EFFECT OF SOME VITAMINS IN REDUCING THE PATHOLOGICAL AND BIOCHEMICAL CHANGES IN RABBIT EXPERIMENTALLY INFECTED BY RABBIT HEMORRHAGIC DISEASE VIRUS

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Abstract

Forty white New Zeland rabbit were divided into 5 equal groups to study the effects of some vitamins (A,E and C) on the pathological alteration of rabbit experimentally infected by liver suspension from rabbits which previously infected by RHDV and recently died and rabbits vaccinated against RHDV and experimentally infected by liver suspension. (G 1) was the control group. (Gs 2, 3 and 4) were administrated vitamin A, in a dose 10000IU/L, vit. E in a dose 500 IU/L and vit. C in a dose 1000IU/L, once daily for 15 days. (G 3) was vaccinated against RHDV and after 15 days from the vaccination, rabbits were experimentally inoculated by liver suspension. (G4) was experimentally inoculated by liver suspension after 15 days from administration of the vitamins. (G5) was experimentally inoculated by liver suspension (positive control). Whole blood and serum samples were collected 36, 48 hr. after the onset of the clinical signs and at the end of the experimental period, for biochemical and virological investigations. Also necropsy and tissue specimens were collected from liver, lungs, spleen, kidneys and heart. The specimens were fixed in 10% neutral buffered formalin for examined microscopically.

Relative improvement in coagulation parameters and serum biochemical analysis in rabbit experimentally infected with RHD virus and supplemented with vitamins (G4) exhibited minimize time of prothrombin, coagulation, bleeding and thrombocyte count compared with group which infected with RHD virus without supplemented vitamins (G 5), meanwhile rabbits in group supplemented with vitamins infected with RHD virus maintained a good general health status compared with group infected without given vitamins reflected by lowering liver functions (AST, ALT, GGT, total bilirubin, glucose), kidney function (urea and creatinine) total protein and albumin.

The histopathological finding of RHD in liver revealed Coagulative necrosis and apoptosis of hepatocytes in addition to proliferation of inflammatory cells. Lungs showed hemorrhage together with focal mononuclear cell infiltration and edema. Spleen showed depletion and necrosis of lymphocytes. Kidneys showed microthrmbi in

renal blood vessel. Hemorrhage and necrosis of renal tubules. The vitamin supplemented groups showed a marked improvement in the most pathological and biochemical changes. It could be concluded that the vitamins ameliorate the dangerous effect induced by RHDV.

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) is an icosahedral, non-enveloped, positive-sense, single-stranded RNA virus within the genus *Lagovirus,* family *Caliciviridae* (Jae et al., 2009). Rabbit hemorrhagic disease (RHDV) was first reported in China in 1984 and rabidly became endemic in most parts of the world. Rabbit hemorrhagic disease virus differentiated into many strains, all highly virulent and with a close genetic relationship (Fauquet et al., 2005). Rabbit hemorrhagic disease virus is an acute or per acute hepatitis of rabbits that is lethal in up to 90% of infections of rabbits older than 8 weeks of age, severe losses are common in unvaccinated animals, on some farms most or all of the rabbit may die (Peter et al., 2009). This disease has also caused dramatic declines in some wild rabbit populations, particularly when it is first introduced. Rabbit hemorrhagic disease spreads very rapidly. The causative virus is very resistant to inactivation if it is protected by organic material, it may persist in chilled or frozen rabbit meat, as well as in decomposing carcasses in the environment, for months (Campagnolo et al., 2003). RHDV in animal tissues such as rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental condition, such as dried excreted virus, is viable for a period of less than 1month (Henning et al., 2005). Due to the rapid course of the disease, the animals usually found in good condition after death. Gross pathological lesions were variable and may include circulatory and degenerative disorders. Liver necrosis and splenomegaly are the primary lesions, the liver usually appears yellowish brown in color, brittle and degenerated, with a marked lobular pattern. The tracheal mucosa hyperemic, containing abundant frothy fluid and the lungs are mottled hemorrhagic and edematous. The spleen enlarged, with rounded edges of an enlarged (splenomegaly). The presence of clotted blood in blood vessels was due to disseminated intravascular coagulation. Such massive coagulopathy was usually the cause of hemorrhages in a variety of organs and sudden death (Brown and Torres, 2008 and Elie et al., 2010). The aim of the present work was directed to study the pathological, biochemical and virological alteration induced by the experimentally

infected RHDV, and to determine the efficacy of some vitamins supplementation in reducing the harmful effect of RHDV.

MATERIALS AND METHODS

Experimental Design:-

Forty white New Zeland rabbits, two months age old weighting from 1 kg to 1.5 kg were randomly divided into 5 equal groups. The rabbits were obtained from private farm with no history of RHDV outbreak and no clinical signs to any other diseases or vaccination against RHDV. The rabbits were kept in stainless steel wire mesh cages under sanitary hygienic condition and fed on balanced commercial pellets and provided with water ad-libitum during the experimental period. Nasal and anal swabs were collected from rabbits to confirm that they were free from any pathogenic bacteria. The rabbits were kept for two weeks without treatment for adaptation. The rabbit groups were, group (1) kept as control (negative control). Groups (2,3and 4), were given vitamins A,E and C at a dose 10000IU/L (Chew, 1987, Sayed and Abd-Elghaffar, 1999), 500 IU/L (Franchini, et al.1991, Sayed and Abd-Elghaffar, 1999) and 1000 IU/L (Lovell, 1989, Beisal, 1982, Sayed and Abd-Elghaffar,1999) respectively in drinking water once daily for 15 days. After 15 days from giving the vitamins, (G3) was vaccinated against RHDV by the local strain obtained from (Vet.Ser& Vacc. Res. Inst. Abbassia-Cairo) and then infected by 1ml/rabbit intramuscularly from liver homogenate suspension previously prepared containing (10 log 2) after 15 days from giving the vaccine. (G4) was infected by 1ml/ intramuscularly from the liver homogenate suspension after 15 days from giving the vitamins. (G5) was experimentally infected by 1ml/rabbit intramuscularly liver homogenate suspension (positive control).

Liver suspension preparation *(Fernando et al., 2010):-* 10%Bacterial free suspensions of liver (+ve for RHDV by post mortem examination and with HA human group O with give 2^{10}) homogenate at concentration of 1:10 (w/v) in saline for antigen preparation. The liver homogenates were frozen and thawed 3 times and finally clarified by centrifugation at 3000 r.p.m for 5 minutes. The supernatant fluid was used as antigen for experimental infection, stored at -20°c until used.

Vaccine: - From (Vet.Ser& Vacc. Res. Inst. Abbassia-Cairo) Sera 0.5 ml, sub cut in neck/rabbit.

Blood sampling:- Blood samples were collected from ear vein of rabbit of each ach group to obtained clear serum for biochemical analysis Platelets count, coagulation time, bleeding time and prothrombin time were determined according to (Coles,1986, Biggs and Macferland., 1962).

Serum biochemical analysis: - Serum samples were subjected to biochemical analysis to determined AST and ALT (Reitman and Frankels, 1957), GGT(Szaz, 1969), glucose (Trinder, 1969), total bilirubin (Monnet, 1960), urea (Faweet and Scott, 1960), creatinine (Henary,1979), total proteins (Weichselbaum, 1946), albumin (Drupt, 1974) and globulin (Doumas and Bigges, 1972). Statistical analysis 0f the obtained data was done by means of soft ware computer program (SPSS 2001).

