIPPRA, 2009). The Kenya Economic Report (KIPPRA, 2009) identifies poultry as the lead livestock enterprise that can contribute the most towards alleviation of abject poverty a suggestion that is in
tandem with Sustainable Development Goal number one (SDG 1). However, growth of the poultry industry is constrained by high mortality associated with respiratory disease conditions (Kiama et al., 2008). Common respiratory viral diseases affecting domestic birds are infectious bronchitis, Newcastle disease, avian influenza, infectious laryngotracheitis and pneumo viruses (Villegas, 1998). Although more than dozen mycoplasma cause respiratory disease conditions in the poultry, the most important ones are *Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasma iowae* and *Mycoplasma meleagridis* (Kleven, 1998). Avian respiratory diseases caused by bacteria are represented by fowl cholera caused by *Pasteurella multocida*, infectious coryza caused by *Haemophilus paragallinarium*, colibacillosis caused by *Escherichia coli*, bordetellosis caused by *Bordetella avium* and infection with *Ornitobacterium rhinotracheale* (Glisson, 1998). All of these agents are capable of causing respiratory diseases of various severities alone though the most serious threat to the poultry are disease conditions caused by multiple agents acting synergistically.

The modern gas exchangers have evolved either as evaginations or invaginations from certain parts of the body. These designs were imposed by factors such as the physicochemical characteristics of the respiratory medium used, the nature of habitat occupied, and the lifestyle pursued (Maina, 1994). To promote gas exchange by diffusion, extensive surface areas and thin gas-blood tissue barriers were contrived. Although these features enhanced the flux of respiratory gases between the body and the environment, the capacity of the body to physically ward off harmful biological pathogens and ameliorate the harmful effects of toxic environmental pollutants at such
sites was innately compromised. Without developing an efficient defense mechanism, the respiratory organs would offer easily assailable areas during the incessant assaults by pathogens and toxic substances. Evolution of gas exchangers was, therefore, accompanied by formation of apt defense devices (Maina and Cowley, 1998). Both mammals and birds have the highest metabolic rates and the most complex lungs of the vertebrates. However, in mammals the functions of ventilation and gas exchange are shared by common structures in the respiratory bronchioles, alveolar ducts, and hundreds of millions of alveoli. Less than 20% of the total lung volume is dedicated to ventilation alone in the conducting airways. In contrast, the avian respiratory system is heterogeneously partitioned and completely separates the functions of ventilation and gas exchange (Dunker, 1994).

In birds, nine air sacs ventilate the small constant volume and they occupy about 90% of the total respiratory system volume while the remaining 10% is comprised of the lung, containing hundreds of gas exchange units called parabronchi (Dunker, 1994). The respiratory surface of the gas-blood tissue barrier in the birds exceeds that of the mammals of equivalent body mass by a factor of 15% and the tissue is 56 - 67% thinner (Maina, 1989). All other prevailing factors being the same, from the perspective of the basis of the basic structural parameters, that is vast and attenuated separation between air and blood, expectedly, the avian respiratory system is more assailable by pathogens than mammalian one (Maina, 1989). Applying a similar corollary, for similar defense competence, more respiratory macrophages may occur on the surface of the avian lung (Nganpiep and Maina, 2002). In birds, respiratory macrophages have been referred to as
free avian respiratory macrophages (Toth and Siegel, 1986; Fulton et al., 1990). Paradoxically, very few free avian respiratory macrophages (FARM) retrievable by pulmonary lavage in birds compared with mammals of equivalent body mass have been reported in domestic fowl, turkey, Muscovy duck, and rock doves (Toth et al., 1987; Ficken and Barnes, 1989; Maina and Cowley, 1998; Nganpiep and Maina, 2002; Kiama et al., 2008). The average number of macrophages in the rock dove is $1.6 \times 10^5$ (Maina and Cowley, 1998) while that in the domestic fowl and turkey is $2.5 \times 10^5$ and $1.15 \times 10^6$ respectively (Ficken and Barnes, 1989; Toth et al., 1987). Some avian immunologists postulate a dearth of FARM on the avian lung air sac system to foreordain a weak innate immunity, a condition that has been purported to predispose domestic birds to respiratory disease conditions hence high morbidity and mortality (Brain and Frank, 1986; Ficken et al., 1986; Fedde, 1998; Maina and Cowley, 1998).

Despite paucity of the FARM on the avian lung air sac system, other investigators have demonstrated a significantly higher phagocytic capacity of FARM in comparison with the corresponding mammalian respiratory macrophages (Kiama et al., 2008; Mutua et al., 2011). Activated FARM generate both reactive oxygen and nitrogen species which can cause severe damage to the lung tissue (Fagerland and Arp, 1990; Maina and Nathaniel, 2001). Chicken, being a gallinaceous bird, is exposed to a highly contaminated air which impacts greatly on the antioxidant defense system of the respiratory tract. Oxidative stress injury is partly dependent on the concentration of oxygen at the site of activity (Maina and Nathaniel, 2001). Among the vertebrates, birds have the most efficient respiratory system which extracts 60% of oxygen in inhaled air compared with mammals.
whose oxygen extraction is 27% (Prosser, 1950) and, therefore, avian respiratory tract is probably more vulnerable to oxidative stress. Broilers exposed to aflatoxins, which are common bioaerosols in poultry settings, suffer oxidative stress mediated inflammation occasioned in part by increasing production of reactive oxygen species (ROS), reactive nitrogen reactive species (RNS) and proinflammatory cytokines such as tumor necrosis factor alpha by immune cells (Yani et al., 2014). Generation of free radicals such as superoxide anion (O$_2^-$) and the hydroxyl radical (OH) as well as ROS such as hydrogen peroxide (H$_2$O$_2$), are directly associated with the oxidative modification of proteins, lipids, carbohydrates, and DNA (Irfan, 2011). Oxidative stress causes cell damage, cell necrosis, apoptosis, autophagy, remodeling of extracellular matrix and blood vessels, endothelial dysfunction, inactivation of antiproteases, cell proliferation, epigenetic changes, autoimmunity, and skeletal muscle dysfunction (Irfan, 2011). Furthermore, oxidants augment inflammatory responses in the lungs through activation of nuclear factor kappa beta (NF-κβ) and mitogen-activated protein kinases (MAPK) signal transduction pathways, chromatin remodeling and transcription of proinflammatory mediator genes. These processes are intricately associated with the progression of inflammatory disease conditions (Irfan, 2011).

Peroxisome is a single-membrane bounded ubiquitous organelle containing a variety of enzymes that catalyze various metabolic pathways, including β-oxidation of very long-chain fatty acids, synthesis of plasmalogens which have immunomodulatory effects, and metabolism of hydrogen peroxide (Rhodin, 1954; Wallner and Schmitz, 2011). Peroxisomes generate oxidants and antioxidants, a balanced redox system, which when
impaired in favor of oxidants results into overproduction and flux of oxidants leading to tissue damage (Marc et al., 2011). Antioxidants react with and detoxify reactive free radicals. In mammals, studies have focused on supplementing or promoting endogenous antioxidants in management and treatment of respiratory conditions. Catalase, the predominant antioxidant peroxisome enzyme, has been targeted in pharmacological antioxidant strategies in therapeutic interventions of the respiratory disease conditions in mammals (Irfan, 2011). Some of the synthetic antioxidants used include enzyme mimetics such as catalase mimetic EUK-179, mucolytic drugs, thiol compounds and dietary agents such as vitamins C and E (Irfan, 2011). In the chicken, alpha-lipoic acid (α-LA) has been shown to reverse systemic oxidative injury induced by aflatoxins in the chicken serum (Yani et al., 2014). Peroxisomes have been localized in the chicken liver and kidney. Morphologically, chicken peroxisomes resemble those found in other animals such as the amphibians and contain catalase, α-hydroxy acid oxidase, D-amino acid oxidase, and urate (Scott et al., 1989). Peroxisomes adjust to changing physiological requirements of a cell, tissue or organism by constantly adjusting their morphology, number, enzyme content and metabolic functions accordingly (Schrader and Fahimi, 2004).

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor super family which also includes retinoid, glucocorticoid and thyroid hormone receptors (Evans, 1988). The term peroxisome proliferator activated receptor was coined after peroxisome proliferator activated receptor-alpha (PPAR α) activation was shown to result in proliferation of
peroxisomes in rodent hepatocytes (Issemann and Green, 1990). Following this discovery, other peroxisome proliferator activated receptors, namely PPAR-gamma and PPAR-beta were later identified (Spiegelman and Flier, 1996). In humans, peroxisome proliferator activated receptor-gamma (PPAR γ) is involved in inflammatory resolution through inhibition of release of proinflammatory cytokines from activated respiratory macrophages (Jiang et al., 1998) and through increased non-phlogistic clearance of apoptotic neutrophils, tissue debris and microbes by respiratory macrophages (Simon and Mariani, 2007). Additional functions of PPAR γ include regulation of monocyte / macrophage differentiation and maintenance of normal lung structure through regulation of epithelial cell differentiation (Simon and Mariani, 2007).

In the chicken, peroxisome proliferator activated receptor-gamma (PPAR γ) has been demonstrated in the abdominal adipose tissue, where expression of the protein has been reported to increase with age. The PPAR γ has been reported to be higher in 6-week old chicken than in 1 to 2 week old chicken (Sato et al., 2004). In addition, PPAR γ is also expressed in the skeletal muscle and the ovaries (Sato et al., 2004). Sato et al. (2004) reported that expression of PPAR γ may be modulated by nutritional states in a similar manner to mammals. In mammals, PPAR γ exist in two isoforms that is: PPAR γ₁ and PPAR γ₂. However, in the chicken, PPAR γ₂ isoform does not exist (Takada and Kobayashi, 2012). The chicken PPAR γ has been shown to be structurally different from the human and mice PPAR γ suggesting possible different functions of the PPAR γ in the mammals and chicken (Takada and Kobayashi, 2012). However, it has been asserted that chicken PPAR γ could have anti-inflammatory roles (Takada and Kobayashi, 2012).
1.2 Problem statement

The avian lung air sac system has extensive surface area, an attenuated gas-blood tissue barrier, and is highly supplied with blood; these properties render the system highly susceptible to oxidative mediated injury occasioned by incessant exposure to endogenous and exogenous oxidants (Maina and Nathaniel, 2001). Oxidants activate FARM to release proinflammatory cytokines that serve to augment inflammation necessary for localization and removal of the injurious stimuli. However, prolonged inflammatory respiratory disease conditions cause high mortality in domestic birds with subsequent economic losses in poultry industry (Pascal et al., 2011; Yani et al., 2014). In mammals, pharmacological approaches target adjustment of peroxisomal enzymes in favor of production and flux of antioxidant enzymes to enhance inflammatory resolution by degrading ROS and free radicals (Irfan, 2011). Effective use of antioxidant therapy requires knowledge of: a) cellular location of antioxidants relative to foci of major sources of oxidants, b) the inherent antioxidant status of the subject being treated and, c) how the antioxidants react to neutralize free radicals (Irfan, 2011).

Activation of peroxisome proliferator activated receptor-gamma (PPAR γ) inhibits production of proinflammatory cytokines in the alveolar macrophages (Jiang et al., 1998), and enhances non-phlogistic clearance of apoptotic neutrophils, tissue debris and microbes in the inflammatory site by respiratory macrophages thus restoring alveolar architecture (Dowling and Simmons, 2009). Despite huge economic losses associated with respiratory disease conditions in the poultry industry, information of the avian respiratory antioxidant defense system remains scarce. Further, there is no empirical data
supporting assertion that chicken PPAR γ induce anti-inflammatory properties in the FARM.

1.3 Justification of the study

This study is based on the rationale that increased levels of the oxidants can exert harmful effects by causing oxidative stress mediated inflammation characterized by accumulation of activated immune cells at the site of inflammation, a condition associated with pulmonary epithelial damage (Dowling and Simmons, 2009). In the mammalian lung, administration of synthetic antioxidants as therapeutic interventions against lung inflammatory disease conditions generally functions to restore the redox system by targeting peroxisome biogenesis and supplementing peroxisomal enzymes such as catalase (Irfan, 2008). To understand the inherent antioxidant defense of the avian respiratory system, this study aimed at carrying out morphological survey and identification of peroxisomes and catalase distribution in the chicken trachea, bronchi and lung tissues.

Non phlogistic clearance of inflammatory sites in the mammalian lungs by respiratory macrophages is enhanced by PPAR γ ligands, (Kazuhiro et al., 2003). It has been postulated that chicken PPAR γ could have anti-inflammatory functions (Takada and Kobayashi, 2012). However, there is no empirical data to support the assertion that PPAR γ agonists have anti-inflammatory roles in FARM yet such information would be of great importance in developing synthetic selective PPAR γ ligands for treatment and management of avian respiratory inflammatory disease conditions.
1.4 Hypotheses

(i) There is no difference in distribution of peroxisomes in the chicken trachea, bronchi and in the lung tissues.

(ii) There is no difference in the distribution of catalase in the chicken trachea, bronchi and lung tissues.

(iii) Troglitazone treatment has no effect on the phagocytic capacity of FARM.

(iv) Troglitazone treatment has no effect on TNF α secretion by the FARM.

1.5 Objectives

1.5.1 General objective

To determine functional characterization of peroxisomes and elucidation of anti-inflammatory properties of peroxisome proliferator activated receptor–gamma ligands in the chicken respiratory system.

1.5.2 Specific objectives

(i) To localize and determine distribution of peroxisomes in the chicken trachea, bronchi and lung tissue.

(ii) To determine distribution of catalase in the trachea, bronchi and lung tissue of the chicken.

(iii) To determine the effect of troglitazone treatment on the phagocytic ability of FARM.

(iv) To determine the effect of troglitazone treatment on TNF α secretion by the FARM.
1.6 Significance of the study

In mammals, chronic inflammation of the respiratory system with subsequent respiratory epithelial destruction is mainly occasioned by uncontrolled production of oxidants by activated respiratory macrophages at the inflammatory site (Irfan, 2008). In the mammalian bronchoalveolar lung system, peroxisomes provide an isolated intracellular microenvironment for detoxification of oxidants prior to their diffusion hence attenuating oxidant destruction of the respiratory epithelium (Irfan, 2011). Further, activation of PPAR γ in the mammalian respiratory macrophages by TZDs such as troglitazone inhibits production of proinflammatory cytokines by immune cells. Additionally, TZDs improve the phagocytic ability of respiratory macrophages which restore alveolar architecture through increased clearance of inflammatory sites (Jiang et al., 1998).

Inflammatory disease conditions cause the highest morbidity and mortality among the domestic birds and are occasioned partly by incessant exposure of the avian respiratory system to endogenous and exogenous oxidants (Pascal et al., 2011). Despite the critical role peroxisomes play in inflammatory resolution by detoxifying oxidants in the mammalian lung, the quantitative and qualitative attributes of peroxisomes in the avian respiratory system are unknown. Further, there is no empirical data on the effects of selective synthetic PPAR γ ligands on the phagocytic capacity and production of proinflammatory cytokines by the chicken FARM.
CHAPTER 2: LITERATURE REVIEW

2.1 The avian parabronchial lung

The basic construction of the avian parabronchial system is derived from multicameral reptilian lungs with three rows of lung chambers, represented by the three rows of secondary bronchi which emanate from the primary bronchus. The secondary bronchi branch to form parabronchial mantle containing air capillaries, the sites for gas exchange by diffusion in avian lungs (Dunker, 1994). Among the evolved air-breathing vertebrates, the lung-air sac system of birds is the most complex and efficient gas exchanger (King and McLelland, 1998).

