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LIGHT, TRANSMISSION AND SCANNING ELECTRON MICROSCOPICAL INVESTIGATIONS OF THE UPPER RESPIRATORY TRACT OF CHICKENS EXPERIMENTALLY INFECTED WITH TURKEY RHINOTRACHEITIS VIRUS

(With 30 Figures)

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فحوص بالميكروسكوب الضوئي والألكتروني الماسح والنافذ للقنوات التنفسية العليا للدجاج بعد العدوى التجريبية بفيروس التهاب الأنف والقصبة الهوائية في الرومي

صلاح سيد البلال

تم في هذا البحث دراسة تطورات الاصابة بفيروس التهاب الأنف والقصبة الهوائية في الرومي وذلك بحقن سلالة معزولة من الدجاج المصاب بظاهرة تورم الرأس عن طريق الأنف والعين في الدجاج البالغ من العمر أربعة أسابيع . لوحظت الأعراض التنفسية بعد أربعة أيام من الحقن كما أمكن قياس الأجسام المضادة للفيروس في أمصال الدجاج المصاب عن طريق إختبار الانتشار في الآجار بعد تسعة أيام من الحقن . أمكن كذلك إعادة عزل الفيروس على أجنة الدجاج بعد سبعة أيام من الحقن . أظهر الفحص الباثولوجي المجهري عن وجود التهاب في الأنف ، الجيوب الأنفية ، الغدة الأنفية الدمعية والقصبة الهوائية والشعب الهوائية . أظهر الفحص بالميكروسكوب الالكتروني الماسح عن فقد موضعي للأهداب من الخلايا الطلائية المهدبة في المفاتيل في الأطوار المبكرة من الاصابة . أما في الأطوار المتقدمة من الاصابة فقد شوهد فقدان منتشر للأهداب من الخلايا الطلائية المهدبة وتساقط لهذه الخلايا كما لوحظ تكاثر للخلايا الطلائية مع التحام الثنيات المتكاثرة على السطح الجوفي لها . أما القصبة الهوائية والشعب الهوائية فقد عانت كذلك من فقد موضعي لأهداب الخلايا الطلائية في المراحل المبكرة من الاصابة والذي أصبح ظاهراً ومنتشراً مع تساقط ملحوظ للخلايا الطلائية مع نهاية التجربة . أثبت الميكروسكوب الألكتروني النافذ ما شوهد بالميكروسكوب الالكترونى الماسح عن فقدان الأهداب وتساقط للخلايا الطلائية في المفاتيل

والقصبة الهوائية والشعب والشعيبات الهوائية كما لوحظ كذلك تكون أهداب غير طبيعية على هيئة أهداب مركبة وأهداب منتفخة وأهداب داخلية . إمتدت التغـــيرات لتشـــمل العضيـــات الخلوية حيث شوهد إتساع وتحوصل في صهاريج الشبكة الاندوبلازمية الخشنة وانتفاخ فـــي الحبيبات الخيطية كما لوحظ كذلك زيادة نشاط الشبكة الاندوبلازمية الخشنة وعلى الرغم مـــن أنه أمكن إعادة عزل الفيروس على جنين الدجاج من الدجـــاج المحقـــون بـــالفيروس إلا أن محاولات العثور على الفيروس عن طريق الميكروسكوب الالكتروني النـــافذ فـــي الخلايـــا الطلائية المهدبة للقنوات التنفسية العليا لم تكن ناجحة .

