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RESEARCH ARTICLE

Pathological, Immunohistochemical and Electron Microscopical Examinations on Chorioallantoic Membrane Lesions in Experimental Fowl Poxvirus Infection

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Summary

The objectives of this study are immunohistochemical detection of the viral antigen and structure of fowl pox virus and developmental stage of inclusion bodies by electron microscopy in experimentally infected chorioallantoic membrane (CAM). Lyophilized fowl pox virus, strain 92 which was obtained from the Veterinary Central Laboratory in Weybirdge, (UK) used in 10^3 titer. Thirty, 10 days old Specific Pathogen Free (SPF) chicken embryonated eggs chorioallantoic membrane that experimentally infected with this strain. Seven days after inoculation of strain, pocks lesions occurred as 0.5-2.5mm diameter in size with grayish-white color and evaluated macroscopically and microscopically. At the histopathological examination, intracytoplasmic inclusion bodies and ballooning degeneration were detected in the hyperplastic and hypertrophic epithelial cells. Positive immunoreactions were also seen in the cytoplasm of the proliferative epithelial cells which may be reflected matrix inclusion bodies that is early form of inclusion bodies and not seen in histopathologically, but seen in ultrastructural examination. Numerous matrix inclusions which consist of developmental stage of virus were detected in effected cells by electron microscopic examination. As a result, this study indicated that, immunoperoxidase technique may be use in the morphologic diagnosis of early stage of the fowl poxvirus (FVP) infection.

Keywords: *Chorioallantoic membrane, Electron microscope, Fowl poxvirus, Immunohistochemistry, Pathology*

Deneysel Tavuk Çiçek Virus Enfeksiyonunda Korioallantoik Membrandaki Patolojik, Immunohistokimyasal ve Elektron Mikroskobik İncelemeler

Özet

Bu çalışmada, tavuk çiçek virusu ile deneysel olarak enfekte edilen korioallantoik membranda immunohistokimyasal yöntemlerle viral antijenin varlığı ve elektron mikroskobik olarak da virus ile inkluzyon cisimciklerinin gelişim aşamalarının incelenmesi amaçlanmıştır. İngiltere Weybirdge Veteriner Merkez Laboratuvarından getirilen liyofilize tavuk çiçek virus 92 suşu 10^3 titrede kullanıldı. Deneysel olarak bu suş ile 10 günlük 30 adet SPF embriyolu tavuk yumurtasının korioallantoik membranına ekim yapıldı. Suşun inokulasyonundan 7 gün sonra membranda grimsi-beyaz, 0.5-2.5 mm arasında değişen çapta çiçek lezyonları görüldü. Bu lezyonlar makroskobik ve mikroskobik olarak değerlendirildi. Histopatolojik incelemede hipertrofik ve hiperplastik epitel hücrelerinde intrasitoplazmik inkluzyon cisimcikleri ile balonumsu dejenerasyona rastlandı. Indirekt immunoperoksidaz tekniği ile yapılan boyamalarda intrasitoplazmik inkluzyon cisimciklerinin yanı sıra proliferatif epitel hücrelerinin sitoplazmalarında da sadece elektron mikroskobik olarak görülebilen histopatolojik incelelemerde saptanmayan makriks inklüzyonlarının varlığını kanıtlayan pozitif immunoreaksiyonlar görüldü. Elektron mikroskobik incelemede virusun gelişim aşamalarından oluşan çok sayıda matriks inkluzyon cisimcikleri saptandı. Sonuç olarak, bu çalışma immunoperoksidaz tekniğinin tavuk çiçek virus (FVP) enfeksiyonda erken devrelerin morfolojik teşhisinde kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Korioallantoik membran, Elektron mikroskop, Tavuk çiçek virusu, İmmunohistokimya, Patoloji*

INTRODUCTION

Avian pox is a disease characterized by cutaneous nodules (dry form) and the lesions vary from smooth surfaces to diphtheritic plaques involving the cavum oris and respiratory tract (wet form) in domestic and wild birds [1,2]. Avian pox virus can be grown on the chorioallantoic membrane (CAM) and in a variety of

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cell culture models [1,3]. Pox virus infection shows the most typical features such as focal and diffuse lesions when grown on the CAM [4-8].