Although mammalian and avian lung systems develop from a common lineage, the avian parabronchial lung differs morphologically from the mammalian bronchoalveolar lung in certain key aspects. While the arrangement of the bronchial airway system of the mammalian lung displays commonly dichotomous bifurcations which terminate in blind-ended air conduits (Horsfield, 1997), in the avian lung, a highly intricate anastomotic system exits (Beillairs and Osmond, 2005). Further, the gas-blood tissue barrier in the avian lung is approximately 56% - 67% thinner (Figure 2.1) than that of a mammal of the same body mass while the respiratory surface is approximately 15% greater (Maina, 1989). Most birds have nine air sacs (Figure 2.2) and some variably extend well beyond the limits of the coelomic cavity, with many bones being extensively pneumatized (King, 1957; Bezuidenhout et al., 2000). It has been reported that some of the air sac diverticulae lie very close to the skin making respiratory system highly vulnerable to
oxidative trauma and the lung susceptible to diffusion of air-borne pathogens after damage to the extensions of the air-sacs as in air sacculitis (Bezuidenhout et al., 2000).

Figure 2.1: Electron micrographs of the chicken (top) and the rabbit lung (bottom) portions. The air capillaries (arrows) in the chicken are thinner than the alveoli (thick arrows) in the rabbit at the same magnification (Watson and West, 2007)
In complete contrast to the tidally ventilated mammalian respiratory system where inhaled fresh air is mixed with residual air, the avian lung is a flow-through system (Figure 2.3). Birds have larger tidal volumes and maintained by a system of air conduits which include the primary bronchus, secondary bronchi, tertiary bronchi and the avian specific air sacs which behave as bellows ventilating the lungs continuously and in a caudal-cranial unidirectional manner (Fedde, 1998; Maina and Van Gills, 2001).
The inspired air completely bypasses the cranial lying openings of the medioventral secondary bronchi, a process which is termed inspiratory aerodynamic valving (Banzett et al., 1968; Brown et al., 1995). In contrast, during the expiratory phase, air flows in the mediodorsal and lateral ventral secondary bronchi. Gas exchange takes place across the air capillaries and blood capillaries within the parabronchial mantle (Sheid, 1979). A functional consequence of this flow pattern is that particles are primarily deposited in the caudal regions of the lung, as was first observed for soot deposition in pigeons captured at train stations (Dotterweich, 1930).

Figure 2.3: Air flow through the avian respiratory system during (a) inspiration and (b) expiration. 1-Interclavicular air sac, 2-cranial thoracic air sac, 3-caudal thoracic air sac, 4-abdominal air sac (Reese et al., 2006)

2.3 The avian respiratory immune system

2.3.1 The nasal associated lymphoid tissue

The mucosa tissue of the avian nose is the first to come into contact with aerosols and microorganisms during inhalation. Lymphocytes have been found in the nasal mucosa, the lateral nasal glands and their secretory ducts (Ohshina and Hiramatsu, 2000).
The CD8\(^+\) cells are distributed in the epithelium and the lamina propria while CD4\(^+\) cells are largely confined to organized lymphoid structures called Nasal Associated Lymphoid Tissues (Ohshina and Hiramatsu, 2000). The major characteristics of the chicken NALT are formation of circumscribed B cells occasionally displaying germinal centers that are covered by a cap of CD4\(^+\) T cells. The surface of these lymphoid follicles is covered by non-ciliated epithelium (Ohshina and Hiramatsu, 2000).

2.3.2 The avian trachea

Although constitutive lymphoid tissue has not been described in the avian trachea, infection models with *Mycoplasma gallisepticum* has shown tracheal mucosa is highly responsive to infections and reacts with extensive lymphocyte infiltration followed by lympho-proliferation (Gaunsen et al., 2006). Production of infectious bronchitis virus (IBV) lesion has been associated with massive lymphocyte infiltration in the tracheal lamina propria (Kotani et al., 2000). A study by Javed et al. (2005) revealed that unvaccinated chicken had infiltration of large numbers of B and T cells and some plasma cells while birds vaccinated with *M. gallisepticum* developed secondary lymphoid follicle-like aggregates with fewer lesions. This indicates that the tracheal mucosa lymphoid tissue can be induced following infection.

2.3.3 The bronchus associated lymphoid tissue

A comparative study of the mammalian and bird lung tissues found that lymphoid nodules in the primary bronchus in birds has much similarity with Peyer’s patches and other gut-associated lymphoid tissues in mammals (Bienenstock et al., 1973).
Bronchus Associated Lymphoid Tissue (BALT) develops at the junctions of the primary bronchus and the caudal secondary bronchi (Fagerland and Arp, 1993) as well as at the ostia of the air sacs (Mayers and Arp, 1987). A mature BALT is covered by distinct layer of follicle-associated-epithelium (FAE) that harbors numerous lymphocytes (Bienenstock and Befus, 1984). Some FAE cells have irregular microvilli on their luminal surface and are in close contact with lymphocytes and myeloid cells suggesting that they may be homologous to the M cells of Peyer’s patches (Fagerland and Arp, 1993). In BALT nodules of 6-8 week old chicken, large B cell follicles are found which are primarily made up of IgM+ cells while IgY+ and IgA+ B cells are present in much smaller numbers (Fagerland and Arp, 1993). BALT development is influenced by age (Jeurissen et al., 1989). Single T cells are already present at predetermined locations in day-old chicks, while B cells are not evident until second week. As weeks progress, increasing numbers of CD4+ cells accumulate and develop into organized structures with T cells at the centre and B cells at the periphery (Fagerland and Arp, 1993). Germinal centers are not observed in the BALT until the end of second week (Fagerland and Arp, 1993).

### 2.3.4 The avian IgA system

In the chicken, an IgA like immunoglobulin was originally described by Orlans and Rose in 1972 and later shown to be polymeric immunoglobulin in serum and several secretions (Wanatabe et al., 1975). Cloning of the constant alpha gene provided structural data allowing the definite designation of this gene as the avian homologue for mammalian alpha heavy chain (Mansikka et al., 1989). The presence of IgA+ B cells in the chicken respiratory tract was demonstrated by immunohistology. The IgA B cells were found
beneath the tracheal epithelium in 6 week old birds but not in younger birds (Jeurissen et al., 1989). Since IgA can be obtained by lung lavage at readily detectable concentrations, it seems likely that IgA+ cells are the prime source of secretory IgA in the chicken lung (Cihak et al., 1991). The known functions of sIgA include to hinder bacterial adherence on mucosal surfaces (Fubara and Freter, 1973), antiviral activity (Ogra et al., 1968), and ability to block release of bacterial enterochelin, an iron chelating agent produced by bacteria and supplies them with iron required for their pathogenicity (Bullen, 1980).

2.3.5 The avian surfactant

Surfactant is a mixture of phospholipids and specific proteins that function to maintain airflow through the respiratory system. In mammals, surfactant protein-D (SP-D) binds to glycoconjugates expressed by a variety of microorganisms, and to oligosaccharides associated with the surface of various complex organic antigens (Crouch, 1998). Alveolar macrophages express surface surfactant receptors that bind to the surfactant protein – microorganism complex and a higher rate of phagocytosis ensues (Wright, 1997; Eggleton and Reid, 1999). Surfactant protein-C is not found in the avian respiratory system but is found in the alveoli of mammals along with surfactant proteins-A and B (Mansikka et al., 1989). Because the mammalian respiratory system includes structures that are collapsible (alveoli) and areas with low airflow, all the three surfactants are important for reducing surface tension and for innate host defense (Bernhard and Floros, 2004). In birds, SP-A and SP-D have been found in the mesobronchi where the lipoproteins regulate inflammation, a function that is important since air flow is slower and small particles could tend to accumulate in the mesobronchi (Reese et al., 2006).
2.3.6 The immune system in the gas exchange area

Interestingly, little is known about the structure and functional relevance of the immune system in the avian lung parenchyma (Reese et al., 2006). Monocyte / macrophage and dendritic cells expressing major histocompatibility (MHC) class II have been identified in the chicken lung parenchyma (Jeurissen et al., 1989). The lung is well equipped with phagocytes and antigen presenting cells mainly located in the interstitial tissue as early as 5 days after hatching (Jeurissen et al., 1989). T cells in chicken lung show the classical cytotoxic T lymphocyte phenotype with the expression of CD8 and α/β TCR molecules. The CD4+ cells and γ/δ T cells are less frequent (Jeurissen et al., 1989).

The origin and distribution of free avian respiratory macrophages (FARM) in the avian lung has been addressed using microscopic techniques. FARM are absent on the surface of air capillaries but present on the epithelial lining the atria and infundibulae (Maina and Cowley, 1998; Maina and Van Gills, 2001). The FARM are also present in the connective tissue below the floor of atria (Maina, 1989) and in the interatrial septa (Reese et al., 2006), suggesting that phagocytic cells are strategically located at the start of gas exchange area to clear air of inhaled particles before reaching the thin and vulnerable air capillaries. In the air sacs, heterophils make up the majority of leukocytes followed by the FARM while lymphocytes are rare (Crespo et al., 1998). Repeated lung lavages lead to an increase in FARM numbers in the lavage fluid, indicating that the cells can transmigrate into air space either from the connective tissue or from the vascular system (Nganpiep and Maina, 2002). Under inflammatory conditions, heterophils and FARM are rapidly attracted to the respiratory surface. The cells are highly adhesive to glass surfaces,
actively phagocytic and cytotoxic to *Escherichia coli* as analyzed by *in vitro* assays (Mayers and Arp, 1987; Ficken and Barnes, 1989). Activated chicken FARM do release TNF α (Zuckerman *et al.*, 1989), the primary cytokine mediator of oxidant mediated lung inflammation triggered by reactive oxygen species, reactive nitrogen species and free radicals (Klasing, 1998).

Phagocytosis is perhaps the best known and, in evolutionary terms, most conserved function of macrophages that has been used to identify these cells *in situ* and to discriminate them from other leukocytes in mixed cell preparations (Klasing, 1998). There are fundamental differences between mammalian respiratory macrophages and FARM. Comparative studies of FARM and the corresponding mammalian cells, the alveolar macrophages, report FARM to have a significantly higher phagocytic ability than the mammalian cells. This is despite the two types of cells having equivalent diameter hence volume (Kiama *et al.*, 2008; Mutua *et al.*, 2011). Further, mobilization of FARM in avian respiratory system does not occur after intravenous application of *Escherichia coli* lipopolysaccharide (LPS), incomplete freunds (IFA) adjuvant, *Saccharomyces cerevisiae* glucan (G) or subcutaneous application of LPS-G-IFA, compounds which are known to induce the migration of macrophages in mammalian respiratory system (Toth *et al.*, 1987). It is not known whether avian macrophages express the cell surface markers CR3 also known as macrophage antigen 1 (Mac 1) and LFA, which are necessary for mammalian macrophage adherence and chemotaxis (Qureshi *et al.*, 2000). Cytokine production is another aspect of the avian respiratory macrophages that has not been well characterized though chicken macrophages do
produce TNF α (Zuckerman et al., 1989) and IL-1 (Qureshi et al., 2000). In mammals, respiratory macrophages have been a major area of focus for drug delivery by aerosolization. Review of drug design with degradation times appropriate for pulmonary delivery to reduce drug build up in the lung upon repeat administration, improved surface chemistry and reduction of drug clearance rates through phagocytosis in the deep lung is a major pharmacological challenge (Edwards and Hanes, 1997). In birds, information on the effect of FARM phagocytic ability on drug administration by aerosolization is scarce (Kiama et al., 2008).

There are, however, some functional similarities between mammalian respiratory macrophages and FARM. In mammals, phagocytosis of particles and micro-organisms is mediated by specific cell surface receptors which have been characterized in great detail (Taylor et al., 2005). In chicken embryos, macrophages with phagocytic activity have been observed as early as embryonic incubation day (EID) 12 in the liver and EID 16 in the spleen (Babior, 1995). Elicited FARM can be obtained in day-old chicks and turkey poults (Qureshi et al., 2000) demonstrating that this part of the innate immune system is functional at hatching. Cultured monocyte-derived macrophages, sephadex-elicited macrophages and the chicken macrophage cell lines HD11, MQ-NCSU and HTC spontaneously phagocytose SRBC, fluorescent micro-beads, bacteria or other particulate matter to different degrees (Beuget et al., 1979; Rath et al., 2003). Although elicited FARM harvested early after inflammatory stimulation of the respiratory system are efficient in phagocytosis, this can be significantly increased if cells are collected at a later time, indicating that FARM undergo functional maturation in response to inflammatory
stimuli (Chu and Dietert, 1988). Interestingly, phagocytosis of non-opsonized particles is restricted to a subpopulation, reflecting the functional heterogeneity of primary cell cultures (Qureshi et al., 2000) but also of macrophage cell lines (Rath et al., 2003). In cultures of elicited FARM, the number of phagocytic cells can be increased from 50% to more than 90% by opsonization with antibodies. This effect correlates with the expression level of FC receptors, as analyzed by the SRBC-rosette assay (Qureshi et al., 2000).

Phagocytosis by FARM has also been demonstrated with a range of bacterial species, including *Escherichia coli* (Miller et al., 1990) and *Pasteurella multocida* (Harmon et al., 1992), and the fungal pathogen *Candida albicans* (Rossi and Turba, 1981). Opsonization clearly increases phagocytotic activity *in vitro* for both bacteria (Myszewski and Stern, 1991) and fungi (Rossi and Turba, 1981). Binding of micro-organisms prior to phagocytosis requires receptor-mediated recognition. Macrophages are equipped with a range of receptor systems such as the scavenger receptors, complement receptors, Fc receptors, C-type lectins and mannose receptors mediating opsonic and non-opsonic recognition (Taylor et al., 2005). Functional studies strongly indicate the presence of some of these molecules on FARM and macrophage cell lines. SRBC opsonized with anti-SRBC serum from chickens or quails strongly adhere to cultured FARM and are more efficiently phagocytosed than non-opsonized erythrocytes (Qureshi, 2003). The observation that *C. albicans* adheres to FARM and is rapidly phagocytosed led to the functional characterization of the chicken mannose receptor and its ultrastructural localization on the cell surface (Rossi and Himmelhoch, 1983; Qureshi et al., 1994;
Detection and uptake of micro-organisms induces the activation of effector mechanisms which, in most cases, lead to pathogen destruction. As already demonstrated, during phagocytosis particles are internalized into phagosomes which subsequently fuse with lysosomes to form a phagolysosome. Lysosomes contain a variety of anti-microbial proteins and enzymes, such as acid phosphatase and β-glucuronidase; which have also been described for FARM (Mast et al., 1998).

Histochemical demonstration of non-specific esterase activity has been used to identify macrophages in tissue sections (Mast et al., 1998). Lysozyme expressed by chicken FARM has attracted some attention, since it is progressively activated during macrophage differentiation and has therefore been used as a stage-specific marker. Enzyme expression has been found to be low in myeloblasts but high in mature macrophages (Grewal et al., 1992), where it is expressed constitutively (Hauser et al., 1981). Furthermore, LPS stimulation of FARM leads to lysozyme gene activation, which may play a role in the antibacterial response of macrophages (Goethe and Loc, 1994).

