Hydrocephalus in Neonatal Rabbits Caused by Reovirus

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SUMMARY

Reovirus was isolated from commercial rabbit colonies in farms at Kafr-Elsheikh and El-Gharbia Governorate with a history of neonatal hydrocephalus and blindness. The affected neonates exhibited hydrocephalus at age from 3-20 days with seasonal incidence at a late summer and early autumn (August, Sept. and Oct.). The gross lesions observed at necropsy were bulging of the skull, collapse of the cerebral hemispheres and replacement of the parenchyma by colorless, transparent cerebrospinal fluid (CSF).

Virus isolation was conducted through chorio-allantoic membrane (CAM) of embryonated chicken eggs. Identification of the virus by electron microscope revealed presence of virion particles having a morphological appearance of Reovirus. Serological tests were also conducted. The pathogenicity of isolates was studied at different ages of rabbits. Histopathological examination of naturally and experimentally infected rabbits revealed marked loss of neurons, severe oedema with dilatation of brain ventricles, degeneration and desquamation of ependymal cells, Eosinophilic intracytoplasmic inclusion bodies were formed in infected CAM of embryonated chicken eggs. This is the first report of isolation of reovirus from neonatal rabbit affected with hydrocephalus.

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INTRODUCTION

Hydrocephalus is a form of oedema in central nervous system and refers to the slow accumulation of excess cerebrospinal fluid (CSF) within the ventricular system (internal hydrocephalus) or within the subarachnoid space (external hydrocephalus) (Jones and Koestner, 1997).

Hydrocephalus was reported as an inherited abnormality or congenital deformity among newborn rabbits (Lieve, 1997 and Sandford, 1988). Pond et al. (1995) reported that signs of vitamin A
deficiency and toxicity are similar with major effects on reproduction, low conception rates, fetal resorption, low survival of newborn kits and hydrocephalus in fetuses occur with toxic level. On the other hand Abd El-Raheem et al. (2000) attributed neonatal hydrocephalus in rabbits to vitamin A deficiency in their does, while Fetah et al. (2001) recorded that ochratoxin A has adverse effects on reproductiveivity of rabbits through inducing stillbirth, abortion and teratogenic lesions in the offspring particularly hydrocephalus.

Some Reovirus strains may cause abortion and congenital abnormalities including hydrocephaly, ataxia and cerebral hypoplasia in sheep and cattle (Fenner et al., 1993 and Murphy et al., 1999). Nathanson et al. (1997) proved that intracerebral inoculation of Reovirus into newborn mice, infects ependymal cells lining cerebral ventricles resulting in hydrocephalus.

The present study aimed to investigate the viral etiology of hydrocephalus in neonate rabbits and its related pathological changes.

**MATERIALS AND METHODS**

**Animals**

A total of 12 rabbit’s colonies in farms at Kafer El-Sheikh and El-Garbia Governorate were investigated during the period of 1999-2004. The newly born rabbits of 3-20 days of age with seasonal incidence at late summer and early autumn (august, Sept., Oct.) exhibited hydrocephalus with blindness.

Post-mortem examination was conducted and gross lesions were recorded CSF was aspirated aseptically and also brain tissues were collected and processed under aseptic conditions for virus isolation trials.

Fertile chicken eggs obtained from private farms and Sakha governmental poultry farm were used for virus isolation, propagation, titration and identification.

**Virus isolation:**

Embryonated chicken eggs 9-12 days-old were inoculated through CAM with 0.2ml/egg of suspected suspension treated with antibiotic, daily candling of eggs for 7 days, deaths recorded within the first 24 hours post-inoculation were excluded as nonspecific, after that embryos died were examined, embryonic fluids and CAM were harvested for further passages. Five blind passages were carried out before titration and identification of the isolates.

Haemagglutination activity of isolated virus was studied through human (O) type erythrocytes at room temperature according to Madbouly (2003).
Avian Reovirus positive control serum:
This was obtained from KPL proflok® Kirkegaard & Perry Laboratories USA (VET LIC. No. 350), for serological studies.

Serological examination:
Serum samples were collected from rabbit does whose progeny exhibited hydrocephalus and also serum samples were collected from the progeny which suffered from hydrocephalus for estimation of antibodies against toxoplasma. (TOXO– HAI, Lab. FUMOUZE-France) As well as Agar gel precipitation test (AGP) and virus neutralization test for Reovirus according to methods described by Beard (1989) were carried on.