SUMMARY

The pathogenesis of turkey rhinotracheitis virus infection (TRTV) in 4-week-old chickens was studied by inoculation of a field strain via occular and nasal routes. This strain has been isolated from chickens with naturally occurring swollen head syndrome .Respiratory signs were observed four days post-inoculation. Antibodies against TRTV infection were detected in the sera of the inoculated birds by agar gel diffusion test at the 9th day post-inoculation. The virus could be reisolated at the 7th day post-inoculation after three blind passages of the infected material in chicken embryos. Histopathology revealed serous rhinitis, sinusitis, catarrhal nasolacrimal adenitis, tracheitis, bronchitis and bronchiolitis. Scanning electron microscopy of the turbinates revealed focal deciliation and desquamation of the luminal surfaces of the ciliated epithelial cells at the early stages of infection. At ten days post-inoculation there were diffuse deciliation, epithelial cell exfoliation, epithelial hyperplasia with fusion of the hyperplastic folds at their luminal surface. The trachea and bronchi showed focal deciliation at the early stages of infection which became diffuse with obvious epithelial cell desquamation at the end of the experiment. Transmission electron microscopy further demonstrated the process of deciliation and exfoliation of the ciliated epithelial cells in the turbinates, trachea, bronchi and bronchioles. Ultrastructural changes involved the cilia and the cytoplasmic organelles of the ciliated cells. There were focal to diffuse deciliation, dilatation and vesiculation of the cisternae of the rough endoplasmic reticulum, and mitochondrial swelling. Abnormalities of the cilia were in the form of swollen cilia, compound cilia and internalization of cilia. Although the virus could be reisolated on chicken embryos, trials for direct demonstration of the

virus by transmission electron microscopy of the ciliated epithelial cells of the upper respiratory tract were unsuccessful.

Key words: *Turkey Rhinotracheitis, TEM, SEM.*

INTRODUCTION

Turkey rhinotracheitis (TRT), an acute respiratory tract infection that affects turkeys of all ages, was first reported in South Africa (Buys and Preez, 1980). TRT is now recognised throughout the world (Lister and Alexander, 1986) and the primary causal agent has been isolated and identified as a pneumovirus (Cavanagh and Barrett, 1988; McDougall and Cook, 1986 and Wyeth et al., 1987) named turkey rhinotracheitis virus (TRTV). TRTV is the cause of a well defined disease in turkeys and can also infect chickens (Cook et al., 1988). TRTV have been isolated from chickens (Picault et al., 1987; Buys et al., 1989; Jones et al., 1991) and Jones et al. (1987) were able to infect chickens with the virus, but without causing apparent disease. Majo et al. (1995) used immunoperoxidase and Jones et al. (1987) used virus isolation, histopathology and immunofluorescence to study the ability of a turkey isolate of TRTV to infects chickens. Catelli et al. (1998) studied the tissue distribution of TRTV and its persistence in chickens using virus isolation, histological and immunochemical methods. However, non of these studies refered to the ultrastructural changes associated with TRTV infection in chickens.

The present paper reports a sequential histopathologic, scanning and transmission electron microscopic study of the respiratory tract mucosa of chickens experimentally infected by TRTV.

MATERIALS and METHODS

Turkey rhinotracheitis virus:

Turkey rhinotracheitis virus strain was kindely supplied by Prof. Dr. A.Ibrahim, Prof. of Poultry Diseases, Faculty of Vet. Med., Assiut University.

Experimental design:

Thirty broiler chickens (4-week-old) were proved to be free from anti bodies to TRTV by agar gel diffusion test and ELISA. The birds were classified into three groups.

Group 1: Ten birds were inoculated intranasally and into the conjunctiva with the virus in a dose of containing 10^6 $TCID_{50}$. 0.3 ml.

Group 2: Ten birds were raised in contact with the inoculated birds to study the possibility of contact infection.

Group 3: The birds of this group act as non-infected control.

The birds were observed daily for 2 weeks for the clinical signs. Sera were collected from all groups after the appearance of clinical signs for detection of antibodies to TRTV by agar gel diffusion test. Trials for virus reisolation was done.

Light microscopy:

Specimens from the nose, turbinate, trachea and lung were fixed in 10% neutral buffered formalin, processed routinely, embedded in parablast, sectioned and stained with hematoxylin and eosin.

Electron microscopy:

Samples from the turbinate, trachea, bronchi and bronchioles were washed gently with physiological saline and fixed in 5% cacodylate buffered glutaraldehyde.

Scanning electron microscopy:

Specimens for SEM were postfixed in 2% osmium tetroxide, dehydrated through a graded series of ethyl alcohol to propylen oxide, and critical point dried. The tissue was coated with a thin layer of gold and examined with a Jeol JSM-5400LV at an accelerating voltage of 15 KV.

