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Assiut Univ., Faculty of Vet. Med., Dept. Animal Med.
Head of Dept. Prof. T.A.A.EL-Allawy

SUDDEN DEATHS IN A SHEEP FLOCK DUE TO MIXED INFECTION WITH COCCIDIA AND CLOSTRIDIUM PERFRINGENS TYPE D.

(With 3 Tables, Fig. A,B,C and 16 Figures)

By

*A.M. ZAITOUN; S.S. EL-BALLAL**
*and Z.M. EL-SAYED***

*: Dept. of Pathology, Faculty of Vet. Med., Assiut Univ
**: Section of Anaerobes, Animal Health Research Institute, Dokki-Cairo

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نفوق مفاجئ في قطيع أغنام بسبب عدوى مختلطة بالكوكسيديا والكولستريديم بيرفرينجينز نوع د

أحمد زيتون ، صلاح سيد البلال ، زينب محمد السيد

كان الهدف من هذا البحث هو معرفة الأسباب التى أدت الى نفوق بعض الأغنام فى قطيع أغنام مكون من ١٢٣ رأس. تسع وعشرون حالة (٢٣,٥٨ %) من القطيع نفقت مفاجأة. كانت أعمار الحيوانات النافقة محصورة ما بين ٥-٨ أسابيع و ٦,٥ شهور ولم يشاهد نفوق في الحملان حديثة الولادة أو البالغين والأعمار الكبيرة. وكانت الأعراض المرضية التي ظهرت على الأغنام المريضة ذو طبيعة عصبية اكثر منها معوية أو تنفسية. وقد أظهرت الصفة التشريحية أن الأمعاء كانت محتقنة وممتلئة بسائل جيلاتينى يميل لونه الى ألبني ويحتوي على قطع من الدم المتجلط. وبالفحص المجهري شوهد بؤر موت خلايا سائلي في الأجزاء المختلفة من أمخاخ الحيوانات النافقة. هذا وقد تم عزل جرثومة الكولستريديم بيرفرينجينز نوع(د) بنسبة ٧١,٤٣ % من العينات المختبرة. وكذلك تم تحديد سم ابسلون " Epsilon toxin " في جميع العينات المجمعة من محتويات أمعاء الحيوانات النافقة وكذلك في مستنبتات العترات الميكروبية المعزولة. وقد أظهرت الفحوصات الطفيلية عن إصابة الحملان والأغنام الشابة بطفيل الكوكسيديا. وقد أجريت محاولة علاجية بنتائج ناجحة في إيقاف النفوق. المشاهدات الحقلية والتحليل الاحصائى أظهرا أن نسبة الإصابة بالمرض ربما ارتفعت عقب حقن الحيوانات بجرعات عالية من دواء الفاشيوليد. من المقترح ان التغيرات الباثولوجية الحادة التى أحدثها طفيل الكوكسيديا في الأمعاء أدت الى تنبيهه ميكروب الكولستريديم بيرفرينجينز نوع (د) الموجود بطبيعة الحال في أمعاء الأغنام وجعلته يتكاثر ويفرز سمومه. ومن المحتمل أن الجرعات العالية من أدوية مضادات الطفيليات تلعب دورا مساعد كبيرا أو صغيرا في إحداث المرض.

SUMMARY

Twenty-nine (23.58%) cases of the investigated sheep flock (123 heads) were suddenly succumbed. Ages of the succumbed animals were restricted between the 5-8 weeks to 6.5 months old. No fatalities were observed in the neonatal lambs or in the adults. Diseased sheep showed neurological manifestations rather than signs of enteritis and respiratory distress. Postmortem findings revealed that the intestines were congested and filled with brownish gelatinous fluid containing shreds of clotted blood. Histopathologically, malacic foci were clearly observed throughout the brains of the succumbed cases. *Clostridium perfringens* type D organisms (pure cultures) were isolated from 71.43% of the tested samples. Epsilon toxin was detected in all samples of the intestinal contents and in the cultures of the isolated strains (toxin-producing strains). Parasitological examinations revealed that the lambs and the younger sheep were suffered from severe coccidiosis. Therapeutic trial was done with successful results to stop the fatality rate. Field observations and statistical analysis revealed that the morbidity % of the disease (enterotoxemia) was apparently elevated prior to inoculation the sheep with higher doses of Fasciolid (nitroxynil), anthelmintic drug. It is suggested that the severe pathological alterations induced by coccidiosis stimulated the quiescent *Cl. perfringens* type D organism that located normally in the intestine of sheep to proliferate and produce its toxins. It is probable that the overdose of anthelmintic drug act as a major and/or minor predisposing factor for pathogenesis of that disease.

Keywords: *Ovine-Sudden death-Enterotoxemia-clinical-bacteriology-panasitology-Antehelmintic drug-pathology.*

INTRODUCTION

Enterotoxemia caused by *Cl. perfringens* type D was one of the major clostridial diseases of sheep characterized chiefly by sudden death of the affected cases with or without premonitory signs (Barker et al., 1996). The spores of pathogenic types of *Cl. perfringens* were commonly found as one of the normal bowel inhabitant microorganisms of sheep but in small numbers without clinical abnormalities (Mahmoud, 1991)

Under certain predisposing factors, the normal bowel inhabitant *Cl. Perfringens* microorganism was rapidly multiplied and increased in number, and produced sufficient amount of epsilon toxin causing enterotoxemic condition

with marked patho-physiological alteration followed by death within few hours (Pathak and Parihar, 1994). Abruptly changes from milk to solid food diet and over-eating on carbohydrate rich diet as much amount of cereal grains were frequently incriminated as the predisposing factors enhancing ovine enterotoxemia caused by *Cl. perfringens* type D (Barker et al., 1996). Enterotoxemia was produced experimentally in sheep with marked patho-physiological alteration by intra-duodenal inoculation (duodenal fistula) of the whole culture broth of *Cl. perfringens* type D with 40 % dextrin (Pathak and Parihar, 1991). Conversely, the latter authors failed to induce ovine enterotoxemia by inoculation the concentrated toxin (alone) of *Cl. perfringens* type D. This may refer to that the carbohydrates substances produce a favorable medium for growing and proliferation of *Cl. perfringens* type D organism in the intestine of sheep. However, under field conditions, ovine cestodes (Moniezia, Thyaniezia and Avitellina species) were incriminated as a direct predisposing factor enhancing clinical enterotoxemia caused by *Cl. perfringens* type D in sheep (Radionov and Islamov, 1988). Consequently, the overdosing carbohydrates may not the only promoting factor for pathogenesis of *Cl. perfringens* type D infection in sheep.