2.3.7 Techniques used for studying phagocytosis

The basic procedure in a phagocytic assay is to incubate FARM with the target particle and then monitor the rate of either target ingestion or its loss from the medium (Absolom, 1986). Beads are often used, particularly if phagocytic index ability is the only parameter of interest (Mark and Christine, 1999). If initial rates of phagocytosis are sought, opsonization should occur before incubation with the FARM so that it does not become a limiting factor. It is also important to mix particles continuously during the assay so that
particle uptake is dependent on phagocytosis and not limited by lack of contact (Absolom, 1986). A major concern in phagocytosis assays is to distinguish between completely ingested particles and adherent ones. This challenge is overcome through differential centrifugation where ingested particles are pelleted while uningested remain in the supernatant (Mark and Christine, 1999).

2.3.8 Respiratory burst activity in the avian

Release of reactive oxygen and nitrogen intermediates has been recognized as an important microbicidal mechanism of activated macrophages. Neutrophils and macrophages reduce oxygen to superoxide, a reaction catalyzed by the NADPH oxidase. Superoxide further reacts with itself to form \( \text{H}_2\text{O}_2 \) from which highly reactive oxidants such as \( \text{HOCl} \) can be formed (Babior, 1995). Stimulation of elicited chicken FARM with phorbol ester has been shown to induce the release of high levels of superoxide (Golemboski et al., 1990), an effect also found with HD11 (Xie et al., 2002). Respiratory burst activity can also be induced with phorbol myristic acid, zymosan A, calcium ionophore (Desmidt et al., 1996) and different Salmonella serotypes (Chadfield and Olsen, 2001). Respiratory burst activity to Salmonella serovar Gallinarum has been shown to be genetically determined and to correspond to a higher rate of elimination of intracellular bacteria (Wigley et al., 2002).

2.3.9 Nitric oxide production in avian

In 1987, nitric oxide (NO) was identified as a highly potent anti-microbial effector molecule secreted by activated macrophages (Hibbs et al., 1987). Subsequent work
showed that NO is generated from L-arginine, which is converted to L-citrulline and NO by the action of nitric oxide synthase (NOS). NOS exist in three isoforms, the constitutively expressed neuronal NOS (nNOS), the endothelial isoform (eNOS) and the inducible enzyme (iNOS). The chicken FARM produce high amounts of NO in response to inflammatory stimuli and cytokines by upregulation of iNOS activity as was first demonstrated by Dietert and colleagues (Sung et al., 1991).

As in mammals, chicken iNOS requires tetrahydrobiopterin as a cofactor (Sung et al., 1994) which is synthesized from GTP in a well-defined enzymatic pathway (Nichol et al., 1985). Inducible NOS activity and tetrahydrobiopterin synthesis are initiated in a coordinated way in chicken FARM in response to stimulation with LPS or IFN γ (Kaspers et al., 1997). Molecular cloning of chicken iNOS revealed between 66 % and 70 % protein sequence identity with mouse and human iNOS and led to the identification of several conserved transcription factor binding sites (Kaspers et al., 1997). The importance of the nuclear factor-kappa beta (NF-κβ) pathway for iNOS induction by LPS has been clearly demonstrated using NF-κβ inhibitors (Lin et al., 1996). This work has subsequently been confirmed by others and extended to demonstrate a common signaling pathway for LPS and CpG oligodeoxynucleotide (ODN)-mediated induction of iNOS involving activated protein kinase C and mitogen-activated protein kinases. A distinctive feature of CpG ODN-mediated NO production is the requirement for clathrin-dependent endocytosis and endosomal maturation, consistent with observations on mammalian macrophages (He and Kogut, 2003). Chicken IFN γ and chicken IFN α were shown to potentiate NO secretion by HD11 cells synergistically (Sekellick et al., 1998).
Nitric oxide secretion has also been used as a readout system for the identification of pathogen associated molecular patterns (PAMP) with macrophage-activating activity. From the initial studies on NO biology in chickens, it became clear that LPS is a potent inducer of NO production (Sung et al., 1991) and iNOS transcription (Lin et al., 1996). Interestingly, repeated stimulation of chicken macrophages with LPS in vitro or in vivo induces a complete block of NO production (Chang et al., 1996). This macrophage response has already been demonstrated in mammals and is known as endotoxin tolerance (Chang et al., 1996). Comparison of LPS derived from different gram-negative bacteria revealed only moderate (Dil and Qureshi, 2002) or no (Okamura et al., 2005) differences in their NO-inducing activities. On the other hand, FARM obtained from chickens with different genetic backgrounds exhibit significantly different iNOS expression activities in response to LPS, thus defining hyper- and hypo-responder lines (Hussain and Qureshi, 1997). This observation has been attributed in part to differences in cell surface expression levels of the LPS receptor, Toll-like receptor (TLR) 4, using anti-human TLR4 antibodies (Dil and Qureshi, 2002).

2.3.10 Inflammation in the avian respiratory system

Inflammation is a complex biological response to harmful stimuli such as pathogens, damaged cells, or irritants such as ROS and RNS and it is a protective attempt by an organism to remove the injurious stimuli as well as initiating the healing process for the tissue (Lee et al., 1996; Ross, 1999). Although inflammation is a useful innate response, prolonged inflammation in the lung has been associated with pathogenesis of respiratory disease conditions such as the aspergillosis in domestic birds (Pascal et al., 2011).
Biochemical markers associated with low grade chronic inflammation include increased levels of C-reactive proteins (CRP) and certain inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF α) (Ross, 1999). IL-6 and CRP have related roles in the inflammatory response, as IL-6 induces CRP production in the liver by activating Janus kinases (JAK) (Heikkila et al., 2007).

In a normal healthy lung, blood endothelial cells (EC) resist adhesion by leukocytes, however exposure to key risk factors such as, pathogens and oxidants, initiates a highly coordinated and well regulated process that increases the expression of adhesion molecules by EC, which promotes the attachment and transmigration of leukocytes into lung parenchyma (Libby, 2006). Oxidative stress in the pulmonary epithelia leads to accumulation of modified lipoproteins, inducing pro-inflammatory pathways involving NF-κβ and the inflammatory cytokines interleukin1β (IL-1β) and TNF α. Inflammatory resolution in mammalian lung involves liquefaction of apoptotic neutrophils but in avian lung, it involves inspissations of apoptotic heterophils which are walled off by epitheloid FARM to form heterophil granulomas (Klasing, 1998).

Two subsets of monocytes with distinct patterns of surface markers and behaviors during lung inflammation have been characterized, the classical subset designated M1 (pro-inflammatory) and the alternative subset designated M2 (anti-inflammatory) (Johnson and Newby, 2009). While Th1 cytokines such as interferon gamma (INF γ), IL1-β and lipopolysaccharide induce M1 phenotype, Th2 cytokines such as IL-4 and IL-13 induce M2 phenotype. Moreover, macrophages are plastic cells with the ability to switch from an activated M1 state back to the M2 state and vice versa upon specific signal (Porchery et al.,
The M1 macrophages are potent effector cells that kill microorganisms and produce primarily pro-inflammatory cytokines, including TNF α, IL-6, IL-12, and ROS and RNS (Gordon, 2003). In contrast, M2 macrophages react to these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors including IL-10, TGF β, and IL-1 receptor antagonist (IL-1 Ra), scavenging debris, and promoting angiogenesis, tissue remodeling and repair, thus resolving inflammatory site (Mantovani et al., 2001; Gordon, 2003). Therefore, the balance between Th1 and Th2 cytokine profile is a critical determinant of inflammation as a successful innate response in elimination of injurious stimuli or in progression to chronic form which is maladaptive and causes lung tissue destruction.

2.4 Particle uptake in the avian respiratory system

In mammals, inhaled aerosol particles are eliminated from the respiratory system by several mechanisms including aerodynamic filtration, mucociliary clearance and phagocytosis (Kiama et al., 2008). Owing to the complexity of its three-tiered bronchial system, aerodynamic filtration may be more efficient in the avian compared to the mammalian respiratory system, where dichotomous bifurcation essentially occurs (Maina and Van Gils, 2001). Detailed study by Hayter and Besch (1974) showed that the deposition and clearance of particles on the avian airways is a function of particle size. Large particles (3.7 – 7 µm in diameter) are deposited and removed in the nasal cavities and proximal trachea, while smaller particles are deposited throughout the respiratory system. Midsize particles (1.1 µm) are trapped primarily in the lung and cranial air sacs while smaller particles (0.091 µm) pass through the entire lung and are finally trapped in abdominal air sacs. Removal of small inert particles (non-toxic iron oxide aerosol, particle diameter 0.18 µm) from the lung was first investigated by Stearns et al. (1986) in
a duck model. It was shown that these particles were not only phagocytosed by FARM but also by epithelial cells of the atria. These observations were subsequently confirmed in chickens, pigeons and ducks by Maina and Cowley (1998). From this work a picture emerges that epithelial cells and FARM play a crucial role in removal of particles from the air on their way to the thin, extensive and highly vulnerable tissue of the gas exchange area (Nganpiep and Maina, 2002).

2.5 Avian lung antioxidant system

The mammalian and avian lung systems, are highly supplied with blood, have vast surface area, and incessantly exposed to both exogenous and endogenous oxidants (Figure 2.4). These factors significantly make the pulmonary epithelia susceptible to oxidative stress (Irfan, 2008). To counter oxidative stress, the mammalian lung antioxidant defense system employs both enzymes and non-enzymes that breakdown oxidants. The major enzymatic antioxidants are catalase, superoxide dismutase, and heme oxygenase (Irfan, 2008). Non enzymatic antioxidants include low molecular weight compounds such as vitamins C and E, carotene, and uric acid (Irfan, 2008). Antioxidants react with and detoxify free radicals and degrade ROS and RNS, thus resolving oxidative stress mediated lung inflammation (Marc et al., 2011).

Oxidative stress occurs when the net flux of ROS/RNS exceeds the capacity of the cells to detoxify these potentially injurious oxidants (Wellen and Thompson, 2010). Oxidative stress induces lung inflammation through activation of NF-κβ, a transcription factor, of immune cells; this activates the cells leading to release of proinflammatory cytokines
(Wellen and Thompson, 2010). The most abundant antioxidant enzyme is catalase released by the peroxisomes; however, the enzyme is present in low quantities in the cytosol and nucleus (Marc et al., 2011). Other antioxidant enzymes released by peroxisomes include superoxide dismutase 1, peroxiredoxin 1 and 5, glutathione S-transferase kappa, and epoxide hydrolase 2 (Marc et al., 2011). In the mammalian lung peroxisomes are the major sources of antioxidant enzymes (Marc et al., 2011). Pulmonary tissue injury due to ROS and RNS is common to much pathology including chronic obstructive pulmonary disease and antioxidant therapeutic interventions in lung disease disorders have focused on increasing endogenous antioxidant release or by enhancing non enzyme defenses (Irfan, 2011).

Figure 2.4: Exogenous and endogenous sources of ROS in the avian respiratory system (Kohen and Nyska, 2002)
2.5.1 Peroxisomes

Peroxisomes are single membrane bound, dynamic organelles of eukaryotic cells (Angermuller and Fahimi, 1981). The organelles were first identified in the electron microscopy images of mouse kidney cells (Rhodin, 1954; De Duve, 1965). Peroxisomes are mostly spherical, 0.1 to 2 µm in diameter, and are surrounded by a single lipid bilayer membrane (Agrawal et al., 2011). The number, size, and function of peroxisomes depend on the cell type and environmental conditions. This is also reflected by their unique variability in enzyme content and their ability to adjust their metabolic capabilities according to cellular needs (Opperdoes, 1988; Oku and Sakai, 2010). Peroxisomes have a large number of oxidases producing H$_2$O$_2$ (Table 2.1) during metabolization of uric acid, D-amino acids and α-hydroxy acids (Purdue and Lazarow, 2001). In addition, peroxisomes contain antioxidant enzymes (Table 2.2) such as catalase, superoxide dismutase, glutathione, and peroxiredoxin 5 (Bonekamp et al., 2009). Therefore, peroxisomes must maintain a balanced redox state in health (Marc et al., 2011).

Catalase removes H$_2$O$_2$ in a catalytic (2H$_2$O$_2$ →2H$_2$O + O$_2$) or peroxidatic (H$_2$O$_2$+AH$_2$→A+2H$_2$O) manner (Fahimi, 1975; Marc et al., 2011). During the peroxidatic process, the potential hydrogen donors (AH$_2$) have been found to include alcohols, formate, and nitrite (Purdue and Lazarow, 2001; Kirkman and Gaetani, 2007). Peroxisomes are also involved in lipid biosynthesis (Lee et al., 1996) and the organelles posses MAVS, an anti-viral signaling protein required for early antiviral protection (Dixit et al., 2010). Peroxisomes are also involved in synthesis of bile acids and side chain of cholesterol (Pederson, 1987) and fatty acid elongation (Horie et al., 1989).
Table 2.1: Peroxisomal enzymes that produce ROS/RNS and free radicals as byproducts of their normal catalytic activity

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein symbol</th>
<th>ROS/RNS</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA oxidase 1</td>
<td>ACOXI</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>Acyl-CoA oxidase 2</td>
<td>ACOX2</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>Acyl-CoA oxidase 3</td>
<td>ACOX3</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>D-amino acid oxidase</td>
<td>DAO</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>D-aspartate oxidase</td>
<td>DDO</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>L-pipeolic acid oxidase</td>
<td>PIPOX</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>L-α-hydroacid oxidase 1</td>
<td>HAO1</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>L-α-hydroacid oxidase 2</td>
<td>HAO2</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>Polyamine oxidase</td>
<td>PAO</td>
<td>H₂O₂</td>
<td>PO</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>XDH</td>
<td>H₂O₂, NO⁻, O₂⁻</td>
<td>PO/MT</td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>NOS2</td>
<td>NO⁻, O₂⁻</td>
<td>PO/C</td>
</tr>
</tbody>
</table>

Table 2.2: Peroxisomal antioxidant enzymes

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein symbol</th>
<th>Substrate</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>CAT</td>
<td>H₂O₂</td>
<td>PO/C</td>
</tr>
<tr>
<td>Superoxide dismutase 1</td>
<td>SOD1</td>
<td>O₂⁻</td>
<td>C/MT/PO/N</td>
</tr>
<tr>
<td>Peroxiredoxin 5</td>
<td>PRD5</td>
<td>H₂O₂</td>
<td>C/MT/PO/N</td>
</tr>
<tr>
<td>Glutathione S-transferase kappa 1</td>
<td>GSTK1</td>
<td>Unknown</td>
<td>PO</td>
</tr>
<tr>
<td>Microsomal GST1</td>
<td>MGST1</td>
<td>Unknown</td>
<td>MT/PO</td>
</tr>
<tr>
<td>Epoxide hydrolase 2</td>
<td>EPHX2</td>
<td>Epoxides</td>
<td>PO/C</td>
</tr>
</tbody>
</table>

PO- Peroxisome; C-Cytosol; MT-Mitochondria; N-Nucleus (Marc et al., 2011)
2.5.2 Peroxisome biogenesis

The half-life of peroxisome is three days, therefore, the organelle is constantly replaced through a process called peroxisome biogenesis or assembly of peroxisome (Wanders and Waterham, 2006). Biogenesis of peroxisomes takes place in three steps: a) formation of peroxisomal membrane b) import of peroxisomal matrix proteins and c) proliferation of peroxisomes (Figure 2.5). The processes involved in assembly of peroxisome membrane proteins are contentious, however, it has been hypothesized that endoplasmic reticulum (ER) could be involved in assembly of peroxisome membrane proteins (Novikoff and Shin, 1964). Other studies report that peroxisome membrane assembly is semi-autonomous process involving trafficking of only some of the peroxisome membrane proteins via the ER (Agrawal et al., 2011).