Transmission electron microscopy:

Fixed tissue blocks were postfixed in 2% osmium tetroxide, dehydrated and embedded in Epon 812. Semithin sections, stained with 0.25% toluidine blue, were examined under the light microscope, and

areas were selected for examination by TEM. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a Jeol-EM100 CXII transmission electron microscope at an acceterating voltage of 80 KV.

RESULTS

1. Clinical signs:

Clinical signs in group 1 appeared 4 days post-inoculation, started with depression, followed by sneezing, nasal secretion, gasping and conjunctivitis. A mild clinical signs were observed in group II at 7th day post-inoculation. These symptoms disappeared within 13 days post-inoculation. Post-mortem findings were congestion of the trachea while the nasal passages and sinuses contained mncous exudate and some cases had congestion of the lung. Group III showed no clinical signs nor post-mortem findings.

2. Serology and virus reisolation:

Antibodies to TRTV could by detected 7 and 9 days p.i in infected and contact birds by ELISA and agar gel diffusion test. Virus could be reisolated 7 and 9 days p.i in inoculated and contact birds after three blind possages of infected material in chicken embryo.

3. Light microscopy:

Histopathological examination of the turbinates of the chickens experimentally infected with TRTV revealed that the early lesions were in the form of serous inflammation with increase in the glandular activity, severe hyperaemia, oedema, and lymphocytic infiltration in the submucosa (Fig. 1). There were focal loss of the cilia from the ciliated cells of the respiratory mucous membrane. These lesions progressed to copious catarrhal exudate, epithelial hyperplasia, hyperaemia and lymphocytic infiltration in the submucosa together with focal deciliation (Fig. 2). The most severe lesions were observed by the end of the experiment where the epithelial cells suffered deciliation, desquamation, marked epithelial hyperplasia, hyperaemia and massive lymphocytic infiltration Marked increase in the glandular activity was also observed (Fig. 3).

The trachea showed lesions varied from mild tracheitis in the early stages of infection to severe tracheitis in advanced stages. The early lesions were in the form of focal to diffuse loss of the cilia, cellular vaculation and exfoliation, increased glandular activity, hyperaemia, oedema and lymphocytic infiltration of the lamina propria. Later in the experiment, there were severe tracheitis with epithelial desquamation, severe hyperaemia, lymphocytic and heterophilic infiltration of the submucosa. Furthermore, epithelial hyperplasia were evident in some cases (Fig. 4).

The primary and secondary branchi showed focal to diffuse deciliation, epithelial cell vacuolation and exfoliation. In the submucosa, there were severe hyperaemia, oedema, lymphocytic and heterophilic infiltration (Fig. 5). In few cases the lesions extended to focal serous pneumonia.

The nasolacrimal gland suffered from catarrhal inflammation with formation of copious catarrhal exudate containing inflammatory cells and cellular debris (Fig. 6). The epithelial cells of the infraorbital sinus showed deciliation, vacuolation, and intraepithelial lymphocytic infiltration (Figs. 7 & 8). The surrounding tissue was sererely hyperaemia and massively infiltrated with lymphocytes and occasional heterophils (Fig. 7).

Scanning electron microscopy:

Scanning electron microscopy (SEM) of the ciliated epithelial cells of the turbinate at the early stages of infection revealed focal deciliation (Figs. 9 & 10). These lesions progressed to epithelial hyperplasia with fusion of the hyperplastic folds at their lumenal surface (Figs. 11 & 12). Hyperplasia of the mucous glands was also observed. By time the deciliated areas enlarged and only the microvilli could be seen on the surface of these cells. Desquamation of the lumenal surface and sometimes the whole cell was the fate of the deciliated cells (Fig.13).

In the trachea, the early lesions detected by SEM were focal deciliation. Later on, there were extensive loss of the cilia and exfoliation of the ciliated epithelial cells (Figs. 14 & 15). Some microvillia were seen on the lumenal surface of these deciliated cells (Fig. 16). Some abnormal cilia were still observed on the surface of some cells (Fig. 17). These lesions progressed to desquamation of the lumenal surface of the deciliated cells. There were widening of the tight

junction between these cells and many of them were in the process of desquamation (Figs. 17 & 18). The ciliated epithelial cells of the primary and secondary bronchi showed similar but less sever lesions (Figs. 19, 20 & 21). Mucous exudate was observed on the lumenal surface of the deciliated cells (Fig. 20).