Transmission Electron Microscopic Examination:
Amnio-allantoic fluid and oedematous chorio-allantoic membranes with clear multiple pock lesions were collected after five blind passages, and stored at –20ºC then ground and homogenized in a sterile mortar, after that freezing and thawing procedure was repeated three cycles, sedimentation of coarse particles by centrifugation at 3000 rpm for 30 min., then the supernatant fluid was collected and checked for sterility to be free bacteriologically.

Ultra centrifugation for supernatant fluid through sucrose cushion to deposit the virions at 30,000 rpm for 30 min. was done (twice). The supernatant was decanted and the sediment was stained with uranyl acetate then the copper grid was coated. Examination and electron micrograph were made using TEM ZEISS EM10 (Germany) at HT 60 kv.

Pathogenicity tests
1- Twenty one-day old New Zealand rabbits from flocks with no history of hydrocephalus were used for testing the pathogenicity of Reovirus isolates, these rabbits were allotted into four groups (A, B, C and D) five in each and kept in a separate unit. Group A, B and C were orally, intramuscularly and intracerebrally infected respectively with 0.2 ml / rabbit of Reovirus isolate containing 10^6 / ml, while group D was used as a control. All infected and control rabbits were observed daily for a period of 20 days for clinical signs and mortality.

2- Five pregnant New Zealand rabbit does, at 10 days of gestation period were experimentally inoculated by intramuscular route with 0.4 ml of Reovirus isolate containing 10^6 /ml, while two pregnant rabbit does were used as a control. All rabbits were observed until parturition and daily clinical examination for newly born rabbits for a period of 15 days was done.
Histopathological examination:

Tissue specimens were collected from the brain of both naturally and experimentally infected neonatal rabbits as well as chorio-allantoic membranes showing pock lesions from embryonated chicken eggs inoculated with the isolated *Reovirus*. Tissue samples were fixed in 10% neutral buffered formalin and then processed routinely for paraffin embedding techniques, embedded tissues were sectioned at 4-6 microns thickness and stained with haematoxylin and eosin (H & E) and examined microscopically according to Bancroft *et al.* (1996).

RESULTS

Clinical and post mortem examination:

The newly born rabbits suffering from congenital developmental abnormalities exhibited hydrocephalus with bulging of the head dorsally (Fig. 1), and blindness as well as nervous manifestation in the form of incoordination, torticollis, circling and inability to stand or walk poorly were observed. The affected rabbits still alive after 15 days showed severe emaciation and poor body condition

Post-mortem examination of hydrocephalic rabbits showed obviously that the brain ventricles were severely distended with serous transparent fluid and the cerebrum became a sac filled with fluid. After aspiration of CSF, the cerebral hemispheres were found collapsed and cerebral parenchyma was limited to a thin layer. In addition to blindness of hydrocephalic rabbits no characteristic lesions were observed in the other organs.

Virus isolation and titration.

The inoculated chicken embryos died 4-6 days post-inoculation, showed subcutaneous hemorrhages and CAM was edematous, hemorrhagic with characteristic pock lesions (Fig. 2). Embryos died after 7 days showed stunted growth, greenish discoloration of the liver with development of necrotic foci (Fig. 3). The isolated virus agglutinates human "O" type erythrocyte rapidly within one minute at room temp. and the titer of isolated virus was $10^6$ / ml (ELD, 50).

Serological examination:

The results of serological examination of serum samples from does and hydrocephalic neonates were negative for toxoplasmosis, and negative in agar gel precipitation (AGP) test for avian *Reovirus*, Furthermore, the virus was not neutralized by specific positive avian *Reovirus* serum.

Transmission electron microscope examination:

Negative staining of the electron microscope revealed that the viral particles were visualized as
bright objects against a dark background. The isolated virion were non-enveloped, nearly spherical in outline with icosahedral symmetry. The capsid was characteristic-ally double–shelled and the viruses occurred as single or double capsid particles about 80 nm in diameter (Fig. 4).

**Pathogenicity tests and Histopathology:**

Experimental oral infection in suckling neonates rabbits with isolated *reovirus* resulted in severe diarrhea and retarded growth without neurological signs, mortality was 60% and the main lesions were catarrhal enteritis. Meanwhile, intramuscular infection showed mild diarrhea and mild nervous manifestation that include drowsiness, in-coordination and tremors. Mortality was 40%, and the main lesions were enteritis and the cut section through the cerebral hemisphere showed varying degree of edema.