The fundamental goal of the following work was to study the probable causative agent(s) responsible for the fatalities of a sheep flock located in Abnoub village, Assiut Governorate-Egypt, and was to clear up the possible predisposing factors that enhanced the target disease. Therapeutic trial was also made to stop or reduce the fatalities of sheep

MATERIAL and METHODS

MATERIAL

History:

Four diseased lambs (6-8 weeks old) were admitted to the Veterinary Hospital, Assiut University, on the last week of Nov., 95, with history of inappetance and extreme loss of condition with brownish diarrhea, and hypothermia. One of the admitted cases died before clinical observation. Symptomatic treatment was ineffective, and those animals died at night on the same day of admittance. Two weeks later, another 3 cases suddenly died without obvious signs with exception of one case showed slightly diarrhea and convulsions immediately before death.

Animals and farm characteristics:

A total number of 116 heads of "balady" sheep of different ages were examined. The investigated flock was consisted of 54 adults and 62 lambs in

different ages (Table 1). The hygienic condition of the sheepfold was clearly sub-level. Feeding of the lambs was dependent mainly on dams' milk for 3-4 weeks and then fed on milk with green fodder and little amount of crushed corn-grains. The total amount of these grains was approximately ranged from 150 to 200 grams / lamb / day. Increasing the age of animal gradually increased the concentrated ration. The investigated sheep flock not vaccinated against any infectious diseases. The owner usually used anthelmintic drugs. On 19/1/96, the owner haphazardly inoculated the sheep with high doses (about 40-50 mg / Kg. B.W.) of anthelmintic drug "Fasciolid" (nitroxynil). According to the pamphlet of this drug the recommended dose is 10 mg/kg B.W. The investigated animals in this flock were divided into five groups according to the ages and the degree of fatalities (Table 3).

Collection of the samples:

The frequent visits (Table 1) to the respective farm were done every week regularly, or according to the owner's calling, some of the clinically diseased animals were successively selected according to the appearance of the clinical signs and subjected to clinical, bacteriological and parasitological examinations. Twenty bacteriological fecal-swabs and fecal samples were successively taken from 20 cases showed abnormal clinical signs and examined bacteriologically and parasitologically, respectively. Urine and blood samples of some infected cases were collected. The freshly dead or emergency slaughtered animals were subjected to postmortem and histopathological examinations.

METHODS

Aerobic bacteriological culturing procedure:

Five percent of sheep blood agar (Oxoid) and MacConkey's agar (Oxoid) plates were used for aerobic bacteriological analysis of the collected fecal swabs. The inoculated plates were incubated aerobically at 37 °C for 18-24 hours. The bacterial colonies were thereafter, biochemically identified as the methods described by Quinn et al. (1994).

Parasitological examinations:

The obtained fecal samples were prepared for parasitological examinations using centrifugal flotation technique for detection of eggs or oocysts as the methods reported by Coles (1980).

Urine analysis:

Fifteen urine samples were aspirated from the urinary bladders of freshly dead and slaughtered cases and tested for the presence of glucose and protein, and for determination of pH of the urine by using urine test reactive-strips (URI-QUIC™ 9SG, StanBio Laboratory, Inc., San Antonio, Texas).

Hematological examinations:

Seven blood samples (with and without anticoagulant) were collected from the clinically diseased sheep immediately before death and from the emergency slaughtered cases (3). The collected blood samples were subjected to blood-sugar analysis and some hematological examinations as shown in Table 2. Blood-serum glucose was estimated colorimetrically by means of specific test kit (Boehringer Mannheim Diagnostica). Erythrocytic (RBCs) and total leukocytic (WBCs) cells were counted by Hemocytometer. The packed cell volume (PCV) was determined by using hematocrite tube methods as described by Coles (1980). Control blood samples was also collected from apparently healthy cases and utilized for comparison (Table 2).

Postmortem examinations:

Postmortem examinations of 15 freshly dead- and 3 emergency slaughtered cases, which showed clinical signs before death, were successively made and described. Of these cases, the grossly hyperemic parts of the intestines and its content were taken as the methods described by Mahmoud (1991) and putted in the ice container and were transported as soon as possible to Anaerobic Section of Animal Health Research Institute (Dokki-Cairo) for isolation of clostridia and detection of their toxins.

Direct smears:

Direct microscopic and mucosal scraping smears from the congested parts of the intestinal walls and from the contents were also made and stained with Gram's stain.

Anaerobic bacteriological culturing procedure:

Culturing procedure for isolation and identification of clostridia from the collected intestinal samples was carried out according to criteria of Quinn et al. (1994). Cooked meat broth medium (Difco) supplemented with 1 % glucose and 10 % defibrinated sheep blood agar (Oxoid) were used for primary isolation. Both media were supplemented with 0.02 % neomycin sulphate (Russell). Five percent of egg yolk agar (Gibco) supplemented with 8 % sterile horse serum was also prepared and used for detection of Nagler's reaction. This reaction was inhibited by specific antiserum (type A). The inoculated plates were incubated at 37 °C for 24-48 hours under anaerobic condition by using anaerobic culture system (GasPack Anaerobic culture System, BBL-BioQuest Division, Becton, Dickinson and Company Cockeysville, Md.). Thereafter, the plates were examined with stereoscopic microscope. The suspected colonies were picked up, and purified and stained with Gram's stain, and subjected to biochemical analysis according to the methods described by Quinn et al. (1994).

Detection and typing of Cl. Perfringens-toxins in the intestinal contents:

The collected intestinal contents were prepared for mice lethality tests to demonstrate the toxins of *Cl. perfringens* infection. For the latter test, 0.4 ml of the clarified supernatant of each intestinal content was inoculated intravenously into each of two mice. If the inoculated mice died within 10 ±2 hours, the clarified supernatant fluid was subjected to intravenous mice toxin-antitoxin neutralization test using specific antitoxins of *Cl. perfringens* types A, B, C and D (Wellcome Diagnostics, Beckenham, England) for typing of the demonstrated toxin according to the protocol described by Quinn et al (1994).