Transmission electron microscopy:

Transmission electron microscopy (TEM) of the ciliated cells in the turbinate and tracheal mucosae have the normal 9+2 pattern of the axoneme. The basal bodies and their striated roottlets, with occasional centrioles were observed beneath the cilia. Interspersed with the cilia were numerous and shorter microvilli. Mitochondrial rough endoplasmic reticulum associations consisting of rough endoplasmic reticulum partially endosing the mitochondria were rare. In TRTV infected group, there were deciliation and various forms of atypical cilia (Fig. 23). These includes compound, swollen and intracytoplasmic cilia. The compound cilia composed of more than one cilium, sometimes there were intracytoplasmic tunnel or vacuole containg several cilia in cut section (Fig. 23 & 24). Intracytoplasmic cilia, the axial microtubule complex is deviated and follows an intracytoplasmic course (Fig. 25). The basal bodies and the striated rootlets were irregularly arranged beneath the cilia. The microvilli on the cell surface showed atrophy or blunting, fusion, branching, Y-shape formation and effacement (Figs. 25, 26 & 27). The rough endoplasmic reticulum was well developed in a whorled or a lamellar configuration. Dilation of the cisternae of RER was frequently observed. Mitochondra were swollen with loss of the cristae. Mitochondrial-RER associations were frequently observed especially in the apical regions of the infected cells (Fig. 22). In more advanced lesions there were mitochondrial swelling, cytoplasmic vacuolation with scanty cytoplasmic organelles, cellular disintegration and desquamation into the lumen through dilatation of the tight junction with the adjacent epithelial cells (Figs. 28 & 29). The nucleus showed irregularity of the nuclear membrane and margination of the nucleolus (Fig. 30).

Viral particles could not be demonstrated by transmission electron microscopy in the ciliated epithelial cells of the trachea and turbinate.

DISCUSSION

In the present work, TRTV could produce lesions in the turbinates, nasolacrimal gland, infraorbital sinus, trachea, and the bronchioles of 4-week-old chicks experimentally infected with TRTV. These lesions were in the form of focal to diffuse deciliation ,epitheilial cell vacuolation and exfoliation, lymphocytic and heterophilic infiltration of the congested epithelia. Similar lesions were described in experimentally infected chickens by Jones et al., 1986; 1987; Picault et al., 1987; Jordan and Pattison ,1996 and Catelli et al., 1998. Majo et al. (1995) described severe lesions in the turbinates of chickens experimentally infected with TRTV and they were able to demonstrate TRTV antigen in the epithelial cells of the turbinate by the immunocytochemical methods. They suggested that turbinate pathology could be a marker for the evaluation of TRTV infection. Furthermore, Jones et al. (1993) hypothesized that massive replication in the turbinates causes severe rhinitis that could enhance the invasion of other infectious agents leading to SHS. In this material, the most consitent and severe lesions were observed in the turbinates of experimentally infected birds. These results may agument the previous hypothesis. The observation of lesions in the lower respiratory tract was previously described by Major et al. (1987). This may indicate that TRTV may have a similar role to respiratory syncytial virus in predisposing poultry to bacterial pneumonia (Bryson et al., 1983).

In this study scanning electron microscopy demonstrated focal to diffuse deciliation and exfoliation of the ciliated epithelial cells in the turbinate, trachea, primary and secondary bronchi. Transmission electron microscopy further demonstrated the process of deciliation and formation of atypical cilia. The forms of atypical cilia seen in this study were compound, swollen and internal cilia. The microvilli suffered from atrophy, branching, fusion and effacement. Among the celluar organelles there were mitochondrial swelling, formation of well developed RER and increased mitochondrial -RER associations. Irregular arrangement of the basal bodies and striated rootllets along with an increase in centrioles.