Hemagglutinationinhibition (HI):-

According to (Fernando et al. 2010): Blood samples were collected from marginal ear vein of rabbits from each group every 12 hours until death or slaughter. Sera were inactivated by incubation at 56° c for 30 minutes, then treated with 25% kaolin (serum final dilution 1/10) at 25° c for 20 minutes and centrifuged to clarify. Dispense 50µL of serum into the first well of round bottom microtitre plate and make 2 fold dilutions into wells PBS. Add 25µL of RHDV antigen (from Vet.Ser& Vacc. Res. Inst. Abbassia-Cairo)) containing 8 HA units to each well and incubate the plate at 25° c for 30-60 minutes. Add 25µL of human group O RBCS at 0.5% concentration to each well and allow settling at 25° c for 30-60 minutes. Titrate the antigen with each test to ensure that 8 HA/ 25µL well used, and include positive and negative serum controls. The serum titer is the end point dilution showing inhibition of HA. The positive threshold of serum titers is correlated to the titer of the negative control sera, it is usually in the rang 1/20 - 1/80.

Hemagglutination test (HA):- It should be performed with human group O (RBCS), freshly collected, stored over night in Alsever's solution, and washed in 0.75% PBS at PH 6.5 (range 6-7.2). HA is less evident or nonexistent when RBCS of other species are used. The test was adapted according to (Fernando et al., 2010) washed RBCS are suspended at 0.75% in PBS. A two fold dilution of the clarified supernatant of a 10% tissue homogenate of liver (pool of each group and examined) is incubated with an equal volume of washed RBCS in a sealed round- bottom microtitre plate at, preferably, 4°c. After 1hour of incubation agglutination at end-point dilution of >1/160 is considered to be positive. Tissue homogenate was prepared according to (Fernando et al., 2010) homogenized in

10% w/v PBS, PH 6.4 and clarified by two consecutive low speed centrifugation (500 g for 20 minutes and 6000 g for 30 minutes). The supernatant, drown form the tube so as to avoid superficial lipid layer, filtered through a 0.22µm pore size mesh titrated by HA and divided into aliquots, which are stored at -20°c.

Pathological examination: - After the end of the experimental period the rabbits were sacrificed, necropsy was performed and all the macroscopic abnormalities were recorded. Specimens were collected from liver, lungs, spleen, heart and kidneys, fixed in 10% neutral buffered formalin. Five micron thick paraffin section were prepared, stained with hematoxylin and eosin, and examined microscopically according to (Bancroft and Stevens, 1996).

RESULTS

Clinical Signs:-

The clinical signs and post mortem examination of the experimentally infected rabbit by RHDV (G5) were showed sudden death without any clinical signs in most cases. Some cases showed elevated temperature 41°c or above, rapid respiration and cyanoses, anorexia and recumbent. Nervous signs may be seen in the late stages with paddling movements. Post mortem examination revealed severe hyperemia of subcutaneous blood vessels. Hemorrhages and edema in the body cavities were observed. Some cases showed hemorrhagic spots in the muscle of the thigh.

Hematological changes:-

Regarding to table(1), there were slight increase in bleeding time, prothrombin time and time associated with thrombocytopenia in (G5) compared coagulation to(G4) supplemented with vitamins and infected with RHDV, however no significant changes in (G1) control, (G2) vitamin, (G3) vaccinated rabbit. Table (2) showed that relative improvement in biochemical parameters' in (G4) compared with (G5) where decrease in serum protein, albumin, glucose while increase in liver enzyme, bilirubin, urea, and creatinine in (G5) infected with virus without addition vitamins compared with (G4) which supplemented with vitamins.

Table 1. Coagulation parameters in rabbit experimentally infected with RHDV.

Parameters	G.1	G. 2	G. 3	G. 4	G. 5
Platlet count(10^3/µl)	183±1.22^c	180.36±1.15^{bc}	182.75±1.03^c	177.5±0.7^b	105.87±1.8^a
Coagulation time(minutes)	3.9±0.07^{ab}	4.05±0.12^b	3.75±0.09^a	4.45±0.12^c	6.35±0.09^d
Bleeding time(minutes)	2.38±0.06^a	2.52±0.09^a	2.40±0.08^a	3.08±0.09^b	4.92±0.12^c
Prothrombin time(second)	74.38±0.94^a	77.37±0.94^b	79.38±0.94^{bc}	81.0±0.80^c	106.5±0.87^d

Means with different alphabetical superscripts in the same row are significantly different at p≤0.05.

Table 2. Biochemical serum analysis in rabbit experimentally infected with RHDV.

Group5	Group4	Group3	Group2	Group1	Parameter
47.75±0.97^c	37.25±1.03^b	30.63±o.95^a	32.63±1.21^a	31.75±0.99^a	AST (U/L)
28±1.00^c	19.13±0.88^b	13.88±0.92^a	14.88±0.9^a	13.36±0.71^a	ALT(U/L)
15.3±0.24^d	9.86±0.30^c	8.81±0.17^b	8.08±0.23^a	7.96±0.21^a	GGT(U/L)
1.44±0.09^b	0.67±0.02^a	0.68±0.02^a	0.65±0.02^a	0.64±0.04^a	bilirubin.(mg/dl)
59.63±1.15^a	75.5±1.19^b	81.25±0.84^c	84.50±1.07^d	82.38±0.94^{cd}	Glucose(mg/dl)
4.87±0.09^a	5.20±0.07^b	5.65±0.08^{cd}	5.75±0.08^d	5.45±0.09^{bc}	Total protein(gm/dl)
1.68±0.07^a	2.33±0.10^b	2.87±0.09^{cd}	3.08±0.07^d	2.76±0.08^c	Albumin(gm/dl)
3.2±0.06^b	2.90±0.08^a	2.75±0.08^a	2.67±0.08^a	2.66±0.08^a	Globulin(gm/dl)
41.13±1.17^c	32.63±1.15^b	26.88±1.27^a	25±1.07^a	25.75±0.94^a	Urea(mg/dl)
2.71±0.10^c	1.78±0.05^b	1.44±0.05^a	1.41±0.05^a	1.39±o.07^a	Creatinine(mg/dl)

Means with different alphabetical superscripts in the same row are significantly different at p≤0.05.

Table 3. HA Log_2 titers of liver of rabbit experimentally infected.

Groups	Treatment	HA Log_2
Gp.1	-ve control	0
Gp.2	Vitamins only	0
Gp.3	Vitamin vaccine infection	3
Gp.4	Vitamin infection	2
Gp.5	Infection	14

Pool of liver of each group and examined rabbit HA with human group O RBCS. Hemagglutination activity:-

As shown in table (3):- Liver homogenate suspensions of (G.1) and (G2), gave –ve HA activity, while in (G.5) the HA activity reached to 14 Log^2 in their liver homogenate suspensions. The other 2 (G.3, 4) gave HA activity 3 Log^2 & 2 Log^2 respectively. The reading of HA, agglutination end point of >4 Log^2 considered to be positive.

Table 4. HI titers with treated group with RHD virus, vitamins and vaccination.

Treatment GPS.	hr/ds post-infection	HI T distribution (log2)													GM
		0	1	2	3	4	5	6	7	8	9	10	11	12	
Gp.5 Infection	Before	8													0
	36 hr					2	4	2							5
	48 hr							1	3	4					7.4
	3d/death										3	1	3	1	10.3
Gp.4 Vitamin infection	Before infection	8													0
	36 hr		4	4											1.5
	48 hr		3	4	1										1.75
	3d			2	5	1									2.88
	4d			1	3	3	1								3.13
	5d				1	3	4								4.37
	6d					2	4	2							5.5
	7d					1	1	5	1						5.75
Gp.3 Vitamin vaccination infection	Before vaccination	8													0
	Before infection								1	2	4	1			8.63
	36 hr							2	2	2	2				7.5
	48 hr							3	3	2					6.63
	3d					1	3	2	2						5.63
	4d						3	2	1						4.2
	5d							2	3	2	1				7.25
	6d								3	2	2	1			8.13
	7d *									2	3	3			9.1
-Ve control	Beginning	8													0
	Slaughter	8													
Vitamin	Beginning	8													0
	Slaughter	8													

Hemagglutination inhibition test (HI.): As shown in table (4), the (G.5), antibody against RHDV began to appear after infection with GM 5 at 36 hr. and increase by the time until death occur at 3rd day post infection and GM 10.3, while in (G4),antibody began to appear after 36 hr.post infection with GM. 1.5 and increased until 7 days post infection and when slaughter GM Reached to 5,75 without clinical signs. The (G3), GM of vaccinated group reached 8.63 and the antibody began to decrease until reached to GM 4.2 at 4th day post infection and increased gradually at 7th day until reached 9.1. The other 2 (G.1&2), there were no detection of antibody against RHDV.