Histopathologically, the most important findings are extreme hyperplasia and hypertrophy of the epithelial cells, ballooning degeneration and eosinophilic intracytoplasmic inclusion body formation in the cells at cutaneous and diphtheritic forms of the disease or infected CAM and chicken embryo fibroblast cell (CEF) [6-8]. Inclusion bodies are generally localized in the mezodermal layer of the CAM [7]. Fowl pox virus and viral particles can be detected by immunohistochemical or ultrastructural techniques [9-11]. Some authors reported that, the fowl pox virus can be expressed at the different stage of chick CAM development [8,12-17].

The aims of this study are to detect the viral antigen by the immunoperoxidase method and to make examination on the viral structure and development stage of fowl pox virus by ultra-structurally in experimentally infected CAM.

MATERIAL and METHODS

In this study, 30 CAM of 10 days old SPF chicken embryonated eggs were used. The chorioallantoic membranes were infected with lyophilized fowl pox virus, strain 92 which was obtained from the Veterinary Central Laboratory in Weybirdge, (UK). Lyophilized fowl pox virus strain 92 was dissolved in sterile PBS at 1 mg/ml dosage, and then 0.1 ml of the inoculum was inoculated to different part of the CAM. This procedure was repeated three times until the virus titer reached to 103 tissue culture infective dose (TCID) per CAM. The virus titer was determined by Agar Gel Precipitation test. The infected CAMs were harvested 7 days post inoculation and fixed in 10% buffered formalin solution. The fixed CAM were embedded in paraffin and cut 4 to 6 µm thick. Sections were stained with Hematoxylin-Eosine (HE), Lendrum's stain (LS) and Mallory Phloxine staining (MPS) in order to demonstrate intracytoplasmic inclusion bodies.

The indirect immunoperoxidase technique was modified from Kovačević et al. [18]. For indirect immuno-peroxidase method after routine deparaffinization and dehydration procedures slides washed in phosphate buffer saline (PBS) and were reacted with 3% hydrogen peroxide (H_2O_2) in methanol for 30 min to quench endogenous peroxidase activity. Following wash in PBS, the samples were blocked with 20% nonimmune rabbit serum for 10 minutes at 37°C. The serum was blotted and the slides were incubated with primary polyclonal chicken antiserum against fowl pox virus (1/64 dilution, Poultry Diseases Research and Vaccine Production Institute, Manisa, Turkey), for 45 min at room temperature. Than washing in PBS, the samples were incubated with peroxidase-labelled (Horse Radish Peroxidase-HRP) rabbit anti-chicken IgG (1/200 dilution, Calbiochem, USA). After 10 min wash in PBS, the slides were incubated for 2 min in DAB (peroxidase substrate-0.05% diaminobenzidine tetra hydrochloride in 0.1 M buffered imidazole/HCI, pH 7.1 solution, 0.05%, Sigma). Than slides dehydrated and counterstained with Mayer's Hematoxylin. Control procedures involved the use of nonimmune chicken serum and PBS instead of antiserum to fowl poxvirus (FPV).

The CAMs were dissected from the surrounding structure and fixed in 2.5% glutaraldehyde in 0.2 M phosphat buffered (pH 7.4) at 4°C. They were postfixed in 1% osmium tetraoxide. After dehydration in acetone, the specimens were embedded in Epon 812. The thin sections were cut at ultra microtome, stained with uranyl acetate and lead citrate, and examined by Carl-Zeiss EM 9 S-2 electron microscopy. Classifications of developmental stages of viral particles in matrix inclusion were made according to Tajima and Ushijama (17).

RESULTS

At the gross examination, typical poxvirus lesions (pocks) were occurred on the CAM, 7 days post inoculation with fowl pox virus. The pocks that formed by the lyophilized strain 92 homogenate were grayish-white and slightly raised. Sizes of the lesions were differed from 0.5 to 2.5 mm in diameter *(Fig 1)*. The infected CAMs were also showed congestion and swelling, accompanied by occasional small hemorrhagic areas. In some CAMs, yellowish coloration and necrotic foci were also seen.