![Figure 2.5: A composite model of peroxisome biogenesis (Nagotu et al., 2012). ADP-Adenosine diphosphatate, PMP-Peroxisome membrane protein](image_url)
Peroxisome matrix proteins, also referred to as cargo, are fully folded oligomeric and cofactor bound proteins. Import of the matrix proteins involves cargo recognition, receptor docking, receptor translocation, cargo release and receptor release and recycling processes (Ma et al., 2009). Proteins imported into peroxisome matrix typically contain one of the two peroxisome targeting signals (PTS 1 or PTS 2). Proteins containing PTS 1 such as catalase are recognized by the cytosol receptor called peroxin 5 (Pex 5) while proteins bearing PTS 2 bind to Pex 7 (Brocard and Harting, 2006). Subsequent to formation of the receptor-cargo complex in the cytosol, the receptor ferries the cargo protein to the peroxisomal membrane and successful docking of the receptor-cargo on the membrane depends on presence of peroxisome membrane bound Pex 13 and 14 (Islinger et al., 2006). Pex 13 and 14 are docking peroxisome membrane bound receptors required for formation of pores in the membrane for successful cargo translocation into the peroxisome matrix (Ma et al., 2009). Once the receptor-cargo complex has translocated into the peroxisome matrix, other intra-peroxisomal membrane bound peroxins such as Pex 8, facilitate cargo release into the matrix and subsequent return of PTS-receptors into cytosol for further import (Kurochkin et al., 2007). After successful assembly of peroxisome membrane and import of the matrix proteins, peroxisomes proliferate by fission, a process that is partly regulated by Pex 11 proteins (Hoepfner et al., 2001).

2.5.3 Cytochemical techniques for investigation of peroxisomes

Cytochemical techniques, including electron microscopy and morphometry, have contributed significantly to present knowledge of peroxisomes (Dariush and Baumgart, 1999). The diaminobenzidine (DAB) method for localization of catalase, a marker
enzyme of peroxisomes, is commonly used in morphological survey for detection and identification of peroxisomes (Dariush and Baumgart, 1999). Fixation of catalase with an aldehyde increases peroxidatic activity of the enzyme and facilitates its detection by DAB, a benzidine derivative which gets oxidized in presence of hydrogen peroxide forming brown precipitate (Herzog and Fahimi, 1974).

The principal of tissue fixation with an aldehyde is also used for distinction between catalase, which requires fixation, and peroxidases, which are highly sensitive to aldehyde fixation (Fahimi et al., 1976). The optimal conditions for detection of peroxisomal catalase with DAB are tissue fixation with aldehyde, incubation with 1 – 2 % DAB at high-alkaline pH of 10.5 (Appendix I) at 37°C to 45°C, and a high concentration of 0.15 % hydrogen peroxide (Angermuller and Fahimi, 1981). The universal buffer of Teorell and Stanhagen with broad pH range of 2 – 12 and strong buffering capacity has proved useful (Fahimi, 1975). However, glycine-NaOH buffer can also be used as well (Dariush and Baumgart, 1999). Cerium technique is presently the method of choice for microscopic detection of peroxisomal oxidases (Dariush and Baumgart, 1999). The optimal conditions of cerium method is short perfusion-fixation with 0.25 % glutaraldehyde in PIPES buffer, pH 7.4, followed by incubation in 3mM cerium chloride with specific substrate such as urate, glycolate or D-proline, in appropriate buffers in presence of 100mM sodium azide (Angermuller and Fahimi, 1981). Sodium azide inhibits peroxisomal catalase activity that would otherwise destroy hydrogen peroxide generated by oxidases (Dariush and Baumgart, 1999). Other methods used for detection
of peroxisomal proteins include immunoperoxidase, immunogold, immunofluorescence and green fluorescent protein detection (Dariush and Baumgart, 1999).

### 2.6 Peroxisome proliferator activated receptors

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor family including also the retinoid, glucocorticoid and thyroid hormone receptors (Evans, 1988). The PPARs consist of an amino-terminal region that allows ligand-independent activation, a DNA-binding domain and a carboxyl-terminal ligand dependent domain (Moras and Gronemeyer, 1998). The term peroxisome proliferator activated receptor was coined when activation of peroxisome proliferator activated receptor alpha (PPAR α) resulted in proliferation of peroxisomes by changing expression of peroxisomal genes (Issemann and Green, 1990). Unsaturated long chain fatty acids and lipid derivatives were later identified as the natural ligands of PPAR α (Dreyer et al., 1993). This discovery lead to further studies on peroxisome proliferator activated receptors resulting into discovery of two related nuclear receptors, peroxisome proliferator receptor gamma and beta (PPAR γ and PPAR δ), which have been shown not to transmit signals for peroxisome proliferation (Spiegelman and Flier, 1996).

A review by Michalik and Whali (2006) reports mammalian PPAR α to causes tissue repair through clearance of inflammatory sites in the kidneys and digestive tract while PPAR γ has been demonstrated to resolve inflammatory disease disorders in the kidneys, digestive tract and in the lungs. The mammalian PPAR γ is expressed in two isoforms γ₁
and \( \gamma_2 \) but these differ only by addition of 30 amino acids at the amino terminus of \( \gamma_2 \). However, PPAR \( \gamma \) isoforms are functionally equivalent (Spiegelman and Flier, 1996). Additional functions of mammalian PPAR \( \gamma \) include maintenance of normal lung structure through regulation of epithelial cell differentiation and regulation of monocyte / macrophage differentiation (Simon and Mariani, 2007). Studies indicate that PPAR \( \gamma \) also functions as an immunomodulator and has potential anti-inflammatory role in asthma (Belvisi et al., 2006). Later studies revealed the mechanism through which mammalian PPAR \( \gamma \) ligands resolve airway inflammation to involve inhibition of release of proinflammatory cytokines from activated respiratory macrophages (Jiang et al., 1998) and airway epithelia cells (Wang et al., 2001).

2.6.1 Molecular mechanism of PPAR transcription

The PPAR is structurally composed of three domains: a ligand-independent domain, DNA-binding domain and a ligand-dependent domain (Figure 2.6). The PPARs bind to the peroxisome proliferator response element on the DNA with sequence AGGTCA \( \text{n} \) AGGTCA, a direct repeat with single nucleotide spacer, (Figure 2.7).

![Figure 2.6: General structure of PPAR (Marx, 2002). AF-Activation function, DNA-Deoxyribonucleic acid, DAB- DNA binding domain](image-url)

Figure 2.6: General structure of PPAR (Marx, 2002). AF-Activation function, DNA-Deoxyribonucleic acid, DAB- DNA binding domain

- Ligand-independent activation domain (AF-1)
- Ligand-dependent activation domain (AF-2)
- DBD - DNA Binding Domain (2 zinc fingers)
- LBD-Dim. - Ligand Binding and Dimerization Domains
Upon activation, PPARs form heterodimers with another nuclear transcription factor, retinoid X receptor (RXR) and bind to specific PPAR response elements (PPREs) in the promoter region of their target genes (Touyz and Schifrin, 2006). This process regulates gene function, which includes activation or repression of gene expression. In the unliganded state, the PPAR / RXR heterodimer is associated with a multiprotein co-repressor complex that contains histone deacetylase activity with subsequent formation of condensed and transcriptionally silenced chromatin (Moras and Gronemeyer, 1998).

Subsequent to PPAR ligand binding to the receptor, the co-repressor complex dissociates and a co-activator complex containing histone acetylase activity is recruited to the PPAR/RXR heterodimer (Touyz and Schifrin, 2006). PPARs have been shown to regulate gene expression through a number of transcriptional activities. Firstly PPARs bind to response elements in the presence of ligands and co-activator proteins and initiate transcription. Secondly PPARs negatively regulate gene expression in a ligand dependent manner by
antagonizing the activities of other signal dependent transcription factors, including NF-κβ (Mattson et al., 2000). The relative contribution of NF-κβ transcriptional activity to either a “good” inflammation (manifested by cell survival and tissue regeneration) or a “bad” inflammation (causing cell death and tissue destruction) rather depends on the kinetics of activation and intrinsic metabolic differences between cell types or within the same cell and on the nature as well as intensity of the activating stimulus (Mattson et al., 2000). The NF-κβ is a widely expressed, inducible transcription factor of particular importance to regulation of cells of the immune system (Karin, 1998). NF-κβ plays a crucial role in the regulation and expression of many genes involved immune and inflammatory responses, including cytokines, chemokines, cell adhesion molecules, complement factors, acute phase proteins, inducible effector enzymes and a variety of immune-receptors (Pahl, 1999). Additionally, NF-κβ has been implicated in regulation of cellular events such as apoptosis, cell proliferation and differentiation and activation of iNOS (Pahl, 1999).

In un-stimulated cells, NF-κβ is sequestered in the cytoplasm as inactive transcription factor complex by its physical association with one of the several inhibitors of NF-κβ (iκβ) (Ghosh et al., 1998). Treatment of cells with extracellular stimuli, including cytokines such as IL-1, TNF α, viral proteins, bacterial lipopolysaccharide, and oxidants leads to rapid phosphorylation and subsequent degradation of iκβ by proteasome. Consequently, dissociation of NF-κβ leads to nuclear translocation and binding of NF-κβ to specific consensus sequences in the chromatin and activation of specific subsets of inflammatory genes (Ghosh et al., 1998). Successful binding of NF-κβ with chromatin requires coactivators such as p50 and p65 (Makarow, 2000). Treatment of cells with PPAR γ ligands, such troglitazone, enhances subsequent binding of PPAR-γ to the NF-κβ coactivators effectively inhibiting NF-κβ induced inflammatory conditions. This mechanism does not involve binding
of PPAR γ to DNA and is referred to as transrepression and is critical in the anti-inflammatory actions of nuclear transcription factors (Moras and Gronemeyer, 1998; Ricote et al., 1998). Activated PPAR isoforms bind to DNA with subsequent different transcriptional DNA activities depending on the PPAR isoform. Activation of PPAR α up-regulates proliferation of peroxisomes and increase of peroxisomal β - oxidizing enzymes (Dreyer et al., 1993). Since PPAR α is predominantly expressed in adipocytes, hepatocytes, cardiomyocytes, kidney cortex and skeletal muscles, its activation is vital in supply for energy in these high capacity fatty acid oxidation tissues (Dreyer et al., 1993). The PPAR δ expression is ubiquitous but is highly expressed in oocytes, nervous system, skeletal and cardiac tissues, and activation of this receptor is associated with normal functions of these tissues (Braissant and Whali, 1998).

Activation of PPAR γ has been shown to cause maturation of preadipocytes to adipocytes, thus increasing adipose tissue content (Tontonoz et al., 1994). Further, PPAR γ exerts antimitotic action to stop cell proliferation during terminal adipocyte differentiation (Dreyer et al., 1993). Activation of PPAR γ stimulates storage of fatty acids in mature adipocytes by increasing: a) release of fatty acids from triglycerides stored in lipoprotein particles, b) intracellular transport of fatty acids to adipose tissue, and c) fatty acids esterification (Olswang et al., 2002). Treatment of activated macrophages with high doses of 15-deoxy-\(^{12,14}\)-prostagladin J\(_2\), a natural PPAR γ ligand, activates PPAR γ in the cells with subsequent inhibition of nitric oxide production. This inhibition is due to an antagonizing activity of PPAR γ directed toward the activity of the transcription factor NF-κβ known for control of cytokine gene expression (Jiang et al., 1998). Furthermore, treatment with PPAR γ ligands of macrophages inhibits the induced
expression of proinflammatory TNF α, IL-6, and IL-1 β and up-regulates expression of Th2 cytokines (Figure 2.8) promoting non-phlogistic clearance of inflammatory site through scavenging of debris, promotion of angiogenesis, tissue remodeling and repair (Jiang et al., 1998; Johnson and Newby, 2009).
Lung interstitial blood monocytes

- Th1 cytokines (IFNγ, IL-1β) and LPS
  - M1 Macrophage
  - Treatment with PPARγ ligand
  - Down-regulates M1 Markers

- Th2 Cytokines (IL-4, IL-13)
  - M2 Macrophage
  - Treatment with PPARγ ligand
  - Up-regulates M2 Markers
Figure 2.8: Monocyte differentiates into M1 or M2 under the influence of Th1 or Th2 cytokines with subsequent upregulation of proinflammatory or anti-inflammatory cytokine profiles respectively. Treatment of MI with PPAR γ ligand alleviates effects of inflammation by down-regulating release of pro-inflammatory cytokines and promoting tissue healing (Gordon, 2003)

2.7 Troglitazone

Troglitazone, the first clinically useful thiazolidinediones (TZDs) class of drugs, was approved for sale in 1997 for treatment of type 2 diabetes (Foneseca et al., 1998). The TZDs lower blood glucose by improving beta cell function (Kumar et al., 1996). Later, TZDs were accidentally found to be selective synthetic PPAR γ ligands (Foneseca et al., 1998). In 2000, troglitazone was withdrawn from the market because of its association with hepatotoxicity and water retention leading to edema but the drug is manufactured for use in in-vitro based research (Lebovitz et al., 2001). Further, treatment of mammalian macrophages with high doses of troglitazone induces cellular protein degradation thus significantly reducing macrophage viability (Kumar et al., 1996).

Two additional members of TZDs class were introduced in US market for treatment of type 2 diabetes mellitus: rosiglitazone (Avadia®, GlaxoSmithKline) and pioglitazone (Actos®, Takeda) (Lebovitz et al., 2001). In the mammalian lung disorders, treatment of respiratory macrophages with TZDs activates PPAR γ in the cells with subsequent inflammatory resolution through trans-repression of genes involved in transcription of proinflammatory cytokines. Furthermore, TZDs activates respiratory macrophage PPAR γ resulting into increased non phlogistic phagocytic clearance of tissue debris, apoptotic neutrophils and microbes by the respiratory macrophages (Kazuhiro et al., 2003). Therefore, the anti-inflammatory properties of synthetic selective PPAR γ ligands could
explain the incremental therapeutic benefits of the ligands in management and treatment of inflammatory disease conditions in mammals (Desvergne and Whali, 1999).

2.8 Chicken antioxidant defense system

Redox regulation of gene expression by superoxide and other related oxidants and antioxidants is beginning to unfold as a vital mechanism in health and disease (McCord, 2000). Unfortunately this subject is much less studied in relation to animals but information is also accumulating which shows the role of free radicals in animal production. In poultry industry, diseases such as nutritional muscular dystrophy, encephalomalacia and exudative diathesis (Combs, 1994), and Pulmonary Hypertension Syndrome (Bottje and Wideman, 1995) are associated with overproduction of oxidants.