Pathological examination:-

Experimentally infected (G.5):-

Liver: Macroscopically: the gross pathology of the examined liver characterized by pale yellow or grayish color with multifocal petechial hemorrhage Fig. (1). Microscopically: The liver of (G.5) showed cytoplasmic vacuolation of the hepatocytes Fig.(2).Coagulative necrosis of the hepatocytes together with intravascular permeation of mononuclear cells were observed Fig.(3). Pericholangiolar fibrosis and portal infiltration of leucocytes were seen Fig. (4). some cases showed cystic dilatation of some bile ducts together with newly formed bile ductules. Focal hepatic necrosis was seen Fig. (5). Apoptosis of the hepatocytes was occurred Fig. (6). In addition hepatocytes showed Coagulative necroses associated with heterophiles infiltration Fig. (7).

Lungs: Macroscopically: The lungs of (G.5) were enlarged in size together with multifocal hemorrhagic areas were noticed Fig.(8). Microscopically: The lungs showed pulmonary edema associated with focal emphysema Fig. (9). Pulmonary hemorrhage associated with deposition of golden brown hemosidrin pigments were noticed Fig. (10).Some cases showed pulmonary hemorrhage together with massive interalveolar infiltration of macrophage laden with hemosidrin pigment as well as focal mononuclear cells aggregation Fig. (11). Hemorrhage and hemosidrin pigment were observed within the cytoplasm of the macrophage Fig. (12). Focal interstitial pneumonia was seen in Fig. (13).

Spleen: Macroscopically: The spleen was enlarged in size with rounded edges and dark red to black in color Fig. (14). microscopically: The spleen showed depletion and necrosis of lymphocyte Fig. (15). in addition hemorrhage, hemosiderosis and multiple megakaryocytes were observed Fig. (16).

Kidneys: Macroscopically: The kidneys were slightly enlarged and dark red in color. Microscopically: The kidneys showed vacuolation of the epithelial lining renal tubules together with micro thrombi inside the renal blood vessels and perivascular edema Fig. (17). Coagulative necrosis of renal tubular epithelial was observed Fig. (18). In addition hypertrophy and vacuolation of the glomerular tuft were seen Fig.(19).

Heart: Macroscopically: the heart was red in color and slightly enlarged. Microscopically: The heart showed intramuscular edema Fig. (20). others showed vacuolation of sarcoplasm of cardiac myocytes as well as Zenker necroses of focal myocytes Fig. (21).

Vitamins and experimentally infected group (G.4):- This group showed marked improvement in the most examined organs. Liver: Macroscopically: do not show any abnormalities. Microscopically: The examined liver of (G.4) showed individual variation in some cases. Coagulative necrosis of some hepatocytes associated with heterophiles infiltration. Others showed no histopathological changes Fig. (22). Activation of Kupffer cells was showed Fig. (23).

Lungs: Macroscopically: the lungs were apparently normal. Microscopically: The lungs showed hemorrhage associated with infiltration of macrophages laden with hemosideren pigments Fig. (24). others showed nearly no histopathological changes Fig. (25).

Spleen: Macroscopically: Apparently normal spleen. Microscopically: The spleen of the most examined cases showed no histopathological changes Fig. (26).

Kidneys: Macroscopically: Do not noticed any abnormalities. Microscopically: The kidneys showed slight necrosis in the epithelial lining some renal tubules Fig. (27). other cases showed no histopathological changes Fig. (28).

Heart: Macroscopically: Nearly normal appearance of heart.Microscopically: Apparently normal cardiac myocytes was seen Fig. (29). others showed vacuolation of sarcoplasm of cardiac muscles Fig. (30).

Vitamins, vaccinated and experimentally infected group (G.3):-

Liver: Macroscopically: The examined liver of this group were nearly normal. Microscopically: The liver of this group not showed histopathological changes. Some cases showed hydropic degeneration of the hepatocytes Fig. (31).

Lungs: Macroscopically: The lungs were red in color in some cases. Microscopically: The examined lungs showed in some cases no histopathological changes Fig. (32). others showed hemorrhage and interalveolar macrophages infiltration Fig. (33).

Spleen: Macroscopically: Do not show any abnormalities. Microscopically: All examined cases showed normal structure.

Kidneys: Macroscopically: The kidneys were apparently normal. Microscopically: Some cases showed normal histological structure. Others showed focal renal hemorrhage Fig.(34). Vacuolation of the endothelial lining glomerular tuft and eosinophilic intratubular protein cast were observed Fig. (35).
Heart: Macroscopically: The heart was apparently normal. Microscopically: Some cases showed intramuscular hemorrhage and edema Fig. (36). others showed no histopathological changes.

The vitamins administrated group (G.2) and the control group (G.1) do not showed any pathological alteration.

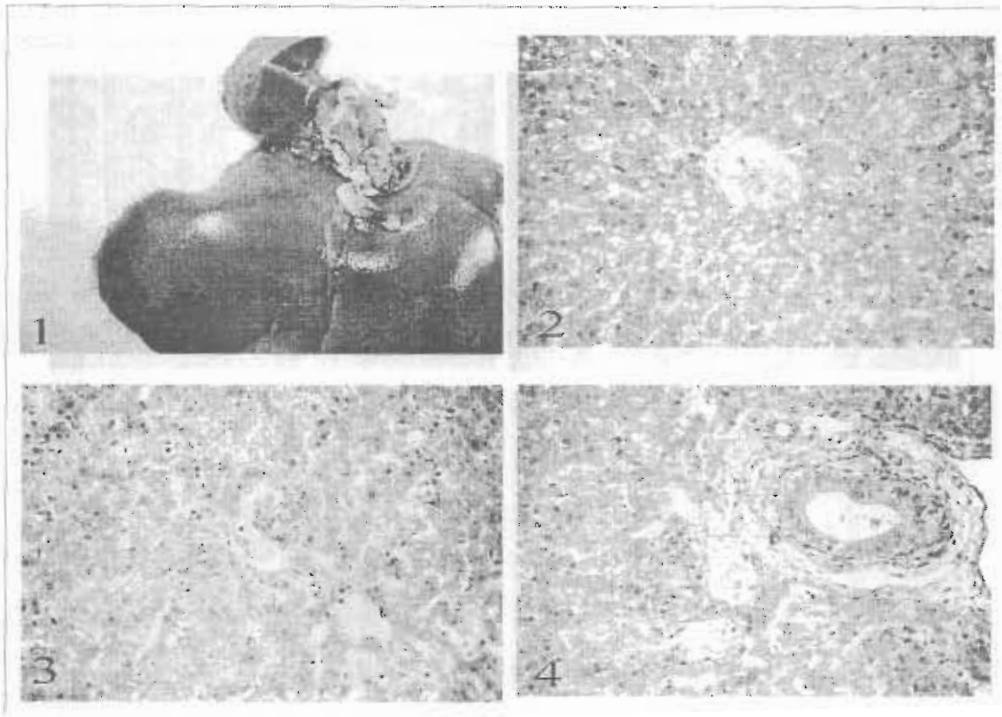

Fig. (1): Photomicrograph of the liver Gp.5 shows pale yellow color with multifocal petechial hemorrhage arrow.

Fig. (2): Photomicrograph of the liver Gp.5 shows cytoplasmic vacuolation of hepatocytes arrow. (H&E. X 400).