Histopathologically, similar findings were seen on all infected CAMs. Epithelial cells exhibited hyper-plasia and hypertrophy with ballooning degeneration and coagulative necrosis in some areas. Chorionic and allantoic epithelium were two or three folds thicker than control CAMs. Several intracytoplasmic inclusion bodies (Bollinger and Borrel inclusion bodies) were found in hypertrophic and hyperplastic epithelium *(Fig 2)*.

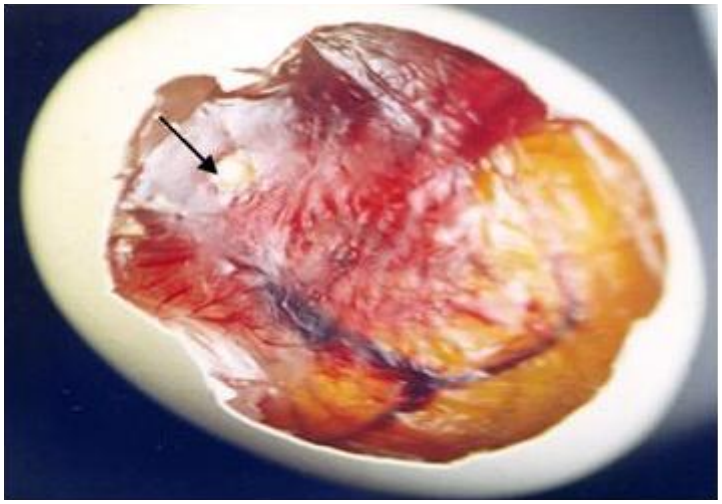

Fig 1. The pock lesion on the CAM of 10 days old SPF CAM embryonated egg (arrow)

Şekil 1. On günlük SPF embriyolu yumurtanın korioallantoik membranında çiçek lezyonları (ok)

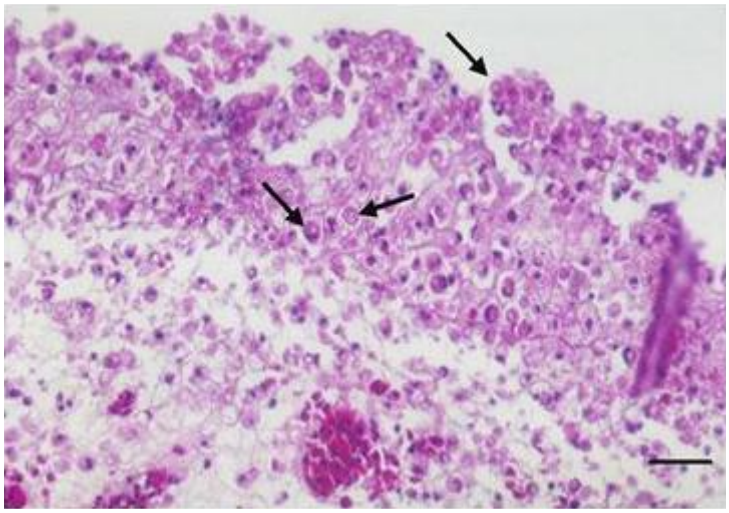

Fig 2. Intracytoplasmic inclusion bodies in the hyperplastic and hypertrophic chorionic epithelial cells (arrows), HE, Bar=100µm

Şekil 2. Korionik epitelin hiperplastik ve hipertrofik epitel hücrelerinde intrasitoplazmik inklüzyon cisimcikleri (oklar), HE, Bar=100µm