Intracerebral infection in suckling rabbits exhibited neurological signs rapidly at 6 days post-infection in the form of in-coordination, tremors, torticollis, spasmodic convulsion and hyper-excitation. Mortality was 60%, and the main lesions were mild enlargement of skull after 15 days post infection. The brain tissue was markedly edematous with prominent dilation of brain ventricles.

Experimental I/M inoculation of pregnant does didn't induce any abnormal signs or behavior, and although their progenies were free from congenital developmental abnormalities, severe diarrhea was observed at 7-10 days of age with 80% mortality of neonates.

Naturally infected neonates showed severe atrophy of nervous tissue. Most of the neurons were completely absent and cerebral tissues were represented by edematous vacuolated neuropil and remnants of granular cell layer (Figs. 5 & 6).

In experimentally infected newborn rabbits, the brain cortex showed prominent neuronal degeneration and necrosis represented by condensed deeply eosinophilic cells with loss of demarcation between nucleus and cytoplasm (Fig.7). The neuronal changes seemed to begin with perineuronal edema especially the Purkinje cells (Fig 8A, 8B). Perivascular edema was constant findings in both cortex and medulla (Fig. 8C), and the latter showed marked vacuolation or status spongiosis (Fig. 9). Focal gliosis and neuronophagia were occasionally detected (Fig. 10). The brain ventricles were severely dilated with focal degeneration, necrosis and desquamation of ependymal cells with focal gliosis of adjacent nervous tissue (Fig. 11). Focal meningial hemorrhages were occasi-
onally observed in subarachnoid space of cerebral cortex (Fig.12).

Microscopical examination of CAM of embryonated chicken eggs inoculated with isolated virus revealed the presences of poorly demarcated eosinophilic intra-cytoplasmic inclusion bodies (Fig. 13).

Figure (1) Hydrocephalus in 4 days old rabbit. Notice bulged head.

Figure (2) CAM of embryonated chicken eggs 7 days post-inoculation showing focal pock lesions.
Figure (3) Chicken embryo 7 DPI with the isolated reovirus showing greenish discoloration of liver with necrotic foci.

Figure (4) Electron micrograph of negative stained reovirus (X40,000).

Figure (5) Cerebral cortex of naturally infected hydrocephalus newborn rabbit showing loss of neuron with pericellular edema around remaining cells (H&E X200).

Figure (6) Cerebral cortex of naturally infected hydrocephalus newborn rabbit showing marked edema and vacuolation (H&E X100).
Figure (7) Cerebral cortex of experimentally infected newborn rabbit showing neuronal degeneration and necrosis (H&E X200).

Figure (8) Cerebral cortex of experimentally infected newborn rabbit showing neuronal degeneration and perineuronal edema (A&B), perivascular edema (C) (H&E X200).

Figure (9) Cerebral medulla of experimentally infected newborn rabbit showing marked vacuolation (status spongiosis) (H & E X 100).
Figure (10) Cerebral cortex of experimentally infected newborn rabbit showing marked focal gliosis in edematous area adjacent to brain ventricle (H & E X 100).

Figure (11) Brain ventricle of experimentally infected newborn rabbit showing dilatation with focal degeneration, necrosis and desquamation of ependymal cells as well as focal gliosis in adjacent tissue (H&E X 100).
DISCUSSION

In the present study, the clinical signs, lesions of naturally infected newly born rabbits, isolation of the viral agent in embryonated chicken eggs, pathogenicity tests and Histopathological findings as well as detection of Reovirus particles by transmission electron microscope, confirm the association of Reovirus with hydrocephalus in neonatal suckling rabbits.

The epidemiology and seasonal incidence of the disease at late summer and early autumn (August, Sep and Oct.) was not fully documented. However many mammalian Reoviruses are transmitted by arthropods and their epidemiology depends on interaction between each of the following host, vector, climate and the virus, which may clarify the common occurrence of this case in the late summer where the vector are numerous. Some Reovirus strains may cause abortion and an epidemic of congenital abnormalities in sheep and cattle characterized by hydranencephaly.
and cerebral hypoplasia, (Fenner et al., 1993 and Murphy et al., 1999).