It has been suggested that oxidant-antioxidant balance is a major determinant of successful chick embryo and early postnatal development (Surai, 2001). The chick embryo brain has high lipid peroxidation due to accumulation of polyunsaturated fatty acids hence extremely susceptible to oxidant stress which is also occasioned by low antioxidant protection (Surai, 2001). The chicken liver is the main site of natural antioxidant accumulation and metabolism. Vitamin E and carotenoid protect the liver against oxidant stress (Surai, 2001). FARM produces ROS following infection and exposure to high oxygen tension and, therefore, a dearth of FARM in the avian lung has been postulated to protect the avian lung against oxidative stress which has high extraction of oxygen than mammalian lung (Maina and Nathaniel, 2001). Chicken serum oxidant and antioxidant system has been reported in broiler chickens. Broiler birds fed on
aflatoxin contaminated diet had significantly high levels of oxidative mediated inflammation characterized by presence of high levels of serum malondialdehyde, tumor necrosis factor alpha, Interleukin-6, and interferon gamma (Yani et al., 2014). However, broilers which had been exposed to aflatoxin, α-lipoic acid supplemented diet had significantly low levels of proinflammatory cytokines in their serum suggesting that α-lipoic acid activates antioxidant defense thus alleviating effects of systemic oxidative mediated inflammation in the chickens (Yani et al., 2014). Antioxidant enzymes in the chicken serum identified in the aflatoxin fed broilers were superoxide dismutase and glutathione peroxidase (Yani et al., 2014).

2.9 Chicken peroxisomes and PPARs

2.9.1 Chicken peroxisomes

Peroxisomes have been shown to be present in the chicken liver and kidney. Morphologically, chicken peroxisomes resemble those described in other species and contain the core structure (Scott et al., 1989). Biochemically, chicken peroxisomes were found to contain catalase, α-hydroxy acid oxidase, D-amino acid oxidase, urate oxidase and xanthine dehydrogenase. However, catalase, the principal peroxisome biomarker, was the most abundant enzyme accounting for 98 % of chicken liver and kidney peroxisomal enzyme content (Scott et al., 1989).

2.9.2 Chicken PPARs

Chicken PPAR δ, γ and α subtype genes were identified through cloning (Takada et al., 2000). For cPPAR γ, amino acids residues predicted for phosphorylation by MAP kinase
are conserved between mammals and chicken PPAR γ. Further cPPAR γ share conserved target amino acid residues (Lys 77 and Lys 365) required for regulation of inflammation with mammalian PPAR γ (Takada et al., 2000). This suggests that cPPAR γ transcriptional activity could have anti-inflammatory properties (Takada and Koyabashi, 2012). While mammalian PPAR γ exists in two isoforms: γ1 and γ2, cPPAR γ2 has not been isolated (Sato et al., 2004; Takada and Kobayashi, 2012). The structural organization of PPAR α and δ genes is largely conserved in humans and chickens but the PPAR γ structure is not. The cPPAR γ gene has 6 exons that extend across 50 kilobases of genomic DNA. In contrast, human PPAR γ gene consists of 8 exons for the γ1 and γ2 subtypes respectively and extends across more than 160 kilobases of the genomic DNA (Takada and Koyabashi, 2012).

2.9.3 Expression patterns of cPPAR m RNAs

Expression patterns of mRNA of cPPARs are similar to rodents and primates. In an adult chicken, cPPAR α is highly expressed in the heart, kidney and liver (Scott et al., 1989). Like in other species, cPPAR δ is expressed ubiquitously (Takada and Koyabashi, 2012). The cPPAR γ protein is predominantly expressed in adipose tissue, lung epithelium and in immune cells such as the macrophages (Sato et al., 2004). Additionally, cPPAR γ expression in the adipose tissue has been reported to increase with age. The protein was reported to be higher in 6-week old chicken than in 1 to 2 week old chicken (Sato et al., 2004). In addition, cPPAR γ is also expressed in the skeletal muscle and the ovaries (Sato et al., 2004).
2.9.4 Regulators of cPPAR mRNAs

In humans, it has been established that cancer, aging and metabolic disorders correlate with PPAR mRNA levels (Irfan, 2008). Human colorectal cancer, for example, correlates with high levels of PPAR γ mRNA, which exerts antimitotic activity of the colorectal cancer cells, indicating cancer related factors regulate PPAR γ expression (Irfan, 2008). Additionally, Sato et al. (2004) reported that expression of cPPAR γ may be modulated by nutritional states in a similar manner to mammals. In fatty broiler chickens, cPPAR γ mRNA expression is induced in liver but not adipose tissue (Takada and Koyabashi, 2012). Since PPAR γ is induced in adipose tissue of obese mammals, the difference in cPPAR γ expression could indicate divergence of PPAR signal transduction mechanisms between avian and mammals (Vidal-Puig et al., 1997). Restriction of feeding induces expression of cPPAR α in the adipose tissue, thus increasing lipid peroxidation to meet energy needs (Takada and Kobayashi, 2012). In spleen, cPPAR α and γ mRNAs are induced by lutein, a plant carotenoid but the expression is reduced by lipopolysaccharide, a finding that is consistent with mammalian PPAR γ (Takada and Kobayashi, 2012). Further, cPPAR γ mRNA expression in chicken ovaries was increased with onset of egg laying, suggesting role of cPPAR γ in egg quality (Sato et al., 2004).

2.10 The amino acid homology between chicken and mammalian PPARs

The amino acid sequence in the DNA binding domain of cPPARs shows high similarity with mammals. The similarities have been reported as a) cPPAR α versus hPPAR α: 95%, b) cPPAR δ versus hPPAR δ: 98 % and c) cPPAR γ versus hPPAR γ: 100 % (Takada and Koyabashi, 2012). The sequence of amino acids in the ligand binding
domain is also high for: a) cPPAR α versus hPPAR α; and b) cPPAR γ versus hPPAR γ: both at 96 % (Takada and Koyabashi, 2012). However, the amino acid sequence of the ligand binding domain of cPPAR δ shows a lower similarity with hPPAR δ at 90 %, a difference that implies different ligand responses (Takada and Koyabashi, 2012).
CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

The study was carried out in the department of zoological sciences, Kenyatta University. All experimental procedures were approved by the Kenyatta University Animal Ethics Committee. Electron microscopy was conducted at the department of Veterinary Anatomy and Physiology Chiromo Campus University of Nairobi.

3.2 Study subjects

Healthy indigenous chicken were used in this study. Peroxisome proliferator activated receptors, a major focus component of this study, are not fully developed in chicken aged six months and below (Takada and Kobayashi, 2012), therefore chicken aged eight months were selected for this study.

3.3 Sample size and sampling

The sample size was estimated using the formula:

\[ n = \frac{z^2 \rho(1-\rho)}{e^2} \]  

(Pascal et al., 2011), where \( n \) = sample size, \( z \) = Z score value, \( \rho \) = proportion of indigenous chicken in Nairobi (190,000) relative to total population of indigenous chicken in the country (26,000,000) (Winrock International, 2010) \( e \) = margin error of the study. Therefore, \( n = \frac{1.96^2 \times 0.0073 \times 0.9927}{0.025^2} \) hence \( n=44 \) chickens. Subsequent to estimating the sample size, table of random numbers was used to select 44 chickens from
a group of 60 indigenous chickens kept by a farmer in Ruiru, Nairobi. The 44 birds were selected from the population of 60 based on assigned wing band numbers.

3.4 Experimental design

In order to acclimatize to experimental environment, the birds were kept in animal house for 10 days in the Department of Zoological Sciences, Kenyatta University under housing conditions of 12-hr light/ dark cycle, temperature of 22 ± 2°C, and relative humidity of 55 ± 15 % . They were fed on a 14 % protein mash (Unga Feeds, Nairobi) and water ad libitum. Using random number tables and cumulative average graphs, the animals were assigned to 4 groups based on their wing-band numbers (group A = 10; group B = 12; group C = 12 and group D = 10). In A, the birds were used for tissue fixation and harvesting for morphological survey and identification of peroxisomes and to characterize catalase distribution in the chicken trachea, bronchi and lung tissues. In B, two groups of 6 birds each were used for recovery of FARM that were quantified and used for morphological and in vitro viability experiments. In C, two experimental groups of 6 birds each, were used for comparison of phagocytic capacity of troglitazone treated and untreated FARM. In D, the birds were used for recovery of FARM for measurement of TNF α production by FARM treated with varying doses of troglitazone. A control experiment of troglitazone untreated FARM was used to determine effect of troglitazone on TNF α production.

3.5 Anesthesia and euthanasia of the chicken
The chickens were anesthetized through intramuscular injection of Ketamine HCl (100 mg / ml) at a dose of 5 mg / kg followed by euthanasia through intravenous injection of Euthanase ® (200 mg / ml sodium pentobarbitone) into brachial vein at a dose of 10 mg / kg.

3.6 Chicken respiratory tract fixation and tissue harvesting
Following anesthesia and euthanasia of the chicken, the respiratory tract fixation was performed as described by Maina and van Gills (2001). Briefly, the bird was placed in supine position and a transverse incision was made at mid-cervical region to reach the trachea which was then exteriorized and cannulated through the larynx. Intra-tracheal installation of a fixative composed of 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 was done from a height of 25 cm and left in the lung air sac system for 4 hours after which the trachea was ligated (Appendix I). The respiratory tract was carefully removed by dissecting carefully from the deep costal attachments and immersed in a fixative under vacuum for overnight to ensure satisfactory infiltration.

3.7 Localization of peroxisomes
To localize peroxisomes, small blocks of wet sections of the trachea, bronchi and the lung tissues measuring 1 mm³ were additionally fixed for 15 min in a 2.5 % glutaraldehyde fixative in 0.1 M phosphate buffer. The blocks were then rinsed three times with 0.01M glycine-sodium hydroxide buffer, pH 10.5, and transferred to freshly prepared alkaline DAB medium (Appendix II) containing 0.2 % 3, 3’-diaminobenzidine, 0.15 % H₂O₂, 0.01M glycine-sodium hydroxide buffer, pH 10.5 (Angermuller and Fahimi, 1981) for 2 hours in a shaking water bath at 45°C. Thereafter, the blocks were washed 3 times with
0.01 M glycine-sodium hydroxide buffer, pH 10.5 in room temperature and post-fixed overnight in 1 % osmium tetroxide at 4°C followed by dehydration in ascending concentrations of ethanol (70 %, 80 %, 95 % and 100 % twice). The ethanol in the tissues was replaced gradually with propylene oxide before infiltration and embedding in epoxy resin. For morphologic examination of peroxisomes, epoxy embedded tissue blocks were trimmed into semithin and ultrathin sections with Reichert ® ultramicrotome (Pennsylvania, USA). The semithin sections, (1 μm thick), were collected on glass slides, stained with 3 % toluidine blue and viewed under a photo light microscope. The ultrathin sections, (80 nm thick), were picked on 200 wire mesh copper grids, stained with uranyl acetate, counterstained with lead citrate and observed with a Philips 2001 TEM (Bonn, Germany) under increasing voltage of 60 Kv. Peroxisomes were identified as organelles containing finely granular matrix in which the diaminobenzidine reaction product was precipitated.

3.8 Morphometric analysis of peroxisomes

The diameters of the peroxisomes were determined under an ocular graticule with a linear scale at a final magnification of × 100. In each field, to avoid bias, the peroxisomes were singled out at random and the diameters of the organelles at the four corners of the fields and one at the middle were counted. The volume density of peroxisomes in the trachea, bronchi and lung tissues was calculated as the ratio of the total volume of the peroxisomes to the total volume of the tissue \[Vv \left(p, t\right)\]. The ratio was estimated by point counting on plane sections (Gundersen, 1977). For this purpose, twelve sections, that is four sections each from trachea, bronchi and lung tissues, were randomly sampled. The
corresponding micrographs were projected on a screen and a transparent square test grid overlaid randomly on each projected image. An estimator of volume density of peroxisomes in the tissues was then calculated thus: \( Vv (p, t) = \frac{P_p}{P_t} \) where \( P_p \) was the total number of points falling on profiles of the peroxisomes and \( P_t \) the total points falling on profiles of the entire tissue.

### 3.9 Localization of catalase

Blocks of paraffin embedded chicken trachea, bronchi and lung tissues were trimmed into semithin (3 – 4 µm) sections using Reichert ® ultramicrotome (Pennsylvania, USA). The sections were deparaffinized with xylene (3×5 min) followed by rehydration in a series of ethanol (2×99 %, 96 %, 80 %, 70 % and 50 % for 2 min each step). For antigen retrieval and improved accessibility of epitopes, rehydrated sections were subjected to 0.01 % trypsin digestion for 10 min at 40°C and additional irradiation for 3× 5 min in a microwave oven (850W, citrate buffer, pH 6) with subsequent cooling for one hour at RM. Blocking of endogenous peroxidase was done by incubating the sections for 5 min with 3 % \( \text{H}_2\text{O}_2 \) in a moist chamber at room temperature (RT). Non- specific protein binding sites and endogenous biotinylated proteins in the sections were blocked for 2 hours with 4 % BSA in PBST (PBS / 0.05 % tween 20, pH 7.4) containing avidin (Avidin/Biotin blocking kit). Washing of sections was done using PBS for 5 min after each of the aforementioned steps. After the last wash, the sections were incubated overnight with rabbit polyclonal antibody against chicken catalase (Abcam, Cambridge Science Park Cambridge, UK). For detection of antigen-antibody complexes, the sections were incubated with biotinylated secondary antibody for two hours at RT. Bound
secondary antibody was detected with peroxidase-coupled extravidin and visualized by histochemical staining of the peroxidase activity using novared as substrate (4 min in RT). This reaction was stopped with water and the sections were dehydrated in series of ethanol and xylene and mounted with depex. Negative controls were incubated in parallel without antibody. The sections were counterstained with 3 % toluidine blue for 10 min, and viewed with a light photo microscope for analysis.

3.10 Recovery of FARM by lavage

Following anesthesia and euthanasia of chicken as described in section 3.5, lavage of the respiratory system was performed with the animal in a supine position (Figure 3.1) at room temperature (22°C). Feathers on the neck region were plucked and the exposed neck area cleaned with gauze bandage soaked in 70 % ethanol. A ventral midline incision was made from the mandible to the thoracic inlet. A folded thread was passed beneath the trachea with the aid of curved forceps and a loose knot made. The trachea was dissected free of surrounding fascia and cut transversely at the mid cervical region. Gently, a cannula was inserted into the trachea through the incision and held tightly in position by fastening the thread. This was done to avoid entry of blood into the lungs. The cannulated trachea was covered with a gauze bandage moistened with physiological saline. Respiratory system was then evacuated of air using a 50 ml syringe. Complete evacuation was indicted by the collapse and a limited retraction of the trachea into the thoracic cavity. The respiratory system was instilled with known volume of prewarmed (40°C) sterilized phosphate-buffered saline (0.1M PBS-pH 7.4) at a pressure head of 25 cm H2O (2500 Pa) until the flow stopped. During instillation, the coelomic cavity was gently
massaged to expel trapped air, ensuring penetration of PBS into small air spaces of the lungs and the air sacs. The instilled fluid was left in the respiratory system for 10 min. Thereafter, the fluid was aspirated with a 50 ml syringe and the recovered volume recorded. The recovered lavage fluid was collected into chilled 50 ml centrifuge tubes. Five washes were performed per chicken. This technique allowed the lavage fluid to reach all parts of the respiratory tract. Preliminary studies with instilled trypan blue PBS mixture, followed by recovery of the lavage fluid and exposure of the chicken respiratory system by dissection, revealed blue stained lungs and air sacs.