In addition to HE, these inclusion bodies were stained with Lendrum's and Mallory Phloxine staining. In the Lendrum's staining they observed red and in Mallory Phloxine they were bright red. In some cells the nucleus located eccentrically due to the presence of intracytoplasmic inclusion bodies and vacuoles. Some cells more than one inclusion bodies were also observed. The chorionic epithelial cells exhibited much more intracytoplasmic inclusion bodies than allantoic epithelial cells. Inclusion bodies in allantoic epithelium were round to oval. In all sections, central areas of the inclusion bodies were empty. Cells with inclusion bodies were swollen and margin of cells was clearly visible. The mesodermal layer of CAM showed edema with few inclusion bodies. Sometimes hemorrhagic areas were found on the mesodermal layer. A few mononuclear cell infiltrations were observed in this layer of CAM. Immunohistochemically, cytoplasmic staining was detected in the epithelial cells of the lesions and reacted with conjugated specific antibody. The inclusion and viral antigens were stained brown to dark color *(Fig 3)*.

In this study, electron microscopic examination of the chorionic and allantoic membranes revealed a number of lipid aggregates containing vacuoles which were found to be surrounded by a limiting membrane. Numerous viruses were observed around the lipid vacuoles. Viral particles at different developmental stages were identified on epithelial cells of the CAM. They were seen in five different forms: Incomplete, spherical, early intermediate, later intermediate and mature stages as mentioned previously by Tajima and Ushijima [17]. Two different types of intracytoplasmic inclusion bodies were also seen in the infected cells in this study. The intracytoplasmic inclusion bodies which were found in this study were identical to the classic inclusion bodies which were visible under the light microscope. These inclusion bodies consisted of a large number of mature forms and an electron-dense stroma. The mature virions had a dumbbell-shaped internal form, with several visible dense-stained coats *(Fig 4-5)*.

The body of other type of intracytoplasmic inclusion bodies was represented by a dense cytoplasmic area composed of fine granules and fibriler materials. In general, it was associated with various kinds of developmental forms and designated as the matrix inclusion *(Fig 6)*. These inclusions were considered to play an important role in the process of viral replication.

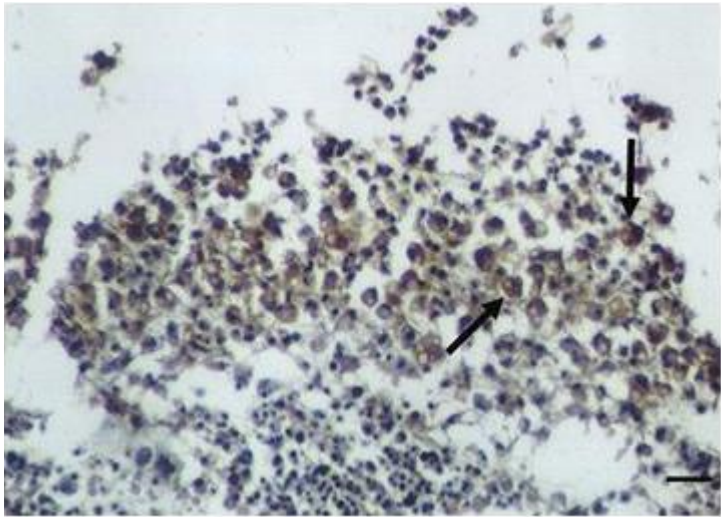

Fig 3. Positive fowl poxvirus antigen in the epithelial cells of CAM (arrows) IIP, Bar= 100µm

Şekil 3. Korioallantoik membranın epitel hücrelerinde pozitif tavuk çiçek virus antijeni (oklar), IIP, Bar= 100µm