Fig. (3): Photomicrograph of the liver Gp.5 shows Coagulative necrosis of hepatocytes arrow 1 and intravascular permeation with mononuclear cells arrow 2. (H&E. X400).

Fig. (4): Photomicrograph of the liver Gp.5 shows Pericholangiolar fibrosis arrow 1 and portal infiltration with leucocytes arrow 2. (H&E. X400).

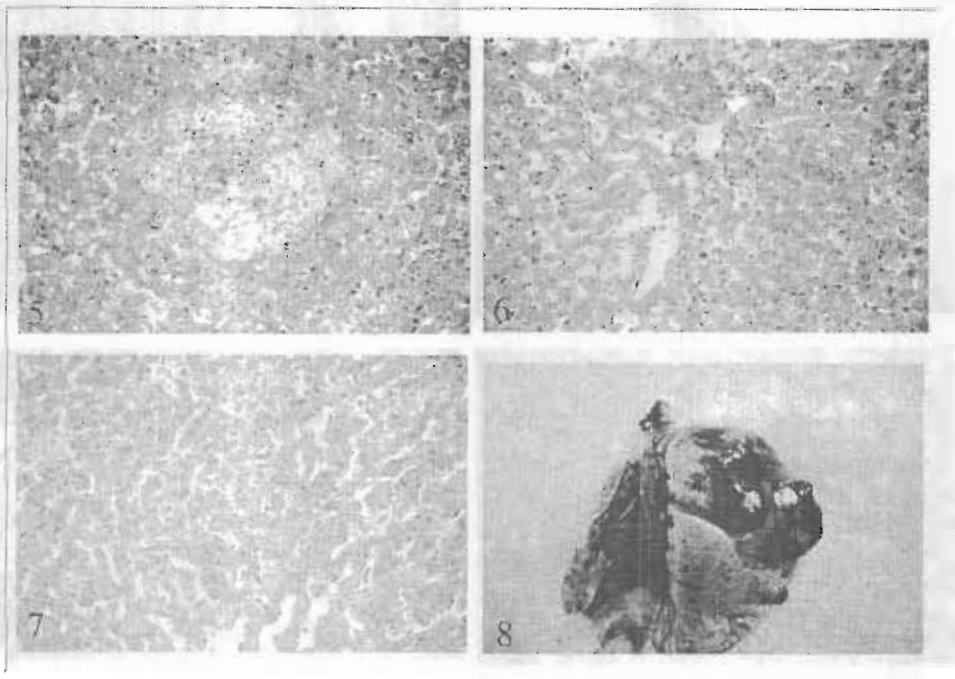

Fig. (5): Photomicrograph of the liver (G.5) shows focal hepatic necrosis. (H&E X400). arrow

Fig. (6): Photomicrograph of the liver (G.5) shows apoptosis of hepatocytes arrow.(H&E. X400).

Fig. (7): Photomicrograph of the liver (G.5) shows Coagulative necrosis of hepatocytes arrow 1 associated with heterphiles infiltration arrow 2 (H&E. X400).

Fig. (8): Photomicrograph of the lungs (G.5) shows enlargement and multifocal hemorrhagic areas arrow.

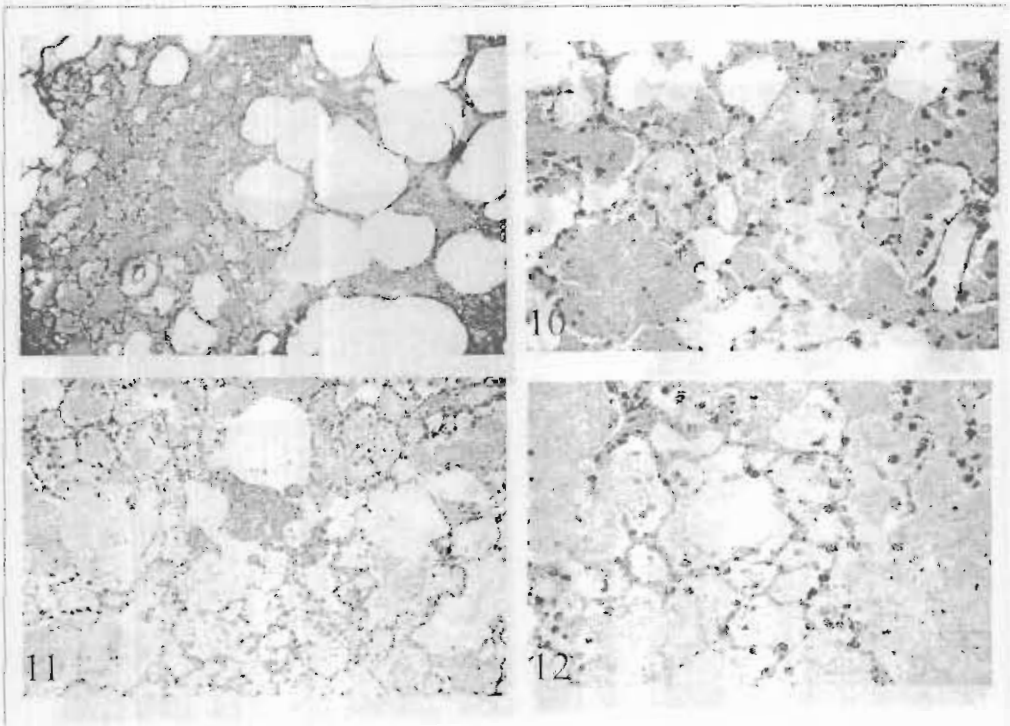

Fig. (9): Photomicrograph of the lungs (G.5) shows pulmonary edema associated 1 with focal emphysema 2. (H&E. X400).

Fig. (10): Photomicrograph of the lungs (G.5) shows pulmonary hemorrhage 1 and macrophage laden with golden brown hemosidrin pigment arrow 2. (H&E.X400).

Fig.(11):Photomicrograph of the lungs (G.5) shows pulmonary hemorrhage 1, massive interalveolar infiltration with macrophages laden with hemosidrin pigment arrow 2 as well as mononuclear cells aggregation arrow 3.(H&E.X400)

Fig.(12):Photomicrograph of lungs (G.5) shows hemorrhage 1 and hemosidrin pigments within the cytoplasm of macrophages arrow 2. (H&E. X400).

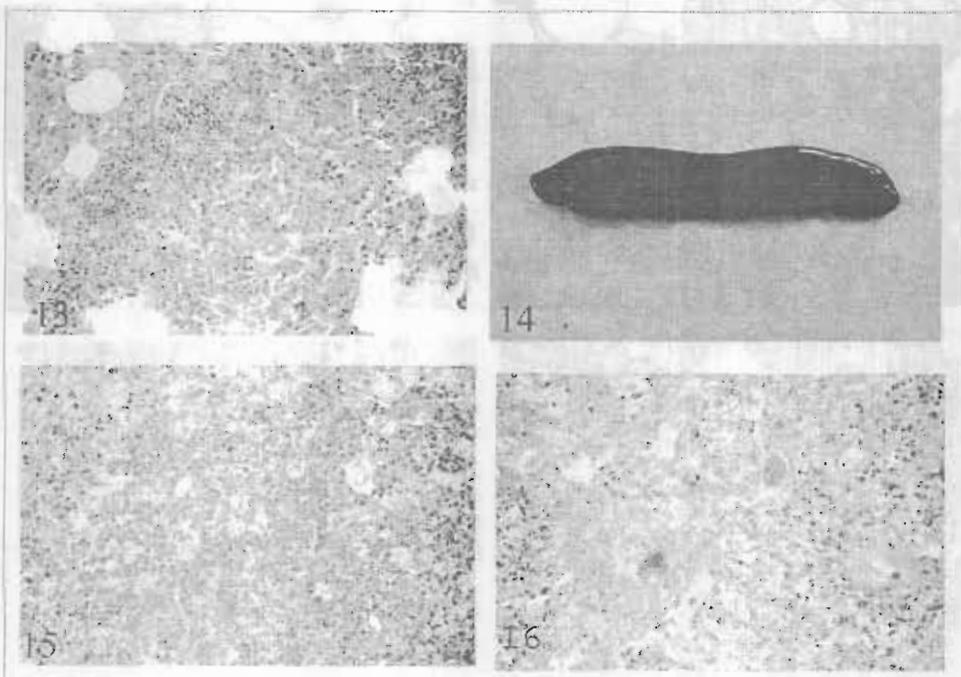

Fig. (13): Photomicrograph of lungs (G.5) shows focal interstitial mononuclear cell aggregation arrow. (Focal interstitial pneumonia). (H&E. X400).