Figure 3.1: Lavage set up of a chicken. Pre-warmed (40°C) sterile PBS was instilled into the chicken lung-air sac system. The top level of the PBS in the lavage bottle was measured to a height of 25 cm from the sternum prior to instillation. On average, 400 ± 10.4 ml of PBS was instilled and 85 ± 1.6 % of this fluid was recovered from the respiratory system

3.11 Counting of FARM

Lavage fluids were centrifuged at 1000 rpm in RT for 10 min in the original tubes using a Hermle Z 200A centrifuge (Marburg, Germany). The supernatant was removed and a
final concentration of 10 ml of the lavage fluid was finally achieved through repeated centrifugations under sterile conditions in a laminar flow unit (China). The concentrated lavage fluids were mixed by vortexing to ensure homogeneous distribution of FARM. Charging of the two chambers of hemocytometer, which was carefully clipped on the stage of an inverted microscope, was done by drawing 200 µl of lavage fluids using a micropipette and counting of FARM was done at final magnification of ×100. Free avian respiratory macrophages were identified as morphologically round or slightly amorphous, refractile, frequently granular cells. Counting of the FARM was restricted to the four large corner squares of each chamber. The total number of FARM per lavage was obtained by getting the average of the counts obtained in upper and lower chambers of the hemocytometer. The number of FARM per bird was then computed as an average of FARM recovered in the five lavages.

3.12 FARM in vitro viability tests
The experiments were performed to determine whether chicken FARM treated with troglitazone would have equivalent in vitro viability with untreated FARM when handled and exposed to similar experimental conditions. Chicken FARM were recovered by lavage and washed three times in PBS. The FARM were subsequently re-suspended at a concentration of 1.5 × 10⁶ cells / ml in RPMI-1640 supplemented with 10 % BSA and 100 U ml penicillin / streptomycin (Bio Whittaker ®) in sterile eppendorf tubes and treated with 9 µM of troglitazone (Abcam, Science Park Cambridge, UK).
A control pellet of FARM was recovered from another group of six birds and processed in a similar manner albeit not treated with troglitazone. The tubes were kept in an incubator with temperature set at 40°C and 5% CO₂ for four hours after which the tubes were centrifuged and the cell pellet washed three times in PBS. Staining of FARM was done by placing and mixing 180 µl of trypan blue (0.3 % in physiological saline), 270 µl of PBS and 50 µl of cell pellet in a sterile eppendorf tube. Cell viability was done by using trypan blue dye exclusion test in hemocytometer.

3.13 Morphology of the FARM

After viability tests were performed, the remaining untreated FARM in eppendorf tubes were washed three times in PBS and fixed in 2.5 % phosphate buffered glutaraldehyde solution in the eppendorf tubes and kept in 4°C for at least 12 hours. The glutaraldehyde fixed cells were centrifuged and post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. This was followed by dehydration in graded replacement of ethanol (70 %, 80 %, 90 %, and 100 % twice). Gradual replacement of ethanol with propylene oxide was then done before infiltrating and embedding the cells in epoxy resin (Appendix III).

The processed blocks of epoxy resin embedded FARM were cut into semithin (1 µm) and ultrathin (80 nm) sections using a Reichter ® ultra microtome (Pennsylvania, USA). The semithin sections were collected on glass slide, stained with 3 % toluidine blue (Appendix IV) while the ultrathin sections were picked on 200 wire mesh copper grids, stained with uranyl acetate, counterstained with lead citrate, and observed with a Philips
201C TEM (Bonn, Germany) under an accelerating voltage of 60 Kv. Light and electron micrographs were developed from the stained respective semithin and ultrathin sections for morphological studies.

3.14 Phagocytosis assays and morphometric analysis

Six chickens were subjected to respiratory tract lavage and the recovered FARM were washed twice in PBS and re-suspended at a concentration of $1.5 \times 10^5$ cells / ml in fresh RPMI - 1640 supplemented with 10 % BSA and 100 U ml penicillin / streptomycin (BioWhittaker ®) in sterile eppendorf tubes. The FARM were treated with 9 µM of troglitazone and placed in incubator with temperature and CO₂ set at 40°C and 5 % respectively for one hour. Another group of six chickens was used for recovery of FARM by lavage. The FARM, at concentrations of $1.5 \times 10^5$ cells / ml, were washed twice in PBS and re-suspended in a fresh RPMI - 1640 culture medium supplemented with 10 % BSA and 100 U ml penicillin / streptomycin (Bio Whittaker ®).

The troglitazone treated and untreated FARM were co-cultured with polystyrene particles of 5 µm diameter (Sigma Saint Louis, Missouri, USA) for 3 hours in incubator which had a slow current of carbon dioxide at 5 % and temperature set at 40°C. Shaking of the tubes was done regularly to avoid sedimentation of the cells and the particles. After 3 hours, the FARM were subjected to centrifugation at 1000 rpm for 10 minutes in RT to separate free particles from the cells. The supernatant was aspirated and the cell pellet washed three times in PBS prior to fixing the cells in 2.5 % phosphate-buffered glutaraldehyde solution for 12 hours. The glutaraldehyde-fixed cells were centrifuged and
post fixed in 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer. This was followed by dehydration in graded series of ethanol (70 %, 80 %, 90 %, and 100 % twice) and gradual replacement of ethanol with propylene oxide before infiltrating and embedding the cells in epoxy resin. Semithin (1 µm) and ultrathin (80 nm) sections were prepared by cutting blocks using a Reichert ® ultramicrotome. The semithin sections were stained with 3 % toluidine blue and used for morphometric analysis which involved estimating diameters of troglitazone treated and untreated FARM. The diameters of the FARM were determined under an ocular graticule with linear scale at a magnification of 100. In each field, to avoid bias, the cells were singled out at random. Only the diameters of the FARM at the four corners of the fields and one at the middle were counted. Twelve ultrathin sections were randomly isolated from the sections obtained from each processed block and picked on a 200 wire mesh copper grids, stained with uranyl acetate, counterstained with lead citrate, for viewing under a Philips 201C TEM (Bonn, Germany) under an accelerating voltage of 60 Kv for analysis of phagocytic capacity of troglitazone treated and untreated FARM.

3.15 Determination of FARM phagocytic capacity

Twelve fields were sub-sampled from each of the randomly selected ultrathin sections using random tables of numbers. The corresponding micrographs of the sampled fields were recorded on a 35-mm TEM film of which the negatives were projected onto a screen at a ×1400. A quadratic lattice was superimposed at a random position onto each projected image. The total number of points falling on the phagocytized particles (P (p)) and on the entire cell (P (C)) were counted and recorded. An estimator of the volume of
the phagocytized particles in a FARM was then calculated as follows: \( V_v(p, c) = \frac{P(p)}{P(c)} \), where \( V_v(p, c) \) is the mean volume of particles per cell, \( P(p) \) is the total number of points falling onto phagocytized particles, and \( P(c) \) is the number of points falling onto the entire cell (Kiama et al., 2008).

3.16 Measurement of TNF α production by the FARM

Chicken FARM were harvested by bronchopulmonary lavage of ten birds. Harvested cells were washed three times in PBS and seeded at a density of \( 1.5 \times 10^5 \) cells / well in RPMI 1640 with 5 % FCS into 24-well tissue culture. The cells were treated with different doses (3 \( \mu M \), 6 \( \mu M \) and 9 \( \mu M \)) of troglitazone for 1 hour before addition of 0.1 ng / ml LPS. After 24 hours incubation at 40°C in 5 % CO₂, the supernatants were harvested for TNF α measurement using chicken TNF α ELISA kit (Bicom biotech, SA). Briefly, the supernatants were diluted appropriately and added to an anti-chicken TNF α monoclonal antibody coated plate and incubated at 40°C for 1 hour. The plate was washed 3 times in PBS-T (Phosphate buffered saline-Tween 20) followed by addition of biotin-streptavidin HRP labeled anti-chicken TNF α. The plate was incubated for 30 minutes at 40°C followed by 3 washes in PBS-T before addition of chromogen solution. Using an ELISA plate reader, the optical density (450 – 655 nm) of the control well was subtracted from each of the values in the test wells and a bar graph generated.

3.17 Data analysis

Student \( t \)-test was used for analysis of paired (FARM \textit{in vitro} viability, phagocytosis assays, and FARM morphometry) experiments. For multiple comparisons (distribution of peroxisomes, peroxisome morphometry, catalase distribution, and assessment of TNF α
production by activated FARM) ANOVA was used followed by Duncan’s multiple range test to determine the level of differences. The level of significance was set at $P < 0.05$ confidence level. The results were presented in form of tables, graphs and micrographs. Means ± standard error of mean (SEM) were used to explain the results in text and tables.

CHAPTER 4: RESULTS

4.1 Morphological characteristics of peroxisomes in the chicken respiratory system

Light and electron microscopy techniques were used to determine morphological traits of peroxisomes in the chicken respiratory system

4.1.1 Light microscopy

Under light microscopy, morphological survey and detection of peroxisomes in the processed portions of chicken trachea, bronchi and lung tissues revealed peroxisomes as small electron dense bodies following reaction with 3’ 3-diaminobenzidine. Further, the organelles were numerous in the trachea ciliated epithelium and in the epithelia lining bronchiolar junctions. In the lungs, the organelles were abundant in the epithelia lining air capillaries (Figure 4.1 A, B and C).

4.1.2 Electron microscopy

Peroxisomes in the portions of chicken trachea, bronchi and lung tissues were revealed at the electron microscopic level following incubation in alkaline DAB-solution to obtain optimal precipitation for sufficient electron-dense reaction product. In all the tissues, peroxisomes were spherical in shape and contained a finely electron dense granular matrix, in which the 3’ 3-diaminobenzidine reaction product was precipitated. In all the
tissues, there were small and mature peroxisomes and some peroxisomes were in close proximity with mitochondria (Figure 4.2 A, B and C).

Figure 4.1: Photomicrographs showing peroxisomes (arrows) in (A) trachea with ciliated epithelium (thick arrow), (B) bronchus with bronchiolar junctions (BJ) and (C) lung with air capillaries (AC). Peroxisomes are localized in the ciliated epithelium of trachea and in the epithelium lining bronchiolar junctions. In the lungs, peroxisomes are uniformly distributed around the air capillaries. The micrographs were prepared from...
semi-thin sections (1 µm) that were processed from tissues embedded in epoxy resin, stained with 3 % toluidine blue and viewed under photo light microscope (× 100)

Figure 4.2: Electron micrographs of chicken peroxisomes (yellow arrows) in portions of (A) trachea rich in endoplasmic reticulum (ER), (B) bronchus and (C) lung. Small peroxisomes (red arrows) were present in all the tissues, an indication of peroxisome proliferation. Some peroxisomes were in close proximity with mitochondria (M). The ultrathin tissue sections (80 nm) were prepared from epoxy embedded tissue
blocks that had been incubated in 3′3-diaminobenzidine, stained with uranyl acetate and counter stained with lead citrate for viewing under TEM (×15000)

4.1.3 Peroxisome morphometry

Under ocular graticule with linear magnification of ×100, the mean diameter of peroxisomes in trachea, bronchi and lung tissues were not significantly different (P > 0.05). The average diameter of peroxisomes was approximately 0.9 µm with mean diameter of peroxisomes in trachea, bronchi and lung tissues measuring 0.87 ± 0.1 µm, 0.89 ± 0.2 µm and 0.9 ± 0.2 µm respectively (Table 4.1). With mean volume density of peroxisomes of 38 ± 1.5 % and 37 ± 1.7 % in the chicken lung and bronchi tissues respectively, there was no significant (P > 0.05) difference in distribution of the organelles in the two tissues (Figure 4.3). However, trachea had significantly (P < 0.05) fewer peroxisomes compared to bronchi and lung tissues. The mean volume density of peroxisomes in the trachea was 16 ± 1.8 (Figure 4.3).

Table 4.1: Mean diameter of peroxisomes

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Diameter of peroxisomes (µm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trachea</td>
<td>Bronchi</td>
<td>Lung</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
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<td>0.8</td>
<td>0.7</td>
</tr>
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<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
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<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
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<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>1.1</td>
<td>0.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Mean volume density of peroxisomes per unit volume of chicken lung, bronchi and trachea tissues. In the lung and bronchi, peroxisomes were equivalently ($P > 0.05$) distributed. However, in the trachea, peroxisomes were significantly ($P < 0.05$) fewer than in the lung and bronchi tissues. The bars show SEM.

**4.2 Localization of catalase in the chicken respiratory system**

Chicken trachea, bronchi and lung tissues revealed specific catalase staining patterns. Tracheal ciliated epithelial lining stained more positive for catalase than non-ciliated epithelial cells. Additionally, FARM on the epithelium stained positive for catalase (Figure 4.4 A and B). The bronchi epithelial cells stained positive for catalase but the staining was abundant in the epithelial cells lining bronchiolar junctions (Figure 4.4 C and D). Catalase was expressed in the epithelial lining air capillaries in the lungs and in the free avian respiratory macrophages (Figure 4.4 E and F). Negative sections which
were not incubated with chicken anti-catalase antibody were used for comparison (Figure 4.5 A and B)

**Figure 4.4: Photomicrographs showing catalase in chicken respiratory tissues.** (A) Trachea ciliated epithelium (E) rich in catalase (CAT). (B) Catalase in the chicken free avian respiratory macrophages (M) on the trachea epithelium, (C) Bronchus with
epithelial cells (E) stained positive for catalase. (D) The epithelia lining bronchiolar junctions (BJ) stained more positive for catalase (arrows to CAT). (E) Chicken lung epithelial cells stained positive for catalase (arrows). (F) A free avian respiratory macrophage with eccentric nucleus (N) stained positive for catalase (arrows). The semi-thin sections (3 – 4 µm) were prepared from paraffin wax embedded tissues (× 100)

Figure 4.5: Photomicrographs of (A) trachea epithelial cells (E) with cilia (arrows), (B) bronchus with bronchiolar junctions (BJ) and (C) lung with air capillaries (AC). The micrographs were prepared from chicken trachea, bronchi and lung sections which were not incubated with chicken anti-catalase antibody and were used to compare distribution of catalase with antibody incubated sections. The semi-thin sections (3-4 µm) were prepared from paraffin wax embedded tissues and stained with 3 % toluidine blue (×100)
4.3 Lavage and numerical characteristics of FARM