The formation of compound cilia seen in this study was previously described in fowls infected with infections laryngotracheitis

virus (Purcell, 1971). The author attributed these ciliary structures to the effect of the virus on allready existing cilia or suggested that it may have been produced by abnormal growth from infected cells. Compound cilia were also reported in the bronchial mucosa of heavy smokers (Ailsby and Ghadially, 1973), and in adult suffering from pulmonary disease with or without bronchitis (Lungarrela et al., 1983) and in children with recurrent respiratory tract infection (Ehouman et al., 1985; Giorgi et al., 1992) and in tracheal epithelial cells infected with cryptosporidium sp. (Tadeja-Simborio et al., 1993). Pathologically altered cilia may cause sluggish movement of mucus which deprives cells of their ability to rid themselves of irritants, carcinogen and other pathogens (Ghadially, 1997). The blunting and Y-shaped formation of microvilli may be an adaptive mechanism of the cell to infection (Tadeja-Simborio et al., 1993). The irregular arrangement of the basal bodies and striated rootlets along with an increase in centrioles, is probably a compensatory mechanism of the cell to infection. The deciliation process and other cilial alterations may have triggered the increase in the number of centrioles which are self replicating organelles. It has been reported that during ciliogenesis, the newly formed single centrioles serveres as the basal body of the developing cilia (Ghadially, 1997).

Swollen cilia have been reported by Duncan and Ramsey (1965) in the porcine nasal mucosa in Bordetell bronchiseptica-induced rhinitis. The mechanism and significance of production of swollen cilia are not evident, but it would seen that such cilia may be functionally incompetent, for the axial microtubule complex may be inadequate to move the increased mass of swollen cilia effectively. It would therfore appear that pathogically altered cilia may constitut yet another factor responsible for the sluggish movement of mucus and the pulmonary pathology that results from an impairment of this vital cleansing mechanism (Ghadially, 1997).

The increased protein synthesis is supported by the presence of well developed RER and increased mitochondrial-RER associations. This indicate a high rate of protein synthesis, not for endogenous metabolic use, but for the use by the virus (Hammersen, 1985; Ghadially, 1997).

Although the virus could be reisolated on chicken embryo from the inoculated birds, trials for direct demonstration of the virus by transmission electron microscopy of the ciliated epithelial cells of the

upper respiratory tract were unsuccessful. This may be due to the small samples taken for EM, or low concentration of the viral particles in the infected tissues.

The results reported here described detailed sequential examination of the upper respiratory tract mucosa by SEM and TEM. From these results it can be concluded that TRTV can infect the upper respiratory tract of 4-week-old chickens and induced light and ultrastructural changes in the cilia of these cells which predispose them for infection by other pathogens and production of SHS.

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FIGURES LEGENDS

Fig. 1: Turbinate of experimentally infected chicken showing serous rhinitis, serous exudate (S) and increased glandular activity (G) (H & E., X 10).

Fig. 2: Turbinate showing epithelial hyperplasia ((↑), hyperaemia and lymphocytic infiltration (L). (H & E., X 10).

Fig.3: Turbinate showing marked epithelial hyperplasia, epithelial desquamation (E), increased glandular activity (G) and massive lymphocytic infiltration (L). (H & E., X 10).

Fig. 4: Trachea showing epithelial desquamation ((↑), deciliation ((↑), epithelial hyperplasia (H), Hyparaemia, lymphocytic and heterophilic infiltration (H & E., X 40).

Fig. 5: Lung showing focal deciliation ((↑), vacuolation (V) and exfoliation of the bronchial epithelium, severe hyperaemia (H), edema, lymphocytic and heterophilic infiltration of the submucosa (H & E., X 40).

Fig. 6: nasolacrimal gland showing catarrhal inflammation (H & E., X 4).

Fig. 7: Infraorbital sinus showing deciliation, intraepithelial lymphocytic infiltration, edema and lymphocytic infiltration in the surrounding tissue. (H & E. X 4).

Fig. 8: Infraorbital sinus showing deciliation, vacuolation, intraepithelial lymphocytic infiltration (H & E., X 40).

Fig. 9: SEM of the nasal turbinate showing hyperplasia of the mucous glands (G), fusion of the hyperplastic folds, cartilagenous surface (S), Lumenal surface (L). The inset is a higher magnification of the lumenal surface to demonstrate focal deciliation ,(○) Cilia (C), (X 2,000, inset X 3000).

Fig. 10: Semithin section of the turbinate to demonstrate focal deciliation and epithelial cell exfoliation, (Toulidine blue, X 40).