Fig. (14): Photomicrograph of spleen (G.5) shows enlarged in size and dark in color.

Fig. (15): Photomicrograph of spleen (G.5) shows lymphocytic necrosis and depletion arrow. (H&E. X400).

Fig. (16): Photomicrograph of spleen (G.5) shows hemorrhage 1, hemosiderosis 2 and multiple megakaryocytes 3. (H&E. X400).

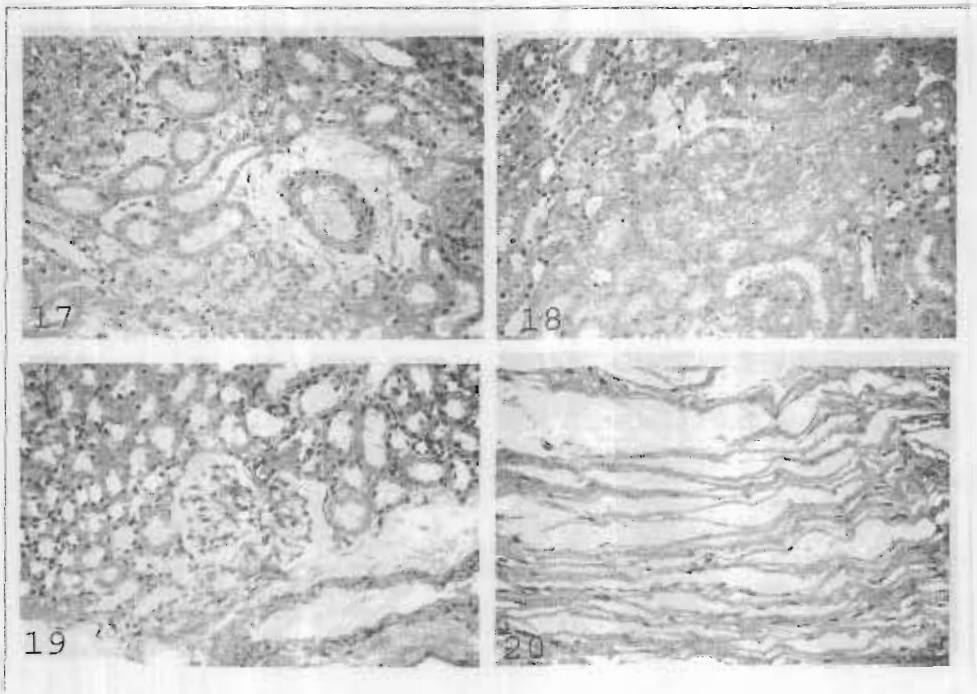

Fig. (17): Photomicrograph of kidney (G.5) shows vacuolation of epithelial lining renal tubules arrow 1 and perivascular edema arrow 2. (H&E. X400).

Fig. (18): Photomicrograph of kidney (G.5) shows Coagulative necrosis renal tubular epithelium arrow. (H&E. X400).

Fig. (19): Photomicrograph of kidney (G.5) shows hypertrophy and vacuolation of glomerular tuft arrow. (H&E.X400).

Fig. (20): Photomicrograph of heart (G.5) shows intramuscular edema arrow. (H&E.X400).

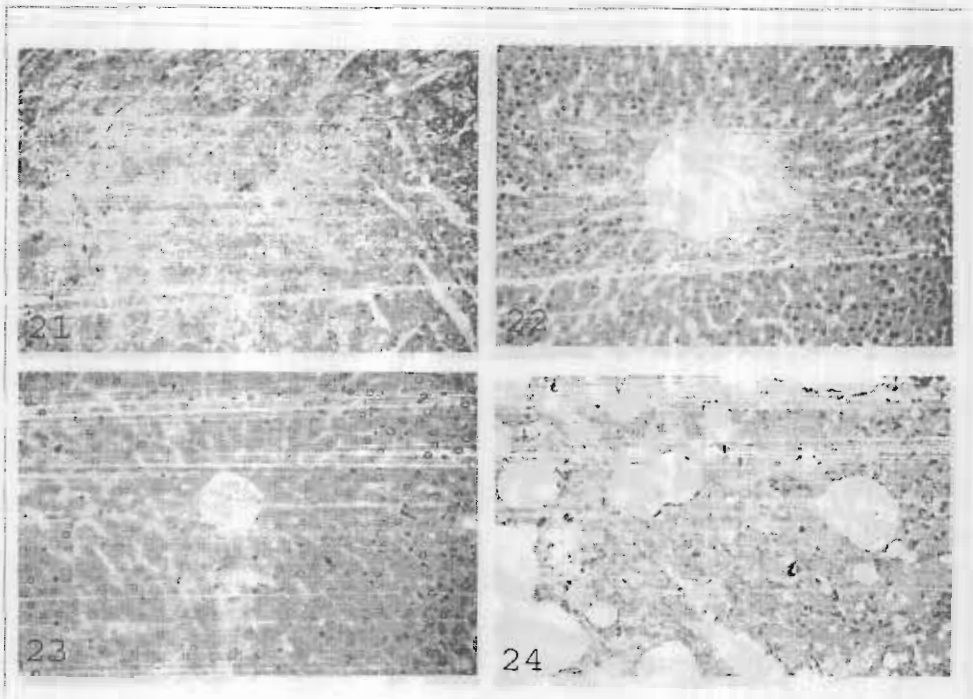

Fig. (21): Photomicrograph of heart (G.5) shows vacuolation of sarcoplasm of Cardiac arrow 1 myocytes as well as Zenker□s necrosis of focal myocytes 2. (H&E.X400).

Fig. (22): Photomicrograph of liver (G.4) shows no pathological changes. (H&E. X400).

Fig. (23): Photomicrograph of liver G.4) shows kupffer cells activation arrow. (H&E. X400).

Fig. (24): Photomicrograph of lung (G.4) shows pulmonary hemorrhage1 associated with infiltration of macrophages laden with hemosidrin pigments arrow 2. (H&E.X400).

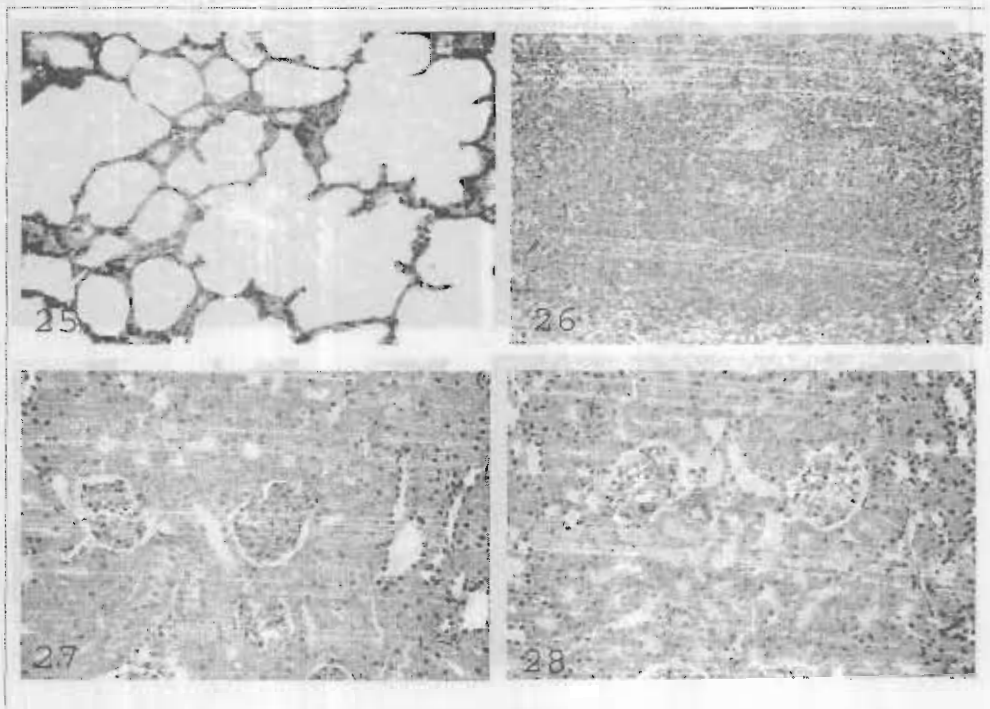

Fig. (25): Photomicrograph of lung (G.4) shows no pathological lesion. (H&E. X400).