To ensure sufficient recovery of FARM, the volume of instilled phosphate buffered saline (PBS) and aspirated lavage fluids were determined. The chicken respiratory system was instilled with an average of $400 \pm 10.4$ ml prewarmed (40°C) PBS and an average of $338 \pm 9.0$ ml of the instilled fluid was aspirated, representing a recovery rate of $85 \pm 1.6\%$ (Table 4.2). The recovered fluid consisted of cells of epithelial origin which exfoliated from the mucosal surface of the respiratory tract and FARM (Figure 4.6). The average number of the FARM harvested by pulmonary lavage was determined for each of the five lavages and per bird. On average, $2.3 \times 10^5$ FARM were recovered per chicken (Table 4.3). Transmigration of FARM was indicated by substantial ($P < 0.05$) increase of FARM during the second lavage. The average number of FARM increased from $1.6 \times 10^5$ to an average of $2.7 \times 10^5$ during the first and second lavages respectively but there was progressive decline of FARM during the subsequent lavages (Figure 4.7).
Figure 4.6: Photomicrograph showing epithelial cells (E) with cilia (arrows) and a collection of FARM (m) recovered by lavage of chicken respiratory system. The photomicrograph was processed from semi-thin (1 µm) sections thick cut from blocks of epoxy resin embedded cell suspensions stained with 3% toluidine blue (× 400)

Table 4.2: Comparison of volume of PBS instilled, aspirated fluid, and recovery rate during lavage of the chicken respiratory system

| Animal No | Lavage 1 | Lavage 2 | Lavage 3 | Lavage 4 | Lavage 5 | Lavage 1 | Lavage 2 | Lavage 3 | Lavage 4 | Lavage 5 | Lavage 1 | Lavage 2 | Lavage 3 | Lavage 4 | Lavage 5 | Lavage 1 | Lavage 2 | Lavage 3 | Lavage 4 | Lavage 5 | Lavage 1 | Lavage 2 | Lavage 3 | Lavage 4 | Lavage 5 |
|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|           | Volume of PBS instilled (ml) | Volume of fluid aspirated (ml) | Recovery (%) |
| 1         | 480      | 390      | 81       |
|           | 470      | 420      | 89       |
|           | 380      | 320      | 84       |
|           | 370      | 320      | 86       |
|           | 370      | 330      | 89       |
| 2         | 400      | 320      | 80       |
|           | 340      | 300      | 88       |
|           | 320      | 280      | 88       |
|           | 300      | 270      | 90       |
|           | 300      | 280      | 93       |
| 3         | 480      | 390      | 81       |
|           | 420      | 380      | 90       |
|           | 400      | 370      | 93       |
|           | 400      | 380      | 95       |
|           | 390      | 300      | 77       |
| 4         | 470      | 240      | 51       |
|           | 380      | 310      | 82       |
|           | 340      | 300      | 88       |
|           | 390      | 340      | 87       |
|           | 380      | 340      | 89       |
| 5         | 480      | 340      | 71       |
|           | 370      | 300      | 81       |
|           | 340      | 320      | 94       |
|           | 340      | 310      | 91       |
|           | 380      | 300      | 80       |
| 6         | 480      | 370      | 77       |
|           | 480      | 440      | 92       |
|           | 430      | 400      | 93       |
|           | 470      | 420      | 89       |
|           | 440      | 370      | 84       |
| Mean      | 400      | 338      | 85       |
| SEM       | ±10.4    | ±9.0     | ±1.6     |
Table 4.3: Summary of the number of FARM recovered per lavage and per chicken

<table>
<thead>
<tr>
<th>Animal No</th>
<th>Lavage</th>
<th>Number of FARM per lavage ($\times 10^5$)</th>
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<td>Lavage 1</td>
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</tr>
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<td>Lavage 4</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lavage 5</td>
<td>2.0</td>
<td>2.34</td>
</tr>
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<td>Lavage 5</td>
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<td>Lavage 1</td>
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<td>Lavage 3</td>
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<td>Lavage 4</td>
<td>2.2</td>
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<td></td>
<td>Lavage 5</td>
<td>2.0</td>
<td>2.25</td>
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<tr>
<td>4</td>
<td>Lavage 1</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lavage 2</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lavage 3</td>
<td>2.3</td>
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<tr>
<td></td>
<td>Lavage 4</td>
<td>2.0</td>
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<td></td>
<td>Lavage 5</td>
<td>1.25</td>
<td>2.05</td>
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<tr>
<td>5</td>
<td>Lavage 1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lavage 2</td>
<td>3.2</td>
<td></td>
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<td></td>
<td>Lavage 3</td>
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<tr>
<td></td>
<td>Lavage 4</td>
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<td>Lavage 5</td>
<td>1.4</td>
<td>2.22</td>
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<td>6</td>
<td>Lavage 1</td>
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<tr>
<td></td>
<td>Lavage 2</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lavage 3</td>
<td>2.75</td>
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<td>Lavage 4</td>
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</tr>
<tr>
<td></td>
<td>Lavage 5</td>
<td>1.8</td>
<td>2.31</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td><strong>2.25</strong></td>
<td><strong>2.25</strong></td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>±0.1</td>
<td>±0.4</td>
</tr>
</tbody>
</table>
Figure 4.7: Mean number of FARM recovered in each of the five progressive lavages. There was substantial ($P < 0.05$) increase of the mean number of FARM recovered during the second lavage than during the first lavage.

4.3.1 FARM in vitro Viability characteristics

Troglitazone treated FARM exhibited equivalent ($P > 0.05$) in vitro viability with untreated FARM under similar experimental conditions. The in vitro viability of troglitazone treated FARM and untreated FARM was $82 \pm 0.7 \%$ and $83 \pm 1.0 \%$ respectively (Table 4.4).

Table 4.4: Summary of in vitro viability of chicken FARM

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Troglitazone treated FARM Viability (%) Score</th>
<th>Untreated FARM Viability (%) Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>83</td>
</tr>
<tr>
<td>5</td>
<td>84</td>
<td>85</td>
</tr>
<tr>
<td>6</td>
<td>81</td>
<td>80</td>
</tr>
</tbody>
</table>
Mean SEM 82 ± 0.7 83 ± 1.0

4.3.2 Morphological characteristics of the chicken FARM

Morphologically, the chicken FARM had plasma membrane ruffled with filopodial extensions and eccentrically located nucleus (Figure 4.8). Ultrastructurally, the FARM had numerous vesicular bodies presumed to be lysosomes (Figure 4.8 B).

Figure 4.8: Photo (A) and electron (B) micrographs of chicken FARM. The FARM have eccentric nucleus (thick arrows) and plasma membrane ruffled with filopodial extensions (thin arrows). In (A), semi-thin sections (1 µm thick) were prepared from epoxy resin embedded cell suspension and stained with 3% toluidine blue for viewing under light microscope (× 400). In (B), vesicular bodies presumed to be lysosomes (red arrows) were present. Ultrathin sections (80 nm) were prepared from epoxy resin embedded cell suspension. The sections were stained with uranyl acetate and counter stained with lead citrate (× 950)

4.3.3 Effect of troglitazone on the phagocytic potential of FARM

Phagocytic characteristics of FARM were performed by treating FARM with troglitazone and analyzing the phagocytic potential of the treated cells. The mean diameters of
troglitazone treated and untreated FARM were not significantly ($P > 0.05$) different. The average diameter of treated and untreated FARM was $11.7 \pm 0.5 \, \mu m$ and $11.8 \pm 0.4 \, \mu m$ respectively (Table 4.5). Despite having equivalent diameters, and therefore volume, the mean volume density of internalized particles in troglitazone treated and untreated FARM were significantly ($P < 0.05$) different. The volume density of internalized particles was $41 \pm 1.1 \%$, and $21 \pm 1.1 \%$ in the treated and untreated FARM respectively (Figure 4.9). Further, under light microscope, treated FARM had intense toluidine staining around the internalized particles (Figures 4.10), presumably an indication of troglitazone improved lysosomes enzyme lytic activity. Quantitative loading of particles in the FARM was assessed using photomicrographs prepared from processed blocks of epoxy resin embedded cell suspensions (Figures 4.10, 4.11, and 4.12).

### Table 4.5: Mean diameter of troglitazone treated and untreated FARM

<table>
<thead>
<tr>
<th>Slide Number</th>
<th>Diameter of treated FARM ($\mu m$)</th>
<th>Diameter of untreated FARM ($\mu m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
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</tr>
<tr>
<td>3</td>
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<td>9</td>
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<tr>
<td>10</td>
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<td>11</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

| Mean         | 11.7                              | 11.8                                |
| SEM          | $\pm 0.5$                         | $\pm 0.4$                          |
The mean diameter of troglitazone treated FARM and untreated FARM were not significantly different ($P > 0.05$).

![Bar chart showing comparison of mean volume density of internalized particles in troglitazone treated and untreated FARM. Troglitazone treated FARM significantly ($P < 0.05$) internalized more particles than untreated FARM. Bars show mean ± SEM.](image)

**Figure 4.9:** Comparison of the mean volume density of internalized particles in the troglitazone treated and in the untreated FARM. Troglitazone treated FARM significantly ($P < 0.05$) internalized more particles than untreated FARM. Bars show SEM.

**Figure 4.10:** Photomicrographs showing internalized polystyrene based particles (thin arrows) in (A) untreated FARM and in (B) troglitazone-treated FARM. In (B), toluidine staining around the internalized particles was intense presumably an indication of increased lysosomes enzyme activity in the FARM following troglitazone treatment. The cells have eccentric nucleus (thick arrows). The FARM and the particles were co-
cultured in RPMI 1640 for 3 hours. The photomicrographs were developed from 3 %
toluidine blue stained semithin sections (1 µm) thick prepared from blocks of epoxy
embedded cell suspension (× 400)

Figure 4.11: Electron micrographs showing internalized polystyrene particles
(arrows) in troglitazone-untreated chicken FARM. The FARM have eccentric
nucleus (N). Three and six particles have been phagocytozed in (A) and (B) respectively.
The FARM and the polystyrene particles were co-cultured in RPMI 1640 culture media
for 3 hours. Ultrathin sections (80 nm) were prepared from cell suspension embedded in
epoxy resin and stained with uranyl acetate and counterstained with lead citrate (× 950)

Figure 4.12: Electron micrographs showing internalized polystyrene particles
(arrows) in troglitazone treated chicken FARM. About twenty and twelve particles
have been internalized in (A) and (B) respectively. Vacuoles containing particles have
irregular shape probably due to lytic action of lysosomes. The FARM were co-cultured
with the polystyrene particles, (5 µm diameter) in RPMI-1640 culture media for 3 hours. Ultrathin sections, 80 nm, were prepared from cell suspension embedded in epoxy resin, stained with uranyl acetate and counter stained with lead citrate (× 950)

4.4 Effect of troglitazone on TNF α production by the FARM

To define the functional role of PPAR γ in chicken FARM, the effect of troglitazone on cytokine production by the FARM was measured by determining TNF α concentrations in culture supernatants of LPS-stimulated FARM after treatment with graded (3 µM, 6 µM and 9 µM) doses of troglitazone. Lipopolysaccharide elicited considerable amounts of TNF α production by FARM at concentration of 0.1 ng / ml. Addition of troglitazone to cultures of LPS-induced TNF α production, significantly ($P < 0.05$) attenuated TNF α production by FARM in a dose-dependent manner (Figure 4.13).

![Figure 4.13: Comparison of TNF α production by LPS-activated chicken FARM treated with varying doses of troglitazone. Troglitazone treatment significantly ($P < 0.05$) attenuated TNF α production in LPS-activated FARM. Bars show SEM](image-url)
CHAPTER 5: DISCUSSION

5.1 Distribution of peroxisomes in the chicken respiratory system

This is the first study on morphological survey and identification of peroxisome distribution in the avian respiratory system, a significant contribution towards understanding the inherent status of the respiratory antioxidant system in the domestic birds. Over the past decades, free radicals and other reactive small molecules have emerged as important regulators of many physiological and pathological processes (Thomas et al., 2008). Currently, it is well established that, at low physiological levels, reactive oxygen species (ROS) and reactive nitrogen species (RNS), serve as signaling messengers for various biological responses including cell proliferation, innate immunity, apoptosis and angiogenesis (Scherz-Shouval and Elazer, 2011). However, increased levels of ROS and RNS exert harmful effects by causing oxidative damage to lipids, proteins and DNA, thus disrupting cellular activities (Acharya et al., 2010).

In this study, peroxisomes were found in the epithelia of chicken trachea, bronchi and the lung tissue. Avian trachea is composed of ciliated epithelium that removes pathogens and particulates by mucociliary escalator mechanism among other immune mechanisms (Kiama et al., 2008). Localization of peroxisomes in the ciliated tracheal epithelium could be crucial in countering oxidative stress burden occasioned by incessant exposure of trachea to particulate matter and pathogens which are inevitable constituents of inhaled air. In bronchi, peroxisomes were abundant in the epithelia lining bronchiolar junctions.
This indicates a strategic location of peroxisomes that provide an antioxidant platform in the bronchi that probably supplements BALT which serves to trap and eliminate pathogens before they penetrate the close but oxidant stress susceptible gas exchange units, the parabronchi (Fagerland and Arp, 1990). The interstitial avian lung tissue is endowed with phagocytes which, on activation, generate ROS and RNS (Toth et al., 1988). Peroxisomes were identified in the lung epithelium, an indication of a well developed antioxidant defense system that probably protects the lung tissue against ROS and RNS generated by the phagocytes and from external sources in the inhaled air.

There are plausible reasons why peroxisomes are abundant in the bronchi and lung tissues than in the trachea as reported in this study. The avian trachea is ciliated and removal of pathogens and particulates that land on this surface is primarily by an efficient mucociliary escalator system (Kiama et al., 2008). In addition, trachea has tight epithelial junctions that offer a physical barrier against invasion by pathogens (Kiama et al., 2008). These apt defenses probably lessen the oxidant burden arising from the pathogens and particulates that land on the avian trachea. Peroxisomes, being the major sources of antioxidant enzymes could be numerous in the distal primary bronchus, secondary bronchi and lungs where they could be critical because ciliated epithelium is lacking in these portions of the avian respiratory system (Maina and Nathaniel, 2001) yet pathogens and particulates impose a lot of oxidant stress on landing on bronchi and the lungs (Kiama et al., 2008).
Peroxisomes are involved in synthesis of lipid bioactive derivates such as leukotrienes, prostaglandins and plasmalogens (Wanders and Waterham, 2006). These lipid derivatives are essential in regulating functions of lymphocytes that are few in the trachea but are the predominant immune cells in the bronchi and lungs. Therefore, abundance of peroxisomes in bronchi and lungs could be indicative of the numerous roles they play in regulating immune homeostasis in these tissues. Oxidative tissue damage is more at the foci of oxygen exposure (Kohen and Nyska, 2002), the lung is more susceptible to oxidative stress mediated tissue damage because exchange of gasses between the environment and the body occurs by diffusion across the lung parabronchial mantle (Maina and Nathaniel, 2001), hence the need for the numerous peroxisomes to ameliorate oxidative damage.

In this study, tissues examined had small and presumably mature peroxisomes. Peroxisomes have a lifespan of three days (Schrader and Fahimi, 2004) and, therefore, the presence of both small and mature peroxisomes in the tissues could be indicative of rapid proliferation of peroxisomes that serves to replace old organelles. It was further observed that some peroxisomes were in close proximity with mitochondria and this could be due to the intimate relationship between the organelles required in β-oxidation of very long chain fatty acids in peroxisomes prior to oxidation in mitochondria to generate energy (Pascal, 2004).