Fig. 11: SEM of the turbinate showing hyperplasia and fusion of the hyperplastic folds at their luminal surface, opening of the mucous glands (G), focal deciliation ((↑), ciliated cells (C), (X 500).

Fig. 12: Semithin section showing epithelial hyperplasia, focal deciliation, profuse catarrhal exudate containing inflammatory and desquamated epithelial cells (Toulidine blue stain, X 40).

Fig. 13: Higher magnification SEM to demonstrate the deciliation and desquamation of the ciliated border ((↑), microvilli on the surface of deciliated cells (M), mucous exudate (MU), sporadic ciliated cell (C). (X 3500).

Fig. 14: SEM of the trachea showing area of ciliated cell (A), area of deciliated cells (B), and area of epithelial cell desquamation (D), (X 500).

Fig. 15: SEM in a higher magnification of the previous micrograph showing ciliated cells (C), deciliated cells (D), only some of the microvilli remains on the luminal surface of some cells ((↑) (X 1000).

Fig. 16: SEM showing deciliated cells (D) among cells with abnormal cilia (C). Inset (A) demonstrate desquamation of the luminal border of ciliated cells ((↑). Inset (B) showed a cell with deciliation (D) and only some microvilli remains on its surface. (X 3500) inset A X 5000 inset B (X 5000).

Fig. 17: SEM showing complete deciliation, widening of the tight junction between the epithelial cells ((↑), epithelial cells in the process of desquamation (D), some ciliated cells (C). Inset showing deciliated cells only few cilia remans ((↑). (X 2000).

Fig. 18: SEM showing complete desquamation of the cilia and microvilli and exfoliation of the epithelial cells. Note remaining ciliated cell ((↑). (X 2000). The inset is a semithin section to demonstrate deciliated cells and some abnormal microvilli on some of them (X40), Toulidine blue (X 40).

Fig. 19: SEM of a primary bronchi (P), Secondary bronchiole (S). (X 1000).

Fig. 20: SEM showing deciliation, only the microvilli remains (M), widening of the intercellular space between the deciliated cells ((↑), mucour exudate (MU). (X 5000).

Fig. 21: SEM of a secondary bronchus focal deciliation (D), ciliated cells (C), tertiary bronchus (T). (X 2000).

Fig. 22: TEM of desquamated epithelial cell of turbinate within the infl. exudate showing activation and proliferation of the rough endoplasmic reticulum (RER) which showed diliation of its cisternae and filled with material of moderate electron density, Mitochondria (M) and Nucleus (N). (Uranyl acetate and lead citrate, X 20,000).

Fig. 23: TEM of the trachea ((↑) showing complete deciliation severe mitochondrial swelling (M), Basal body (B), transversely sectioned tunel lying within the cell containing cilia in cut section (T), Compound cilia ((↑). (Uranyl acetate and lead citrate , X 10000).

Fig. 24: TEM of the trachea, higher magnification to demonstrate more than cilia in cut section contained in intracytoplasmic tunnel or vacuole (T). (Uranyl acetate and lead citrate, X 20000).

Fig. 25: TEM of the trachea showing complete deciliation into the lumen, swollen cilia ((↑), abnormal microvilli (atrophy or blunting, fusion, branching or effacement of microvilli and Y-shaped microvilli irregular arrangement of the basal bodies (B) absence of the striated rootlets, internal cilia ((↑), tight junction (T). (Uranyl acetate and lead citrate). (X 28000).