Mammalian Reovirus exhibited different degree of neurotropism in suckling mice, but restricted to newborn, (Flamand et al., 1991). The pathogeneses of hydrocephalus observed in the present study could be explained as a results of sloughing of infected ependymal cells in brain ventricles with subsequent obstruction of the aqueduct of Sylvius as well as blockage of cerebrospinal fluid outflow from the fourth ventricle. The neuronal changes detected could be attributed in part to mechanical pressure exerted by accumulated CSF in brain ventricle (specially in naturally infected cases) as well as to actual infection by the viral agent as it had been reported that reovirus has a tropism to both ependymal cells producing hydrocephalus and/or neurons producing meningo-encephalitis in mice (Van der 1977).

Similar opinion have been suggested by Nathanson et al., (1997) who found that in Reovirus infect ependymal cells lining the cerebral ventricle in newborn. Serological techniques including AGP test and virus neutralization test between isolated Reovirus and specific avian Reovirus serum revealed that no serological cross reaction between mammalian and avian Reovirus strains, this may be attributed to that avian Reoviruses possess a group-specific antigen discernable with gel diffusion techniques and a serotype specific antigen demonstrable with neutralizing antibody in plaque reduction or chicken embryo assays (Van der, 1977 and Rosenberger and Olson, 1977).

Absence of clinical signs and lesions in experimentally infected pregnant Does could be returned to the age related susceptibility differences of the mammalian reovirus as have been reported by Flamand, et al. (1991). Conrat et al. (1988) also reported that mammalian reovirus exhibits different degree of neurotropism in suckling mice, but restricted to newborn.

Experimental infection of newborn rabbits induced brain oedema with prominent dilatation of brain ventricles, resulting in varying degrees of cavitations of cerebral hemispheres but not fully predominnate hydrocephalus. Hydrocephalus in newborn rabbits from experimentally infected pregnant does was absence. This may be attributed to multiple etiologies inducing congenital hydrocephalus. These factors may includes vitamin A deficiency and toxicity (Pond, 1995), vitamin A deficiency (Abd El-Raheem et al., 2000) and intoxication with Ochratoxin A (Fetaih et al., 2001). In addition Dellepiane (1990) and Benko (1991) recorded hydroceph-
alas in rabbits due to encephalitozoonosis, Toxoplasmosis and Listeriosis. Moreover Jubb and Huxtable (1993) mentioned that the inducing factors of congenital hydrocephalus are usually obscure. Meanwhile Saif (1992) reported that immuno-suppressive agents have been shown to exacerbate the pathogenesis of Reoviruses in chickens.

Conclusions

It could be concluded that Reovirus may be acting in association with other factors (nutritional factors, immunosuppressive agents, vectors and climate) in induction of neonatal hydrocephalus in rabbits. According to available literature this is the first report of isolation of reovirus from neonatal rabbit affected with hydrocephalus in Egypt. Further studies are required to clarify more about this infection in rabbits.

REFERENCES


الملخص العربي

تم إجراء هذه الدراسة على بعض مزارع الأرانب في محافظتي كفر الشيخ والغربية بعد ملاحظة ظهور الأعراض على صغار الأرانب من عمر عشرون يوم حتى ثلاثة أشهر على شك عمي كلي وتضخم الرأس المائي. تركزت الإصابات في شهور الصيف من عام 1999 وتكررت في الأعوام التالية وحتى الآن. تم عزل فيروس الريو من جميع الحالات في أجنحة ببعض الدجاج المخصب وتم إجراء عدء تجريبي بالفيروس المعزول في أمضات أرانب حاملة وعلى صغار الأرانب. حدد نفس الإصابات وتم التعرف على الفيروس بالفحص الميكربيوة الكهروإلكتروني والفحص النسيجي للأرانب المصابة نتائج احصائية وتعريبيا وأغشية الجنينية لأجنحة الدجاج. وشملت النتائج وجود استحالة مرشدية، واحتقان وكتكز خلوي في الخلايا والأنسجة العصبية. تم الاستدلال على وجود أجسام احتوائية في الخلايا المصابة في الأغشية الجنينية لأجنحة. وقد خلصت الدراسة إلى وجود ارتباط وثيق بين حدوث الإصابات والعدو بفيروس الريو إن لم يكن هو المسبب الوحيد لها إلا أن ذلك يحتاج لمزيد من الدراسة.

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