5.2 Distribution of catalase in the chicken respiratory system
Catalase is the principal peroxisomal antioxidant enzyme (Irfan, 2008). In this study, catalase was predominantly expressed in the trachea ciliated epithelium, epithelial lining bronchiolar junctions in the bronchi and in the epithelial lining air capillaries in the avian lung. The avian lung parabronchi mantle is highly vulnerable to oxidative stress (Maina and Nathaniel, 2001; Powers and Jackson, 2008). Consequently, successful elimination of oxidants in the bronchi prior to diffusion into the susceptible lung is crucial.

Presence of catalase in the epithelial tissue lining bronchiolar junctions could be critical in neutralizing exogenous oxidants in inhaled air and in supplementing BALT, which is also located at the junctions of primary and secondary bronchi, and whose immune functions involves trapping and eliminating pathogens before reaching the vulnerable air capillaries (Reese et al., 2006). Avian respiratory macrophages produce hydrogen peroxide as an integral component of their anti microbial innate immunity against invading pathogens (Golemboski et al., 1990; Victoria, 1994) and, therefore, identification of catalase in the chicken FARM in this study is indicative of an antioxidant enzyme that degrades hydrogen peroxide to avoid oxidative damage of the phagocytes and the surrounding tissue.

5.3 Effect of troglitazone on the phagocytic capacity of FARM

Some avian immunologists postulate a dearth of FARM on the avian lung air sac system to foreordain a weak innate immunity thus the reason for high mortality and morbidity associated with respiratory diseases conditions in domestic birds (Fedde, 1998; Maina and Cowley, 1998). Without unequivocal empirical evidence, a dearth of FARM in the
avian respiratory system should not, *ipso facto*, imply avian pulmonary cellular defenses are inadequate or even compromised. As phagocytosis is the most important defense mechanism in all phyla of the animal kingdom (Kavet and Brian, 1997; Nicod, 2005), it is particularly important that the contribution of the FARM in the clearance of particles and neutralization of pathogens in the lung should be well understood.

This study reports a mean volume of internalized particles per unit volume of troglitazone untreated FARM of 21 %. In a study that compared *in vitro* mean volume density of internalized particles per unit volume of a FARM in the chicken and the rat, chicken FARM exhibited a significantly higher phagocytic capacity than rat respiratory macrophages with phagocytic indices of 23 % and 5 % respectively (Kiama *et al*., 2008). In another comparative *in vitro* study of the phagocytic abilities of the domestic duck FARM and rabbit respiratory macrophages, the mean volume density of internalized particles per unit volume of a cell was 20 % and 9 % in the duck and rabbit respectively (Mutua *et al*., 2011). These data indicate that FARM have significantly higher phagocytic capacity in comparison to the corresponding mammalian cells, the respiratory macrophages.

An understanding of the mechanisms and molecules that enhance FARM to regulate immune and inflammatory responses may permit the development of products, diets, or husbandry techniques to modulate immunity for the enhancement of the productivity of poultry (Rosen and Spiegelman, 2001). Specific rationales for modulating FARM function in poultry include: a) providing enhanced or sustained immune response to
infectious organisms; b) enhancement and direction of vaccination responses; c) mitigation of immunosuppression arising from infectious diseases, dietary toxins, or stress; d) accelerating the development and maturation of the immune system; e) inducing tolerance to nonpathogenic environmental immunogens; and f) mitigating the catabolic consequences of an immune response (Klasing, 1996).

This study demonstrates for the first time that selective synthetic PPAR γ ligands improve the ability of freshly harvested FARM to internalize particles. Troglitazone treatment of chicken FARM approximately enhanced the phagocytic ability of the cells by almost two fold, from 21 % to 41 %. This data indicates that synthetic PPAR γ ligands could be used to improve non-specific cellular immune responses by enhancing phagocytic clearance of particulate matter in avian respiratory system. Engulfment of Aspergillus fumigatus conidia by chicken FARM, for instance, has been shown to lower the phagocytic and killing capacity of the cells (Van Waeyenberghe et al., 2012). In such infections, administration of synthetic PPAR γ ligands could be evaluated to ascertain restoration of the phagocytic and killing abilities of the FARM. In this study, troglitazone treatment of FARM not only enhanced the phagocytic capacity, but irregular vacuoles formed around ingested particles indicating up-regulated destruction of internalized particles by the FARM.

5.4 Effect of troglitazone on TNF α production by FARM

For the first time, this study demonstrates that synthetic PPAR γ ligands have inhibitory effects on TNF α production by the chicken FARM. Troglitazone treatment of the FARM
inhibited TNF α production in lipopolysaccharide (LPS) activated FARM in a dose dependent manner. Tumor necrosis factors are a family of cytokines that produce a range of biological effects. Studies report TNF α as the primary regulator of inflammation (Zhang et al., 1995). In mammals, TNF α is produced by macrophages, T cells and NK cells and causes both inflammation and endothelial activation (Zhang et al., 1995). In the chicken, TNF α activity has been reported. After infection with *Eimeria* (Zhang et al., 1995) or Marek’s disease virus (Klasing, 1991), release of TNF α from chicken macrophages can be detected in cross-reactive mammalian cellular cytotoxicity bioassays. Injection of chickens with TNF like factors enhances weight loss due to *Eimeria* infection, which is partially reversible by treatment with antihuman TNF antisera (Zhang et al., 1995).

### 5.5 Hypotheses testing

Based on the outcome of this study:

(i) Peroxisomes are not evenly distributed in chicken trachea, bronchi, and lung tissues. The organelles were abundant in lung and bronchi tissues but few in the trachea.

(ii) Catalase distribution is not uniform in the chicken trachea, bronchi, and lung tissues. Catalase was abundant in trachea ciliated epithelial cells, in the epithelial lining bronchiolar junctions but evenly distributed in the lung epithelium.

(iii) Troglitazone treatment of FARM significantly enhanced the phagocytic potential of FARM.
(iv) Troglitazone treatment of FARM significantly attenuated TNF-α production by activated FARM.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study concludes that:

(i) Peroxisomes are strategically located in the chicken trachea ciliated epithelial cells, in the epithelia lining bronchiolar junctions and in the epithelia lining air capillaries in the lung. However, peroxisomes were few in the trachea but abundantly expressed in the lungs and bronchi tissues.

(ii) Catalase, the principal antioxidant peroxisomal enzyme, was abundantly expressed in the ciliated epithelia cells in the trachea, in the epithelia lining bronchiolar junctions and in the epithelia lining of air capillaries in the lungs. Catalase was also expressed in the free avian respiratory macrophages.

(iii) Treatment of chicken FARM with troglitazone, a selective synthetic PPAR γ ligand, increased the phagocytic capacity of the FARM significantly. Formation of irregular vacuoles around internalized particles in the troglitazone treated FARM indicates increased killing ability of the FARM.

(iv) Troglitazone treatment of chicken FARM significantly attenuated TNF α production in lipopolysaccharide activated FARM.
6.2 Recommendations

(i) Peroxisomes are strategically distributed in the avian respiratory system. This offers possible targets for application of antioxidant therapeutics aimed at resolving oxidant stress mediated respiratory disease conditions in poultry industry by targeting peroxisomes.

(ii) Catalase staining depicted a strategically located antioxidant enzyme expression in the epithelial ciliated trachea epithelia, the enzyme was also abundantly expressed in the epithelia lining bronchiolar junctions and in the epithelia lining of air capillaries in the lungs. Therapeutic agents could be designed targeting catalase to improve avian respiratory antioxidant defense system.

(iii) Troglitazone increased the phagocytic ability of FARM. A major challenge in administration of drugs by aerosolization is the high absorptive capacity by alveolar macrophages in mammals. It is worthwhile to consider the improved phagocytic capacity of FARM treated with PPAR γ ligands in administration of vaccines by aerosolization as a means of treatment and management of avian respiratory diseases.
From this study, troglitazone inhibited release of proinflammatory cytokine in activated FARM. This suggests that synthetic peroxisome proliferator activated receptor gamma ligands could be designed and used as therapeutic agents in resolving inflammatory respiratory diseases of the domestic birds.

6.3 Suggestions for further research

There is need to carry out research to determine:

(i) Distribution of other peroxisomal antioxidant enzymes in the avian respiratory system.

(ii) Whether peroxisome and catalase distribution observed in the chicken respiratory system is similar in other domesticated birds.

(iii) The effect of troglitazone-enhanced phagocytic potential of FARM on vaccine application by aerosolization.

(iv) The effect of troglitazone treatment of FARM on production of other proinflammatory cytokines.
REFERENCES


### APPENDICES

#### Appendix I

**Protocol for tissue fixation for morphological survey and detection of peroxisomes**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-tracheal instillation of 2.5 % glutaraldehyde</td>
<td>4 H</td>
</tr>
<tr>
<td>Ligate trachea, dissect and remove the respiratory system and fix in 2.5 % glutaraldehyde</td>
<td>overnight</td>
</tr>
<tr>
<td>Cut 1 mm³ blocks of trachea, bronchi and lung tissues</td>
<td></td>
</tr>
<tr>
<td>and fix in 2.5 % glutaraldehyde</td>
<td>15 min</td>
</tr>
<tr>
<td>Wash 3 times in glycine-NaOH buffer</td>
<td>10 min each</td>
</tr>
<tr>
<td>Put blocks in 0.2 % DAB in a shaking water bath (45°C)</td>
<td>2 H</td>
</tr>
<tr>
<td>Wash 3 times in glycine-NaOH buffer</td>
<td>10 min each</td>
</tr>
<tr>
<td>Post fix in 1 % osmium tetroxide</td>
<td>overnight</td>
</tr>
<tr>
<td>Wash 3 times in glycine-NaOH</td>
<td>10 min each</td>
</tr>
<tr>
<td>Counter stain in uranyl acetate</td>
<td>1 H</td>
</tr>
<tr>
<td>Wash 3 times in glycine-NaOH</td>
<td>10 min each</td>
</tr>
<tr>
<td>Dehydrate in 70, 80, 96, 100 ethanol</td>
<td>20 min each</td>
</tr>
<tr>
<td>Infiltrate Ethanol: Propylene oxide</td>
<td></td>
</tr>
<tr>
<td>3:1, 1:1, 1:3, 100 % propylene oxide</td>
<td>30 min each</td>
</tr>
<tr>
<td>Embed. Propylene: Epon</td>
<td></td>
</tr>
<tr>
<td>3:1, 1:1, 1:3, 100 % Epon resin</td>
<td>1 H each</td>
</tr>
<tr>
<td>Centrifuge (all at 850 rmp for 10 min)</td>
<td></td>
</tr>
<tr>
<td>Put accelerator and embed</td>
<td>5 days at 60°C</td>
</tr>
</tbody>
</table>
Appendix II

Protocol for 0.2 % diaminobenzidine (DAB) solution

(a) 1 % DAB

Add 1 g DAB in 100 ml distilled water

Add HCL (drop wise) until the solution turns light brown in colour

Vortex until DAB dissolves completely (about 10 minutes)

Aliquot and store in -20°C

(b) 0.3 % Hydrogen Peroxide

Add 2 ml of 30 % hydrogen peroxide to 198 ml of distilled water

Aliquot and store at -20°C

(c) 0.01M glycine-NaOH buffer

Add 50 ml of 0.1 M glycine-NaOH to 450 ml of distilled water

To make 0.2% DAB, 0.15% H₂O₂, 0.01 M glycine-NaOH Buffer pH 10.5 (100 ml)

Add 20ml of 1% DAB, 50 ml of 0.3 % H₂O₂, 30 ml of glycine-NaOH buffer solution and mix well
## Appendix III

**Protocol for transmission electron microscope**

**Fixation and processing of cells for electron microscopy**

<table>
<thead>
<tr>
<th>Activity</th>
<th>period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation in 2.5% glutaraldehyde in phosphate</td>
<td>At least 12 H</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1 Na-cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1 M Na-cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1M Na-cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Post fixation in 1% OsO₄ in 0.1M Na-cacodylate buffer</td>
<td>90 min</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1 M Na-Cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1 M Na-Cacodylate buffer</td>
<td>5 min</td>
</tr>
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<td>Centrifugation at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.1 M Na-Cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>Centrifuge at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>0.5% uranyl acetate in 0.05 M Maleate buffer</td>
<td>90 min</td>
</tr>
<tr>
<td>Centrifuge at 1000 rpm</td>
<td>10 min</td>
</tr>
<tr>
<td>Washing with 0.05 Maleate buffer</td>
<td>5 min</td>
</tr>
</tbody>
</table>
Centrifugation at 1000 rpm 10 min
Washing with 0.05 M Maleate buffer 5 min
Centrifugation at 1000 rpm 10 min
Washing with 0.05 M Maleate buffer 5 min
Centrifugation at 1000 rpm 10 min

**Dehydration**

70, 80, 96, 100 (twice) % ethanol 20 min each
Centrifuge at 1000 rpm 10 min

**Intermediate: ethanol: propylene oxide**

3:1, 1:1, 1:3, 100 propylene oxide 30 min each

**Propylene oxide: Epon-resin**

3:1, 1:1, 1:3 1 H each
Epon–Resin overnight
Centrifugation at 1000 rpm 10 min
Embedding in fresh Epon with 1.5% catalyst 5 days at 60°C

**Preparation of transmission electron microscopy materials**

(i) **Epoxy-resin mixture**

Prepared by making:

Solution A of 60 ml of Epon-812 + 100 ml of DDSA and
Solution B of 100 ml of Epon-812 + 90 ml of MNA

Then mixing 40 ml of solution A and 60 ml of solution B

(ii) **Embedding media**

Prepared by mixing:
100 ml of the epoxy-resin mixture and 1.8 ml/3 drops of DMP-30 (accelerator) in embedding capsules (eppendorf tubes can be used instead of capsules)
Appendix IV

Stains and staining method

(i) Preparation of 3% toluidine blue in 3% borax stain

Weigh 3gm of toluidine blue in 100ml of water and into these add 3gm of borax.
Filter the solution every time before use.

(ii) Preparation of uranyl acetate stain

In a glass bottle put:

Uranyl acetate 5 gm
Methanol 100 ml

Add methanol, close the bottle and shake until the contents dissolve.
The solution is sensitive against light.
Filter through 200 nm Millipore filter and store.

(iii) Preparation of lead citrate stain

Weigh:

Lead nitrate 1.33 g
Sodium citrate 1.76 g

Mix the two in 30ml of distilled water
Shake for 2min, allow stand for 1.30 hours, shaking at intervals
Add 8ml 1N NaOH and dilute to 50 ml with distilled water. Mix by inversion.
Stain for 15-30 min. Use solution for 1-1.5 weeks.

N.B It is advisable to first dissolve lead citrate in the 30ml water and then add sodium citrate. The solution should be milky. After adding NaOH the solution should turn colorless.
Staining of sections

(i) Staining thick (Semithin) sections using toluidine blue

Sections are picked on glass slide with drop of water and dried for 30 min at 80°C. This is done to ensure complete dehydration. Cover the sections with the stain and wait until it steams. Wash the stain in a jet of distilled water. Dry and examine under light microscope.

(ii) Staining ultrathin sections using uranyl acetate and lead citrate stains

Immerse grid having the sections in a drop of uranyl acetate and stain for 30 seconds. Rinse grid thoroughly with freshly distilled running water using a plastic syringe. Bring one drop of lead citrate stain on wax paper for 20 min.
Appendix V

Publications from this research