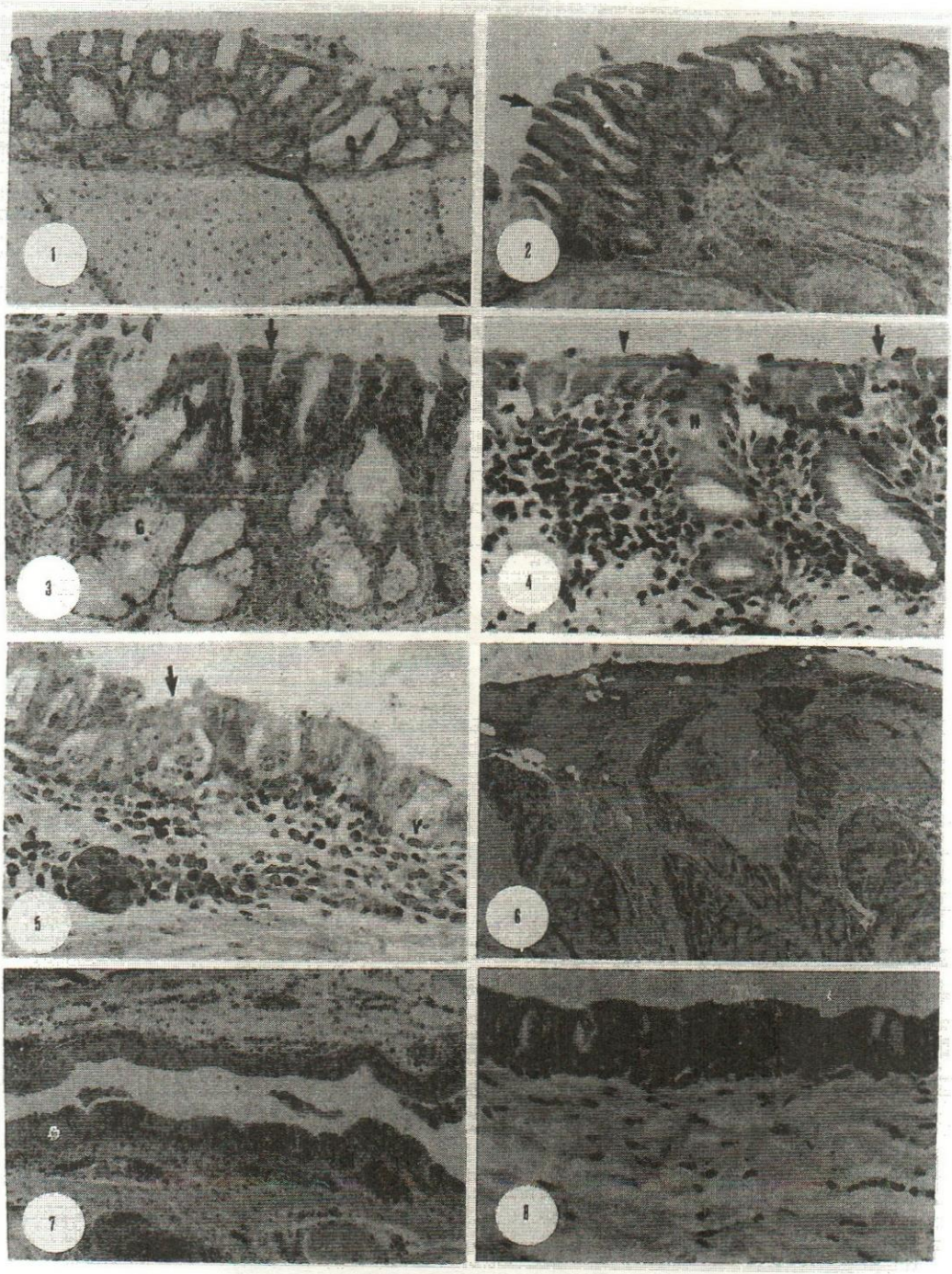
Fig. 26: Higher magnification showing swollen cilia ((↑), atrophy, fusion of the microvilli. (Uranyl acetate and lead citrate, X 54000).