Detection of Cl. Perfringens-toxins in the cultures:

Detection of *Cl. perfringens*-toxins in the whole growing culture was also made; the isolated strain was inoculated into cooked meat medium and anaerobically incubated for 12-18 hours. Thereafter, the broth medium was centrifuged and filtered through triple layers of sterile cheesecloth, and the pH was adjusted to 7.3 by adding 1N sodium hydroxide. This filtrate was divided into two halves. The first half was letting without additives. The second half was treated with trypsin 0.1 % (Trypsin 1: 250, Difco) in sterile peptone water and was incubated at 37 °C for 30 min. Thereafter, the both halves (treated and untreated) were subjected to intravenous toxin-antitoxin neutralization test in mice as the method reported by Quinn et al. (1994)

Histopathological examinations:

For Histopathological examinations, different specimens of the internal organs of the recently dead and slaughtered animals that showed clinical sings were collected. These specimens included: different parts of the intestines particularly the grossly congested portion, livers, kidneys, spleens, lungs and brains. The collected specimens were fixed in 10 % neutral buffered formalin, dehydrated, embedded in parablast and then sectioned. The tissues sectioned slides were stained with different stains, hematoxlyin and eosin (H&E), periodic Acid-Schiff (PAS, for demonstration of the hepatic glycogen) and Gram's stains.

Therapeutic trial:

On 26-01-96, after monitoring the causative agents, therapeutic trial was carried out in the 2nd, 3rd and 4th groups (Table 3). Sulphadimidine sodium (200 mg/kg BW) coupled with 1 gm tannic acid/head, and 1 gm of activated vegetable charcoal/head was applied by oral route for three successive days. Three days later, this treatment was repeated again. On 15-02-96, the therapeutic course was reapplied. The hygienic conditions were corrected. The investigated flock was kept under observation for 6 successive months as a follow up.

Statistical analysis:

The statistical analysis of the obtained data was done by means of a Microstat Computer Program (1984) copyrighted by Ecosoft, INC., USA.

RESULTS

Clinical examinations:

During the period of investigations, 29 (23.58 %) animals of the investigated sheep flock (123 heads) were suddenly succumbed. The morbidity and the case fatality rates were tabulated in Table 1. Of these succumbed animals, 18 cases showed clinical signs before death (Fig. A). The time interval between the observed clinical signs of the diseased animal and death was varied according to age of the affected animal. This time was approximately 6–8 hours in small aged lambs (1.5-3 months) and was about 10-24 hours in young growing animals (>3-7 months-old). No fatalities were observed in the 2 days-3 weeks old lambs or in the adults and aged cases (Fig. B).

The majority of the clinically diseased animals (15 of 18) (83.33 %) showed moderate degree of enteritis with recurrence of unpleasant, reddish watery diarrhea. This diarrhea was pasty in consistency in some cases. The remained cases (3) (16.67 %) of the clinically diseased animals were defecates semi-soft dark pellets surrounded by much amount of viscous mucous secretion mixed with blood or shreds of clotted blood.

Signs of respiratory distress including harried respiration with irregular rhythm of the respiratory cycle were seen on 10 animals (55.56 %) of the clinically diseased animals. Of these animals, 7 cases showed oral breathing, and by auscultation moist gargling sounds were heard on the lower thirds of the lungs areas. The heart sounds were clearly muffled.

Before death, staggering gait while walking (which induced by force) followed by left or right lateral recumbence with pedaling movements of the fore- and the hind limbs with violent muscular contractions in the abdominal muscles and head pressing, were clearly observed in 88.9 % of the clinically diseased animals. These affected cases were unable to get up again, and they appeared as blind animals and the responsiveness to the pupillary light reflex tests was incomplete. The time interval between the beginning of the nervous signs particularly severe convulsions and death was within few minutes (30 ± 12min.). Two cases showed extremely depression (without motor irritability) and dullness till the point at which the affected animal did not response to the external stimuli.

Postmortem examinations:

The postmortem examinations of the freshly dead or emergency slaughtered cases revealed that all examined animals had similar gross lesions that differed only in severity with minor exceptions. These lesions were more severe in the small aged lambs (5-8 weeks old) than others. The intestines particularly the posterior portions of the small intestine (ileum which was clearly distended) were congested and filled with brownish gelatinous fluid containing shreds of clotted blood in the most examined cases (12 of 18). This intestinal fluid was bloody in 6 cases (5-8 weeks old). Severe congestion of the livers, which had mottled appearance, was noticed. The gall bladders were distended with bile secretion and its walls were thickened. The kidneys were severely congested in all examined animals. Forty percent (6 of 15) of the freshly dead animals, their kidneys had, apparently, a normal consistency while the remained cases (9) had soft friable kidneys. Cut sections were made in these softened kidneys and showed whitish gelatinous sticky material was concentrated centrally (renal pelvis). This friability and softening renal lesions were not found in the kidneys of the emergency slaughtered cases. The examined spleens had a normal consistency and appeared to be normal. The lungs (particularly of animals, which showed oral breathings and other respiratory signs) were suffered from congestion and marked edema particularly in the diaphragmatic lobes, which appeared as small plastic bags filled with water and air bubbles. The pericardial sacs in all examined animals were filled with watery fluid. By aspiration, this pericardial fluid was partially clotted forming parts similar to clots of coagulated white-eggs. The brains of the freshly dead or slaughtered animals showed no gross lesions and appeared to be normal.

Direct smears:

The direct microscopic- and the mucosal scraping smears from the intestinal walls of the congested portions of the intestines revealed shorts fattened Gram positive rods with different stages of eimerial gamonts and oocysts, and traces of Gram negative coccobacilli.

Urine analysis:

The collected urine samples showed acidic pH (6 to 6.5) and the presence of glucose (250-500 mg/dl) and protein (30-100 mg/dl) indicating glucosuria and proteinuria.

Hematological examinations:

Results of blood and blood-sugar analysis declared that there were highly significant increase ($p<0.01$) in the PCV and in the levels of blood-glucose (Table 2).

Bacteriological examinations:

The aerobic bacteriological culturing of the collected fecal swabs showed non-significant pathogenic bacteria (proteus spp., citrobacter spp. and pseudomonsa spp.). Conversely the anaerobic bacteriological culturing of the collected intestinal samples (11 freshly dead and 3 slaughtered animals) revealed that the *Cl. perfringens* type D microorganism was isolated from 10 (71.43 %) cases.

Detection and typing of Cl. Perfringens-toxins:

Mice lethality and toxin-antitoxin mice neutralization tests referred to the presence of the lethal toxin (epsilon) of *Cl.. perfringens* type D in all samples of the intestinal contents and in the supernatant solutions of cultures of the isolated strains (toxin-producing types).

Parasitological examinations:

The parasitological examinations of the collected fecal samples revealed the presence of oocysts of coccidia species. The degree of infection was considered severe in the second group (5-8 weeks-old lambs) than the third- and the fourth-groups (Table 3). In the fifth group the coccidial infection was traces (Table 3). No eggs of helminthiasis were found in the collected samples with exception of 3 cases showed few eggs of gastrointestinal nematodiasis.