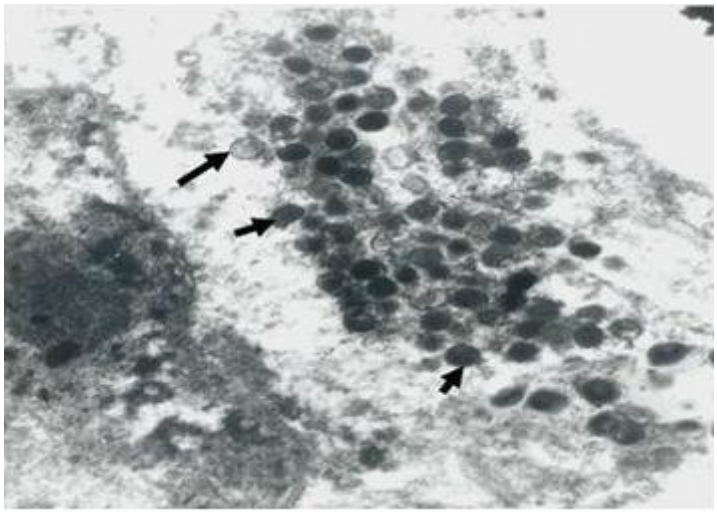

Fig 4. The matrix inclusion body that contained developmental stages of the virus in the cytoplasm of epithelial cells in the CAM of 10 days old SPF embryos chicken egg (arrows), Transmission electron microscope, X16.000

Şekil 4. On günlük SPF embriyolu tavuk yumurtasını korioallantiok membranında virusun çeşitli gelişim aşamalarını içeren matriks inklüzyon cisimciği (oklar), Transmission elektron mikroskop, X16.000

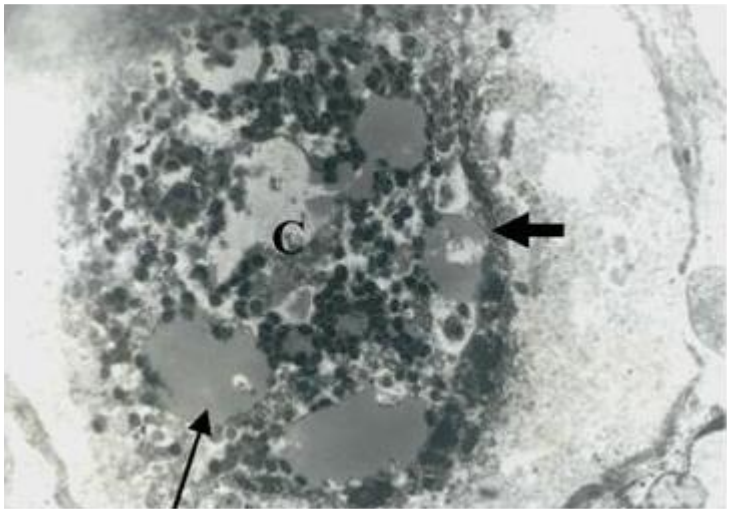

Fig 5. The classical inclusion body in the cytoplasm of epithelial cells in the CAM of 10 days old SPF embryos chicken egg (thick arrow) and lipid vacuol (thin arrow), C: centarl of the inclusion body, Transmission electron microscope, X 5.500

Şekil 5. On günlük SPF embriyolu tavuk yumurtasının korioallantoik membranında klasik inklüzyon cisimciği (kalın ok) ve yağ vakuolü (ince ok), C: İnkluzyon cisimciğinin merkezi, Transmission elektron mikroskop, X 5.500

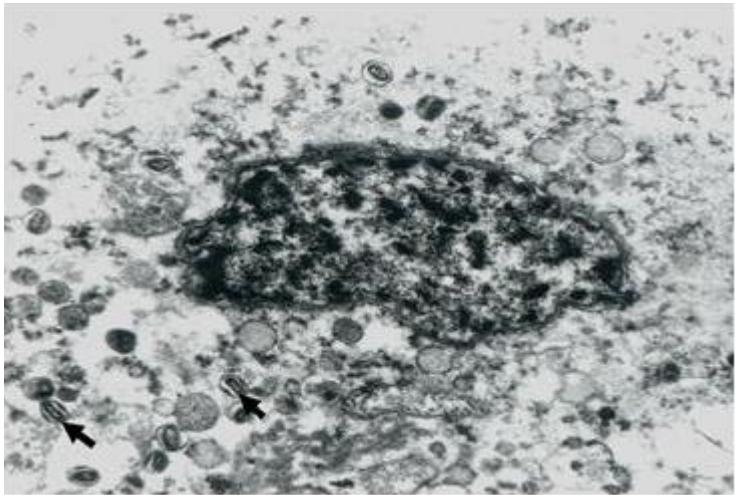

Fig 6. The free mature virion in the cytoplasm of epithelial cell in the CAM of 10 days old SPF embryos chicken egg (arrows), Transmission electron microscope, X 11.500