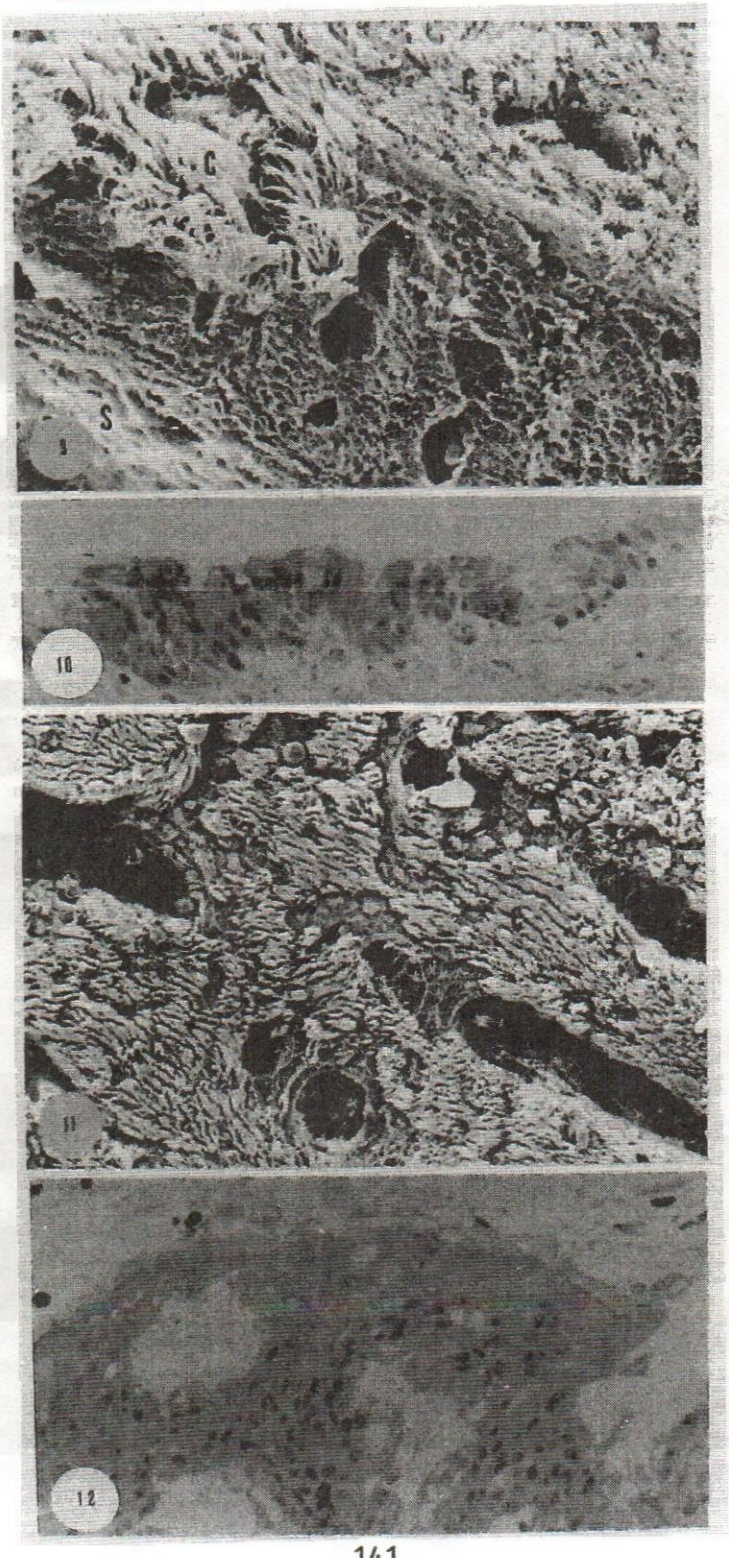
Fig. 27: Higher magnification showing Y-shaped, fusion and blunting of the microvilli (Uranyl acetate and lead citrate, X 13400).

Fig. 28: TEM of the trachea showing deciliation, the cilia showing the typical (9+2) pattern (() of microtubules, some were swollen ((↑), swollen mitochordria (M). dilation of the tight junction between the two epithelial cells. (Uranyl acetate and lead citrate, X 28,000).

Fig. 29: TEM of the trachea showing complete deciliation and desquamation of the microvilli, mitochondrial swelling (M), diliatation of RER, Rough endoplasmic reticulum-mitochondrial association, Lumen (L), cytoplasmic vacuolation (V), viral like particle ((↑). The adjacent cell (C) showed cellular disintegration and desquamation into the lumen. (Uranyl acetate and lead citrate, X 8000).

Fig. 30: TEM of the trachea showing desquamation of the ciliated border (↑), mitochondrial swelling (M), Nucleus (N), loss of the junctional complex with the adjacent cells, Nucleolus (↑), irregularity of the nuclear membrane. (Uranyl acetate and lead citrate, X 13400).

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