Histopathological examinations:

The light microscopic examinations of the intestines revealed that the tips of the intestinal villi were necrotic and eroded (Fig.1), and the sub-epithelial blood capillaries and the sub-mucosal vessels were hyperemic. The epithelial cells of the ileum showed different stages of Eimeria gamonts, desquamation of the parasitized cells, severe hyperemia and hemorrhage (Fig.2). Numerous typical shorts fattened Gram positive bacilli mixed with the desquamated epithelial cells were clearly detected (Fig.3). The livers had centrilobular hepatic necrosis and congestion of the sinusoids with numerous apoptotic cells with no inflammatory cellular reactions (Fig.4&5). These livers were completely exhausted from the glycogen contents as indicated by its negativity for PAS reaction (Fig.6). The gall bladder showed cholicystitis with necrotic epithelial cells and intense lymphocytic infiltration with follicular formation. The kidneys showed degeneration and necrosis of the epithelial cells of the proximal convoluted tubules (Fig.7) with edema, congestion and interstitial hemorrhage (Fig.8). The glomeruli were congested with transudation of protein rich plasma in the Bowman's space whereas the collecting renal tubules were filled with protein rich fluid (Fig.8). Kidneys of the slaughtered animals showed no alteration excepting congestion. The spleens were congested with exhaustion and lymphocytolysis of the white bulb (Fig.9). The

lungs showed marked edema, congestion and atelectasis (Fig.10). The brain showed perivascular edema and perivascular accumulation of plasma protein (Fig.11&12). Congestion and perivascular hemorrhage were also obvious in all brain regions (Fig.13). Brain edema was clearly evident in the white matter. The neurons were suffered from chromatolysis, loss of the dendrites and neuronophagia. Neuronal pyknosis with perineuronal edema was also observed (Fig.14). Malacic foci were distributed throughout the brain (Fig. 15&16).

Therapeutic trial:

The morbidity % of the investigated flock was greatly declined (from 14.43 % to 2.10 %) (Table 1) after the first therapeutic trial. This percent reached to zero after the second therapeutic trial and no fatalities were observed thereafter for 6 months follow up (Fig. C).

DISCUSSION

In the present work, the clinical investigations (particularly the shortness time from the beginnings of the clinical signs until death), the macroscopic and the microscopic lesions, and the laboratory examinations, revealed that the disease responsible for the fatalities of sheep in the investigated farm was enterotoxemia caused by *Cl. perfringens* type D.

Sheep normally harbor the *Cl. perfringens* type D organism in their intestines without harmful effect (Mahmoud, 1991). This organism was profusely proliferated by abruptly pathological changes in the intestinal environment of sheep and secreted epsilon toxin, which regarded as enterotoxin and neurotoxin. The latter secreted toxin penetrated the intestinal wall and circulated in the blood damaging the vascular endothelium of the whole body vessels leading to fluid effusion and edema, and subsequently death of the host (Pathak and Parihar, 1991 and Quinn et al., 1994). This may interpret the obtained results concerning the general vascular lesions of the various organs of the dead sheep.

Enterotoxemia caused by *Cl. perfringens* type D was often predisposed by abrupt change in feeding to diet rich in carbohydrates as grains that probably changed the physiology of the intestinal environment and produced a vital chance for over-growth of this organism secreting its toxins (Barker et al., 1996). However, severe ovine enterotoxemia caused by *Cl. perfringens* type D in association with heavy infestation with different types of tap worms (cestodes) was recorded by Orynbaev (1981) and Radionov and Islamov (1988). Furthermore, the migratory stage of *Muellerius capillaris* (nematodes) and fascioliasis due to *Fasciola hepatica* (trematodes) in sheep were also

encountered as predisposing factors enhancing clinical ovine enterotoxemia (Gardinar and Pale, 1960 and Arru and Deiana, 1973, respectively). The latter opinions may reveal that the heavy intestinal parasitism plays a pivotal role in the pathogenesis of ovine enterotoxemia through changing the intestinal environment.

In the present study, according to history taking and the frequent field observations, neither dietary changes nor over-feeding with carbohydrate-rich diet had been noticed. Conversely the amount of the applied grains was apparently low. The obtained results of the mucosal scraping smears from the intestinal wall and the histopathological examinations of the ileum showed severe pathological changes induced by intra- and extracellular eimerial infection and numerous typical shorts fattened Gram positive bacilli. These changes may suggest that heavily coccidiosis altered the intestinal environment of sheep and enhanced the pathogenesis of enterotoxemia through disruption of the mucosal cells and increase the vascular permeability (Pout, 1976). The observed pathological alterations induced by coccidial infection may also increased the facilitates of the absorption of the secreted epsilon toxin from the intestine to the liver through the portal circulation causing hepatic lesions and subsequently "epsilon-toxemia" occurred in the infected host. This opinion may interpret the obtained histopathological lesions of the examined livers (marked centri-lobular hepatic necrosis). Similar picture could not be traced in the available literature. However, severe outbreak of enterotoxemia caused by Cl. perfringens type D in association with heavily coccidiosis in a flock of goats was reported by Uzal et al. (1994).

Clinical examinations of the examined sheep revealed that not all enterotoxemic sheep showed signs of enteritis and respiratory distress but all diseased cases had signs of nervous manifestation (particularly severe convulsions before death). This may suggest that the central nervous system be overwhelmingly affected than other systems. Such suggestion was supported by the histopathological examinations of the brains, which revealed perivascular proteinaceous edema and hemorrhages (vascular lesions) in association with neuronal degeneration progressing to malacic foci (alterative lesions). Similar lesions were reported by Uzal and Kelly (1997) who suggested that the malacic foci in sheep's brain were a characteristic finding to ovine enterotoxemia caused by *Cl. perfringens* type D. The obtained histopathological results also revealed that the brain edema was diffuse and this probably was compressed the optic tracts. Consequently the observed signs of apparently blindness and result of the pupillary light reflex test of the diseased cases immediately before deaths were probably explained.

The histopathological results of the lungs of sheep that showed signs of respiratory distress revealed severe pulmonary edema particularly in the inter-lobular septa, which probably resulted from the action of epsilon toxin on the pulmonary blood vessels. This may interpret the irregularity of the respiratory cycles and marked oral breathing of the infected sheep.