Fig. (26): Photomicrograph of spleen (G.4) shows no pathological changes. (H&E. X400).

Fig. (27): Photomicrograph of kidney (G.4) shows nearly no pathological lesion. (H&E.X400).

Fig. (28): Photomicrograph of kidney (G.4) shows no pathological changes. (H&E.X400).

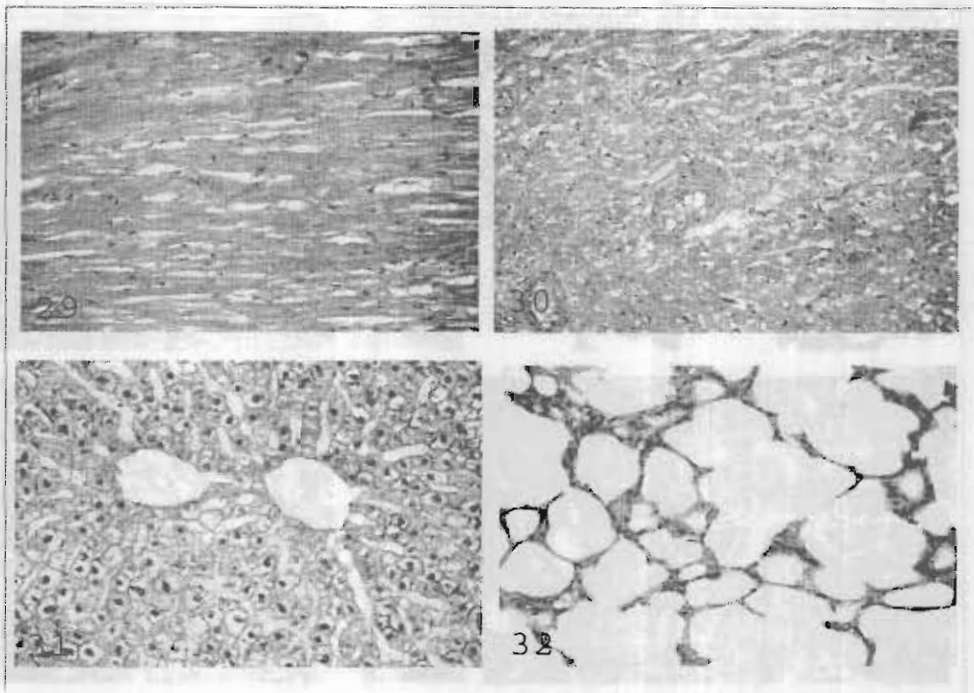

Fig. (29): Photomicrograph of heart (G.4) shows apparent normal cardiac myocytes. (H&E. X400)

Fig. (30): Photomicrograph of heart (G.4) shows vacuolation of sarcoplasm of cardiac myocytes arrow.(H&E. X400).

Fig. (31): Photomicrograph of liver (G.3) shows hydropic degeneration of hepatocytes arrow. (H&E. X400).

Fig. (32): Photomicrograph of lung (G.3) shows no pathological changes. (H&E. X400).

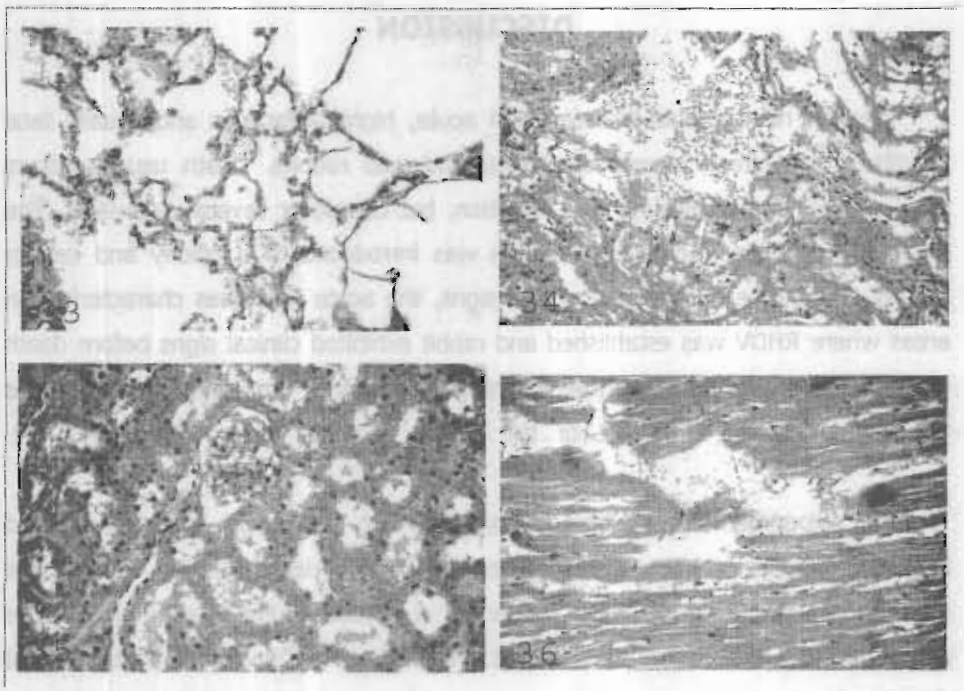

Fig. (33): Photomicrograph of lung (G.3) shows interalveolar macrophages infiltration arrow. (H&E.X400).

Fig. (34): Photomicrograph of kidney (G.3) shows focal renal hemorrhage arrow. (H&E.X400).

Fig. (35): Photomicrograph of kidney (G.3) shows vacuolation of endothelial lining glomerular tuft arrow 1 and eosinophilic intratubular protein cast 2. (H&E. X400).

Fig. (36): Photomicrograph of heart (G.3) shows intramuscular hemorrhage arrow 1 and edema 2. (H&E.X400).

DISCUSSION

Rabbit hemorrhagic disease is an acute, highly infectious and usually fatal condition that affects domestic, farmed and wild rabbits. Death usually occurs between two and three days post infection, but can occur several days later. The peracute form occurred when infection was introduced to a colony and rabbits usually died suddenly with few clinical signs, the acute form was characteristic in areas where RHDV was established and rabbit exhibited clinical signs before death and the subacute form was found in the later stages of an epidemic where there were clinical signs but most of the rabbits survived (David Chasey, 1997).