Şekil 6. On günlük SPF embriyolu tavuk yumurtasının korioallantoik membranın epitel hücrelerinde sitoplazmik yerleşimli serbest haldeki olgun virus (oklar), Transmission electron mikroskop, X 11.500

DISCUSSION

Avian poxviruses can cause disease in domestic and wild avian species. The disease occurs throughout the world but the occurrence varies in different geographical regions. Fowl pox infection can cause significant economic loses in domestic poultry flocks by causing a transient drop in egg production in layers and a retarded growth in young birds. Mortality and morbidity rates in fowl pox virus may be higher than 50% [1,2,6]. Avian poxviruses can be isolated by inoculation the suspected material to developing chicken embryos, susceptible birds, and cells cultures of avian origin [3,6]. Primary CAM, chicken embryo, chicken embryo kidney, chicken embryo dermis, duck embryo, or quail cells have been used to propagate avian poxviruses [6]. Diagnosis of the fowl pox infection is difficult before macroscopical lesions developed. Because of the economical importance early diagnosis of this disease can prevent to lose of production and transmission in the flock. For early diagnosis of this disease, we used CAM because fowl pox virus exhibit most of the typical lesions on CAM as occurred in natural infection in chickens and than immunohistochemical diagnosis of the disease were made and compared and supported ultrastructural examination.

Macroscopically, the pocks lesions developing on the CAM appear in various color and sizes [1,2,19,20]. In this study, the CAM lesions varied in diameter and appeared grayish-white color. While some lesions were round and regular and the others were irregular. Histopathological examination of the pox virus lesions in our study was similar those of previous study. Inclusion bodies were found similar to classical type inclusion bodies that previously described [6-8]. Additionally, in this study, contrary to Tsukamoto et al.[7], we encountered the inclusion bodies much more in chorionic epithelial cells than mezodermal layer of CAM. Histochemically, the inclusion bodies were stained with Lendrum's and Mallory Pheloxine staining as stated previously [20].

Fowl pox inclusions are composed of lipids. Previous studies suggested that the inclusion bodies include fowl pox viral particles with an outer layer which may be lipid or lipoprotein in nature [13,20]. This study showed that the marked increase in lipid vacuole may play a role in the formation of inclusion bodies since we observed the initial virus localization were around the lipid vacuoles. In addition, the staining with HE and indirect immunoperoxidase showed that the center of inclusion bodies had empty vacuoles. Thus, it may be concluded that the center of inclusions may contain lipid vacuoles and lipid granules which might have an important role for inclusion body formation. Electron microscope was used for to examine of viral agent and developmental stage of viral inclusion bodies especially for matrix inclusion bodies. The classical inclusions might have been formed by the aggregation of viral particles accomplished by maturation of matrix inclusions and by a secondary addition of dense lipid materials. The cellular alterations observed in the infected cells could not be considered as specific for viral replication. These findings were similar to the previous studies [12].

In previous studies viral particles were described in various development stages during electron microscopic examination [8,13,14,20]. In this presented study two types of intracytoplasmic inclusion bodies

were detected and it was seen that these resemble the classic inclusions detectable easily by light microscope and matrix inclusions which are easily detectable by electron microscope. It was found that matrix inclusion contains five developmental forms of the virus [1,6,16,17]. Our result also supported to these findings. Furthermore, the presence of the matrix inclusion was determined by the immunoperoxidase technique.

This study indicated that, immunoperoxidase technique may be used in the morphological diagnosis and detection of the pox virus infection in the early stage of disease. Although ultrastructural examination are expensive and take for a long time but especially examination of viral particles and comparative studies like as immonuperoxidase, this technique can be use for support to immunoreaction in experimental studies. This study showed that immunohistochemisrty were supported by ultrastructural examination. Especially in big poultry flocks pox virus infection can be detect by immunohistochemically methods in case of lost production. Our study also indicated that this technique is very sensitive for early diagnosis of fowl pox infection.

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