Concerning the ages of the infected animals, Fig. (B) indicated that the percent of infection with the disease was high in the small aged lambs (5-8 weeks old) and then decline with increasing the ages of animals until 6.5 months old. This may reveal that enterotoxemia was related to coccidiosis where the coccidial infection in the 5-8 weeks-old lambs was superior in comparison with the fourth and fifth groups (Table 3). Feeding of the 0-3 weeks-old lambs in the investigated farm was depending completely on the dams' milk (colostral milk) in the first days of their life. The colostral milk contained certain inhibitory substances inhibiting the action of the intestinal trypsin (Kulkarni and Pimpale, 1989) which responsible principally for activation of the epsilon toxin as reported by Pathak and Parihar (1994). The latter authors concluded that the epsilon toxin was secreted as a prototoxin (inactive form) and was converted into the active toxin in the duodenum under the effect of the digestive enzymes particularly trypsin. This may explain the absence of clinical enterotoxemia in the neonatal lambs. On the other side, the coccidial infection in the 7.5 months-old and aged sheep was traces and they were appeared as healthy sheep. Clinical coccidiosis was chiefly confined to young sheep up to four and six months of age and the older animals being immune (Soulsby, 1982). This may give an account for the absence of the fatalities in the sheep of the fifth group (Table 3), and this may also be proved that the heavy coccidial infection play a vital role in the pathogenesis of enterotoxemia.

From hematological point of view, the obtained results listed in Table 3 revealed that the value of the PCV was significantly increased and the erythrocyctic count was relatively increased in the positive enterotoxemic cases indicating hemoconcentration. This was probably due to loss of fluid from the blood into tissues (pulmonary and brain edema) and cavities (pericardial and peritoneal serous effusion) through the injurious action of the circulating epsilon toxin on the endothelial cells of the blood vessels increasing the vascular permeability (Pathak and Parihar, 1991 and Ual and Kelly, 1997). Table 3 also showed that the enterotoxemic cases were hyperglycemic. Such finding was supported by the results of histopathological studies which revealed that the examined livers of those animals were free from glycogen contents as demonstrated by negative PAS reaction. Consequently the hyperglycemic state

was due to depletion of the glycogen contents from the livers, which possibly stimulated by hepatocyte-bound epsilon toxin (Barker et al., 1996). Other possible account for hyperglycemia in ovine enterotoxemia caused by *Cl. perfringens* type D was described by Worthington et al. (1979) who concluded that rapidly developing brain edema caused large increases in catecholamine which activate the adenyle cyclase. The latter elevate the production of the cAMP, which stimulates glycogenolysis leading to hyperglycemia. Brain edema was evident in our histopathological results. The level of blood-glucose was highest and spilled into the urine and this may interpret the results of glucosuria of enterotoxemic cases.

Microscopically, renal degenerative changes (nephrosis) with interlobular hemorrhages and edema were clearly observed in the examined kidneys of the dead animals whereas no pathological alterations could detected in the kidneys of the emergency slaughtered cases excepting congestion. This probably indicates that the observed lesions were due to autolysis. The renal degenerative changes increased the vascular permeability of the glomerular capillaries leading to shedding of protein into the urine. Consequently the results of proteinuria of the enterotoxemic cases were explained.

Destruction of the predisposing factor (coccidial infection) that probably stimulated the quiescent normal bowel inhabita nt bacteria (*Cl. perfringens* type D) in sheep to proliferate and produce the disease (enterotoxemia), was the fundamental purpose of the applied therapeutic trials. Coccidia species were sensitive to sulphadimidine drug (Soulsby, 1982) and the therapeutic trials revealed that the morbidity %, and subsequently fatality %, of the disease was reduced to zero and no fatalities were recorded during 6 months follow up (Fig C). This may refer to that heavily coccidiosis was act as predisposing factor to proliferation of *Cl. perfringens* type D.

The field observations and the statistical analysis (Table 1 and Fig. C) showed that within 38 days (from 27-11-95 to 4-1-96), 12 cases were succumbed (with average 0.31 case per day). Thereafter, on 19/1/96, high doses of Fasciolid drug was injected, and six days later post injection, another 14 animals died (with average 2.3 cases per day). This probably reveals that the overdoses of the inoculated anthelmintic drug increased the susceptibility of sheep to the disease. No accounts could be obtained in the available literature and the authors could not interpret the possible reason for this point. However, similar observation was reported by Uzal et al (1994) who encountered the overdoses of anthelmintic drugs were one of the predisposing factor of caprine enterotoxemia caused by *Cl. perfringens* types D. Concerning nitroxynil, Bineva et al. (1980) reported that although anthelmintic Dovenix (nitroxynil)

had no effect on the proliferation of *Cl. perfringens* types C and D, it increased the susceptibility of animals to infection. They also reported that some sheep were succumbed following inoculation of a normally sublethal dose of *Cl. perfringens* type C culture at the same time as s/c injection of this drug at 10 mg/kg body weight.

In conclusion, enterotoxemia caused by *Cl. perfringens* type D can possibly be considered as opportunistic disease of sheep depending principally on the predisposing factors that stimulate the quiescent organism, which located normally in the intestine of sheep to proliferate and produce its toxins. Heavily infection with coccidiosis can possibly be considered as one of the major predisposing factors of ovine entertoxemia. It is probable that the overdoses of anthelmintic drug act as a major and/or minor predisposing factor to the pathogenesis of that disease. For the latter note experimental research work is noticeable.

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LEGENDS

Fig. 1: Ileum showing severe hyperemia of the propial and submucosal blood vessels and denuded villi (H&E, x 132)

Fig. 2: Higher magnification of Fig. 1 to demonstrate coccidial stages in the enterocytes (↑), notice denuded villi and severe hyperemia (H&E, x 1320).

Fig. 3: Intestine showing Gram positive bacilli mixed with desquamated epithelial cells, denuded villi (↑) (Gram stain x 3300).

Fig. 4: Liver showing centrilobular hepatic necrosis (H&E, x 132).

Fig. 5: Higher magnification of Fig. 4 to demonstrate apoptosis of the hepatic cells around the central vein (H&E, x 1320).

Fig. 6: Liver showing completely negative PAS reaction in the hepatocytes, notice reaction in the epithelial cells of the bile ducts (PAS stain, x 250).

Fig. 7: Kidney showing nephrosis of the proximal convoluted tubules and leakage of plasma proteins into the Bowman's space (H&E) x 1320).

Fig. 8: Kidney; medullary area showing intertúbular hemorrhage, and engorgement of the collecting tubules with protein rich fluid (H&E, 250).

Fig. 9: Spleen showing depletion and lymphocytosis of the white pulp (H&E, 528).

Fig. 10: Lung showing congestion, alveolar and interlobular edema (H&E, 528).

Fig. 11: Brain showing perivascular edema and collapse of the blood vessels (H&E,250).