The experimentally infected rabbit (G.5) in this work presented rapid respiration, anorexia, recumbeny, nervous signs and death. Similar findings are reported by (David Chasey, 1997, Shahera et al., 2000 and Elie et al., 2010). These results may be due to the non specific responses of the rabbit to limit the viral dissemination and replication this aid the specific immune response to release and attack the viral infection (Carter, et al., 2005). The nervous signs observed may be due to hepatic encephalopathy which may result from the increase of the circulatory levels of toxins such as ammonia or mercaptans or abnormal amino acid. Also hepatic encephalopathy was characterized by development of cerebral edema (Swain, et al., 1992, Marcato, et al.,1991 and Krogstad et al., 2005). The sudden death occur in this work may be attributed to the multiple or diffuse necrosis in the liver and disseminated intravascular coagulation (Carter, et al., 2005, Plassiart, et al., 1992). The post mortem finding observed in this work was hyperemia of the subcutaneous blood vessels and hemorrhage in the body cavities. These results are agreed with (Sammin, et al., 1995, Nawar, et al., 1996 and Shahera, et al., 2000).

Regarding to component of coagulation parameters that considered valuable diagnostic and prognostic indicators in RHDV infection(Izumis,et al.,1996).The results represented by prolonged prothrombin time this may be due to decrease in factor V,V11and X with high levels of soluble fibrin as well as reduction of thrombocyte number similar results were reported by (Plassiart, et al., 1992).Other coagulation defects recorded in this work in the form of thrombocytopenia and increase in bleeding time and coagulation time. (Maria, et al,. 2003) discussed that thrombocytopenia may occur secondarily in association with some viral infection such as RHD.Prolonged whole blood coagulation and bleeding time may be due to severe liver damage, thrombocytopenia and disseminated intravascular coagulation. Present study revealed some improvement in biochemical analysis in rabbits infected

virus supplemented with vitamins suggested that some evidence indicate vitamin C reduce virus activity by inhibition viral replication (Johnston,2001) on the other hand other previous studied by (Beisal,1982, Chew,1987,Lovell, 1989, Franchini, et al.,1991, Sayed and Abd-Elghaffar,1999) reported that the use of vitamins as immune stimulant, antibacterial and antiviral agents were succeeded. Enzymatic study of liver revealed that RHD infection increase the activity 0f AST, ALT and GGT these were specific to hepatic disease allow to escape of large quantity of these enzymes into blood similar results were previously obtained by (Ferreira, et al., 2004 and Sanchez, et al., 2004). Agree with (Maria, et al., 2003) hypoglycemia recorded in this work was due to failure of both gluconeogenesis and insulin in the damaged liver .Significant increase in serum urea and clearance creatinine could be due to vascular changes as hemorrhages, congestion ,thrombosis and degenerative changes in liver and kidneys(Nowotny, et al., 1993 and Alonso, et al., 1998). Total protein and albumin of infected rabbit had lower figures in comparison with normal and group received vitamins these attributed to deficient synthesis of albumin reflect RHDV induced pathology in liver also albumin loss is a common cause of hypoalbuminemia due to renal damage caused by RHD infection similar result were recorded by(Maria, et al., 2003). However decrease in serum albumin is often accompanied by a relative hyperglobulinemia (Coles, 1986).

Tissue obtained from infected rabbits, mainly the liver, which contains the highest concentration of the viral particles is the only source of viral antigen used in laboratory diagnostics and vaccine production, thus testing for hemagglutination activity is processed liver samples is one of the cornerstones for rapid diagnosis of RHDV outbreaks in rabbits (Fitzner, and Gromodzaka, 2007). RHDV incubation period varies from 1to 3days, and death usually occurs 12-36 hours after the onset of fever. That happened in our experiment in G. 5.The signs appeared at 3rd day of infection ,then death with high level of antibody against RHDV(GM 10.3) and HA activity of liver pool homogenate of this group is more than 2^{12} (14 log^2),and this agrees with(Yang,1989 and Yang and Du, 1989), they said that hemagglutination titers have been detected in liver tissue of affected rabbits(mostly liver) as early as 12 h post-infection and observed to rise rapidly, reaching a maximum as high as 14 log^2 at death. Our findings in G.4, antibody began to appear after 12hr. of infection. (GM 0.5), gradually increase, reach (GM 5.75) at 7 day post infection and HA activity of collected liver 2 Log^2, while in G.3 HI titer at the end of vaccination period before infection giving (GM 8.63). After infection GM decreased gradually and reached to 4.2 at 4 day post infection, then began to increase gradually at 7 day post infection till reach 9.1, with HA activity of pooled liver homogenate is 3 Log^2.

This results is agreed with (Fernando et al., 2010), requirements for vaccines for indirect control of the disease is achieved by vaccination using a killed vaccine prepared from clarified liver suspensions of experimentally infected rabbits and subsequently inactivated and adjuvant. Vaccinated animals quickly produce solid protective immunity against RHDV infection (within 7-10 days) and experimental data indicate that protection lasts for a long period over 1 year (Fernando et al., 2010).

The gross lesions of the experimentally infected group (G.5) revealed yellow or grayish with multifocal petechial hemorrhage in liver and swollen with multifocal hemorrhagic areas in lungs. In addition the spleen was enlarged and dark in color together with enlarged kidneys and heart. Our obtained results are confirmed with (David Chasey, 1997, Shahlera, et al., 2000, Adel, 2006 and Elie, et al., 2010). The microscopic findings of the liver in this work showed vacuolation of hepatocytes together with Coagulative necrosis associated with heterophiles infiltration. Apoptosis of the hepatocytes and intravascular permeation of mononuclear cells were observed. Pericholangiolar fibrosis and portal infiltration of leucocytes. These results are confirmed with (Marcato, et al., 1991, and Elie, et al., 2010). The liver cells macrophages and monocytes represent a very important cellular targets for RHDV and the infection of these cell types could be a start in the progress of the disease pathogenesis (Romario, et al., 1999 and Elie, et al., 2010). RHDV characterized by two basic types of the hepatic cell death, necrosis and apoptosis. The significant loss of hepatocytes that occurs due to the infection was leading to disseminated intravascular coagulation and hemorrhage and this might be due to the release of large amounts of thromboplastins due to hepatolysis (Alonso, et al.,1998 and Elie, et al., 2010). The programmed cell death (apoptosis) was correlated with the appearance of RHDV induced pathology in tissue as detected in situ by DNA fragmentation. Hepatocytes apoptosis in RHDV could produce extensive paranchymal destruction, resulting in an acute and lethal fulminant hepatitis (Park, et al.,1995). The positive cells were degenerated hepatocytes associated with macrophages presence in the cellular aggregates which might be due to phagocytosis of viral -replicated necrotic hepatocytes(Alexandrov, et al.,1992, Park and Itakura, 1992 and Park et al.,1995). Necrotic hepatocytes were characterized by many cytopathological changes including formation of cytoplasmic vesicles, shrinkage of cell body vacuoles and complete dissolution of chromatin matter of dying cell. A large number of viral particles was accumulated in the membrane bound vacuole of necrotic hepatocytes (Park, et al., 1992). Necrosis of hepatocytes

are consistent with a lytic viral infection causing hepatocytes disruption with subsequent release of viral and heterophil infiltration (Park, et al., 1997).

The microscopic changes of lungs in the present study demonstrated edema, hemorrhage together with massive infiltration of macrophages as well as mononuclear cells aggregation. Interstitial pneumonia was also seen. These findings are correspond to those found previously in RHDV- infected rabbits by (Fuchs and Weissenbock, 1992, Prieto, et al., 2000, Leghans, et al., 2001 and Van, et al., 2006). (Carrasco, et al.,1991) explained that the stimulation of pulmonary macrophages occurs by congestion, edema and hemorrhage and they plays an important role in retention and removal of the viral or bacterial particles and cell debris from the blood stream. The microscopic findings of the kidneys in this work showed microthrombi inside renal blood vessels together with perivascular edema and necrosis of renal tubular epithelium. Similar results were obtained by (Alnoso, et al., 1998 and Adel, 2006).