Fig. 12: Brain showing perivascular protein rich plasma (H&E, 330).

Fig. 13: Mid brain showing severe edema and hemorrhage, chromatolysis and perineuronal edema (↑) (H&E, x 250).

Fig. 14: Brain stem showing severe edema of the white matter, neuronal pyknosis and perineuronal edem (H&E, x 250).

Fig. 15: Cerebrum showing malacic foci (H&E, x 250).

Fig. 16: Brain stem showing edema of the white matter and focal malacia (H&E, x 160).

Table 1: The morbidity % and the case fatality % of the diseased sheep.

Visit	Date	Nr. of sheep	Nr. of the adults	Nr. of the lambs	Nr. of the diseased cases	Nr. of the dead cases@	Morbidity %	Case fatality %
0	25-11-95	123	54	69	0	0	0	0
0	27-11-95	119	54	65	4	4	3.36	100
1st	12-12-95	116	54	62	3	3	2.59	100
2nd	04-01-96	111	54	57	5	5	4.50	100
3rd	25-01-96	97	54	43	14*	14*	14.43	100
4th	15-02-96	95	54	41	2	2	2.10	100
5th	07-03-96	94	54	40	1	1	1.03	100

N.B.: Before the thrid visit (shadow row) the owner inoculated the sheep with higher doses (about 40 - 50 mg / kg BW) of "Fasciolid" (nitroxynil) 25 % (Chemical Industries Development, Giza - A.R.E. under license of Phrmachium-Bulgaria).

@: Epsilon toxin was identified from the intestinal contents.

+: Three younger sheep of these cases were slaughtered due to severe neurological signs.

*: Significant ($p<0.05$).

Table 2: Blood and blood-serum analysis of the positive enterotoxemic sheep and control cases.

Parameters	Positive cases (n = 7) X ± S.D.	Control cases (n = 6) X ± S.D.
RBCs	$10.92 \pm 2.00 \times 10^6$ Cmm^3	$09.73 \pm 1.8 \times 10^6$ Cmm^3
WBCs	$11.95 \pm 2.81 \times 10^3$ Cmm^3	$12.80 \pm 3.0 \times 10^3$ Cmm^3
PCV	48.50 ± 1.0 %**	37.50 ±5.00 %
Glucose	19.72 ± 0.5 mmol/L**	02.10 ±0.45 mmol/L

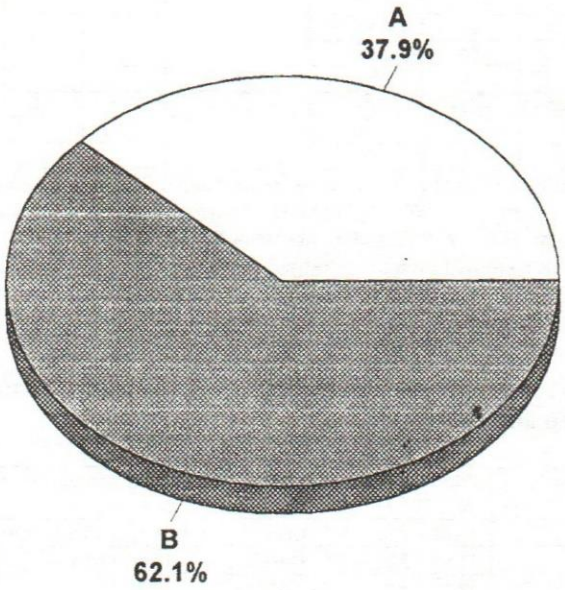
**: Highly significant ($P<0.01$)

Table 3: Age of the diseased cases, degree of coccidial infection and Nr. of the dead Cases.

Groups	Age (weeks)	Nr of the examined sheep	Degree of coccidial infection	Nr of the dead cases (enterotoxemia)
I	0-3	9	(-)	0
II	5-8	30	(+++)	22 (73.33%)**
III	9-13	18	(++)	5 (27.78%)
IV	15-26	12	(+)	2 (16.67)
V	>30	54	(-)/ traces	0

**: Highly significant ($P<0.01$).

Fig. (A): Shows that 11 (37.9 %) cases of the dead sheep died without obvious signs and 18 (62.1 %) cases showed clinical signs before death>



A- Animals died without obvious clinical signs
B- Animals showed clinical signs before death

Fig. (B): shows % of infection with enterotoxemia and age of the animal

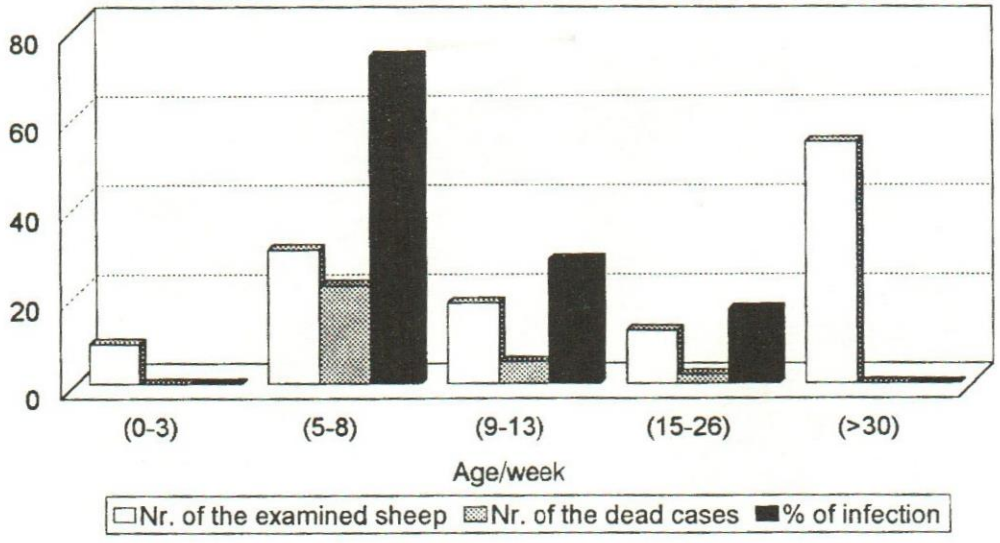
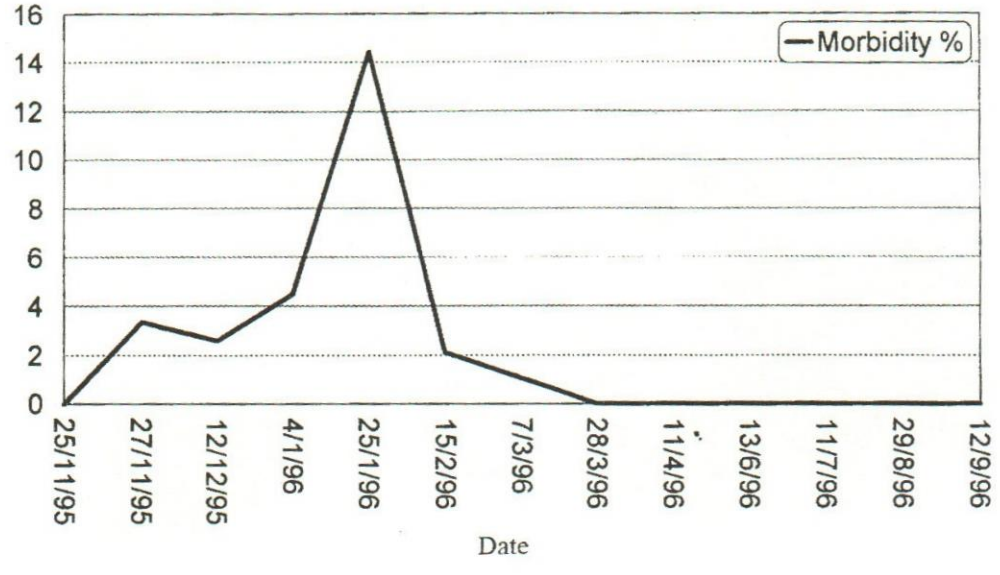
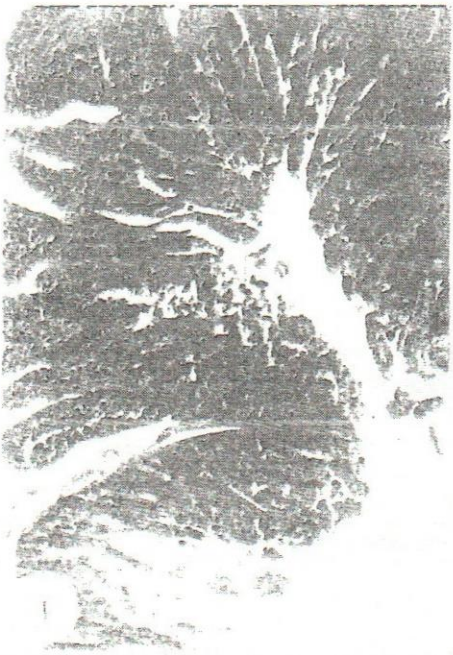
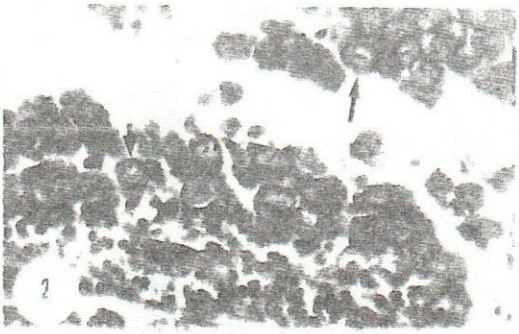
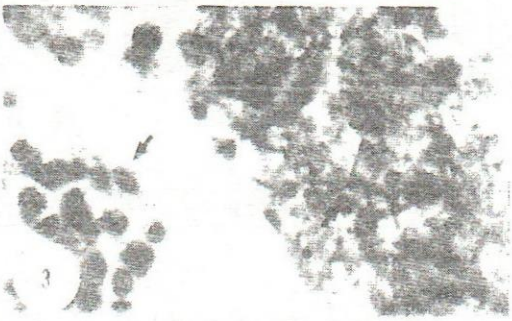


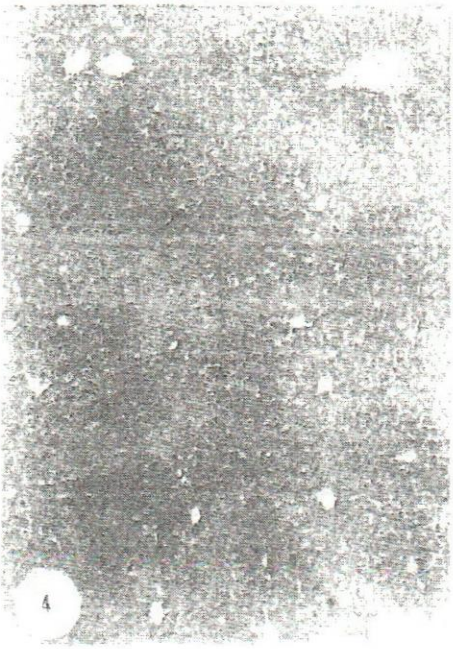
Fig. (C): Shows the morbidity % of the investigated flock during the periods of investigation and follow up

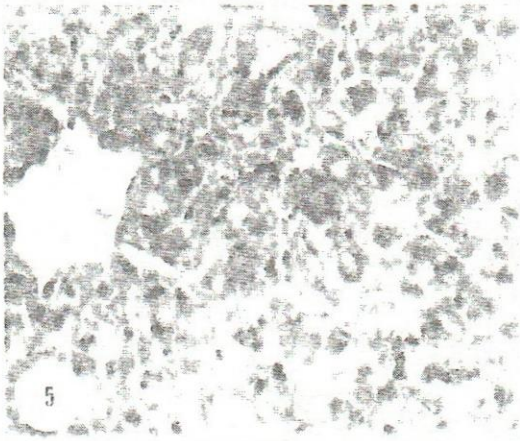


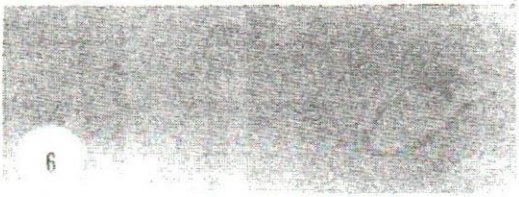


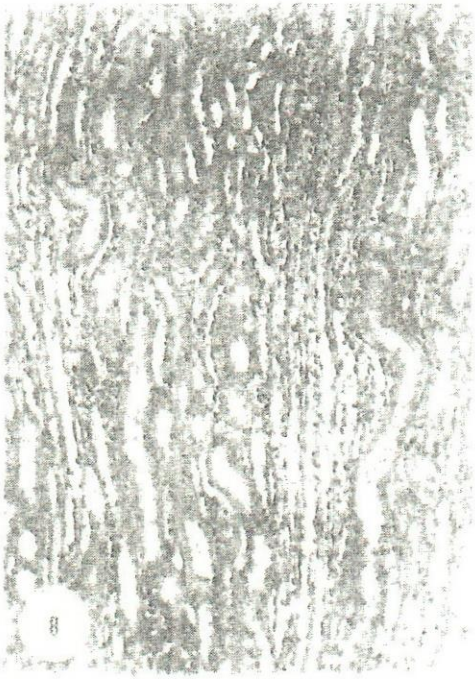


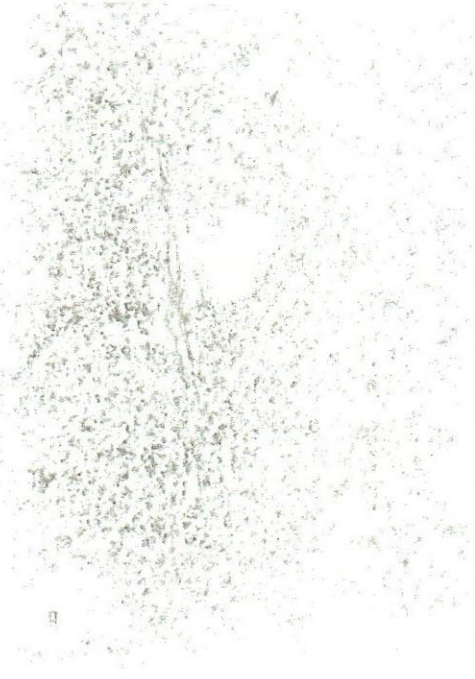


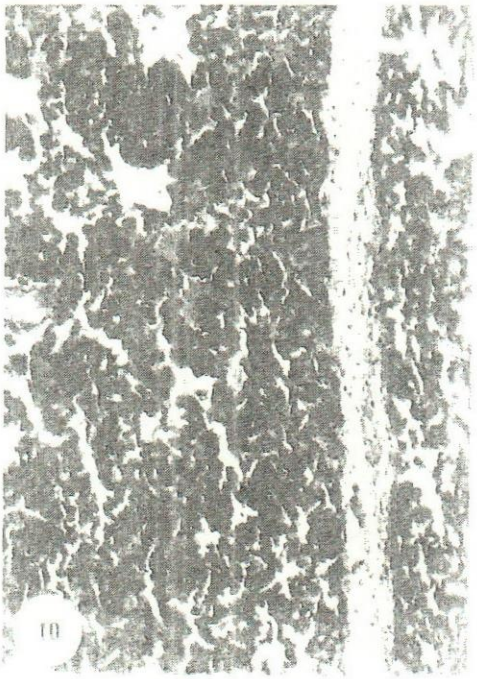


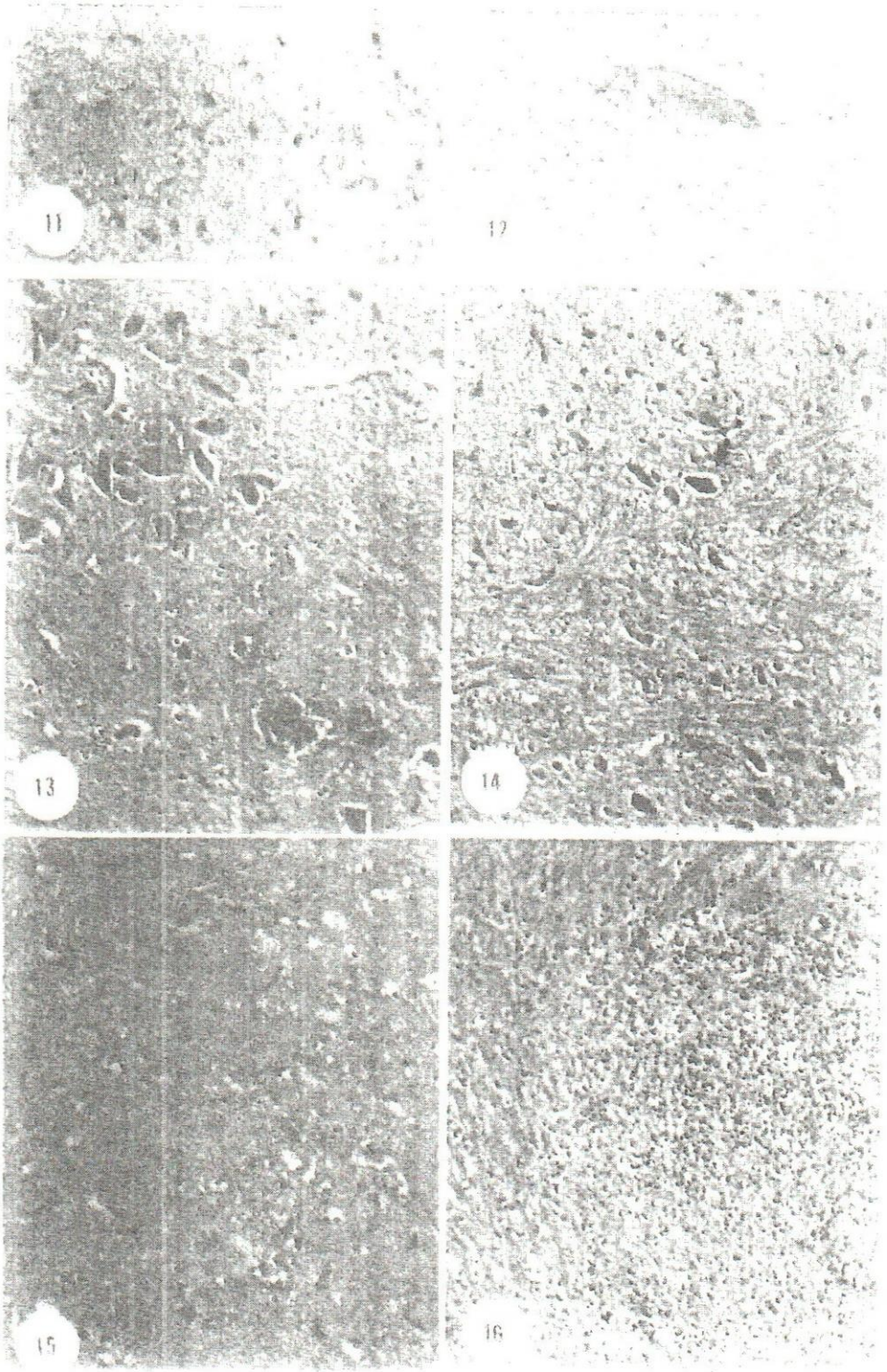








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