The microscopic alterations in spleen and heart in the present study revealed depletion and necrosis of lymphocyte together with hemorrhage and multiple megakaryocytes. The heart showed edema and Zenker necrosis. Our findings are confirmed with (Romario, et al., 1999) who mentioned that viral specific antigens were detected in liver, lung, and spleen and lymph nodes cells. Also intravascular infected cells were detected in most organs including kidneys, myocardium, thymus and central nervous system. Circulating viruses are removed by reticuloendothelial system, primarily by Kupffer cells of the liver, and to a lesser extent in the lung, spleen and lymph nodes (Mims, 1964 and Adel, 2006).

The histopathological changes of the vitamins supplemented groups(gr.3,gp,.4): The vitamins administrated groups represented a marked improvement in the features of most examined organs(liver, lungs, spleen, kidneys and heart), our results are agree with (Chew,1987, Franchini, et al., 1991, Sayed and Abd-Elghaffar,1999). The vitamins ameliorate most pathological, biochemical and virological changes occurred by experimentally infected virus group in this work.

Moreover, (Eduardo and Wafaie, 2005) mentioned that vitamin A is maintaining the integrity of epithelia and stimulate the immune responses of animals against viral infection. On the same context they used vitamin A supplementation (retinoid compounds) to decrease the risk of mortality and morbidity in children was infected by measles, human immunodeficiency virus (HIV). The experiment was performed in vitro and animal studies, they suggest that retinoid are important regulators of monocytic differentiation and function. Retinoic

acid promotes cellular differentiation (Breitman, et al., 1980, Geissmann, et al., 2003 , Jiang, et al., 2003 and Mohty, et al., 2003), and influences the secretion of key cytokines produced by macrophages, including tumor necrosis factor(TNF-α), IL-1B, IL-6 and IL-12. All trans- retinoic acid skewed the differentiation of human peripheral blood monocytes to IL-12 secreting dendritic cells in the vitro study (Mohty, et al., 2003). Whereas in the animal study it inhibited lipopolysaccharide induced IL-12 production by mouse macrophages (Kang, et al., 1999). All –trans-retinoic acid was shown to decrease secretion of TNF-α in murine peripheral blood mononuclear cells (Kim, et al., 2004) and myelomonocytic (Mou, et al., 2004) and macrophage cell lines (Mathew and Sharma, 2000 and Motomura, et al., 2001). On the other hand, retinoid appear to enhance the secretion of IL-1B (Hashimoto, et al., 1998) and IL-6 by macrophage and monocytes (Arena, et al., 1997). In rats (Hatchigian, et al., 1989) and in vitro experiments (Dillehay, et al.,1988), vitamin A increased phagocytic capacity of macrophages.

The obtained results in this work are in a partial agreement with (Nwaigwe, et al., 2010), on birds administrated vitamin E supplemented diets during the infection by infectious bursal disease virus (IBD). He found that vitamin E could be beneficial in lowering their lipid peroxidation levels and its combination with vaccination may enhance the feed intake in birds during and after IBD infection and quicken their recovery through improved immunity. On the same context (Melinda, 2007) recorded that vitamin E a lipid soluble antioxidant are important mediators for protection against oxidative stress. Also he mentioned that the deficiencies in either selenium or vitamin E results in increased viral pathogenicity and altered immune responses, furthermore results specific viral mutation. (Weber, et al., 2008) reported that antibody formation against Newcastle disease was improved by vitamin E treatment in drinking water.

On the same context our results are supported by (Tantcheva, et al., 2003) on male mice infected with influenza virus, he reported a successful preventive effect of vitamin E and C, alone and in combination on the damage caused by influenza virus (IVI) when administrated in single once daily doses of vitamin E (60 mg/kg b.wt.) and vitamin C (80mg/kg b.wt.) intraperitonealy 3 days before virus inoculation. He discussed that, after virus inoculation, animals decapitated, monoxygenase enzyme activity(ethyl morphine N-demethylase, amidopyrin N-demethylase, analgin N-demethylase, analine dehydroxylase, cytochrome P-450 content and NADPH- cytochrome C reductase {CCR}) was determined in liver 9000 x g supernatant. Primary and secondary products of lipid peroxidation (LPO, conjugated dienes [CD] and TBA- reactive substances) were measured in blood

plasma, lung and liver 9000 x g supernatant. Vitamin E effectively restores LPO-levels increased by IVI. The effect of vitamin C was similar but slighter. The combination (vitamin E+C) had greater effect on LPO levels. P-450 dependent monooxygenase activity was significantly restored and more pronounced cytochrom p-450 content and NADPH- CCR activity was noted. On the same line (Harri Hemila, 2009) reported that vitamin C is an efficient water soluble antioxidant and may protect host cells against the oxidants released by phagocytes. Upon activation, phagocytes release a set of oxidizing agents intended to kill viruses and bacteria.

Moreover, (Dr. Thomas Levy and Dr. Robert Ctheart, 2010) he is one of the world 's experts in effect of vitamin C on viruses and he is treated thousands cases of infectious diseases with high dose of vitamin C (Ascorbic acid). Neuramidas inhibition which prevents viral release from infected cells in one of more than ten ways that vit. C helps knock out viruses, both by inhibiting the virus itself and by strengthening the body 's own immune response by improving immune cell function (macrophages and lymphocytes), upping interferon and nitric acid oxide and making more antibodies which target viruses.

Conclusion: - Finally, it could be concluded that vitamins is highly effective in alleviation of the pathological changes induced by RHDV.

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تأثير بعض الفيتامينات فى تقليل التغيرات الباثولجية، البيوكيميائية و الفيرولوجية في الارانب المصابة معمليا بمرض النزف الفيروسى

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استهدف هدا البحث دراسة تأثير بعض الفيتامينات (ا ،هـ ،ج) على التغيرات المرضية للارانب نتيجة للاصابة المعملية بمعلق كبد ارانب مصابه بمرض النزف الدموى الفيروسى سابقا ونافقه حديثا وعلى الارانب التى يتم تحصينها ضد مرض النزف الدموى الفيروسى بعد ١٥ يوم من اعطاء الفيتامينات، وبعد ١٥ يوم من اعطاء التحصين يتم احداث عدوى صناعية بمعلق كبد الارانب.

تم استخدام عدد٤٠ أرانب أبيض نيوزيلاندى وتم تقسيمهم الى ٥ مجموعات بالتساوى. المجموعة الاولى: تركت ضابطة للتجربة، والمجموعات ٢،٣،٤ تم اعطائهم فيتامين(أ) بجرعة تساوى ١٠٠٠٠ وحدة دولية/لتر، وفيتامين (هـ) بجرعة تساوى ٥٠٠ وحدة دولية/لتر، وفيتامين (ج) بجرعة تساوى ١٠٠٠ وحدة دولية/لتر. بجرعة واحدة يوميا فى مياه الشرب لمدة ١٥ يوم، ثم تحصن المجموعة (٣)بالتحصين المحلى ضد مرض النزف الدموي الفيروسى، وبعد ١٥ يوم من التحصين يتم احداث عدوى صناعية بمعلق كبد الارانب المحضر معمليا. بعد ١٥ يوم من اعطاء الفيتامينات يتم احداث العدوى الصناعية للمجموعة الرابعة. اما المجموعة الخامسة يتم احداث عدوى صناعية بمعلق الكبد (كنترول ايجابى). يتم اخذ الدم وفصل السيرم عند ظهورالاعراض ونهاية التجربة وذلك لاجراء الفحوصات البيوكيميائية والفيروليجية. كما يتم عمل الصفة التشريحية وتسجيل التغيرات المرضية الملحوظة. كما يتم اخذ عينات من الكبد ،الرئة ،الطحال،الكليةو القلب ووضعها فى فورمالين ١٠% لعمل الفحص الباثولوجى. لوحظ تحسن نسبى فى اختبارات التجلط و التغيرات البيوكيميائية فى الارانب التى اعطيت الفيتامينات ثم احدث بها عدوى النزف ؤالدموى الفيروسى و قد تبين ذلك فى تقليص وقت النزف والتجلط ووقت البروثرومبين وكذلك عدد الصفائح الدموية وذلك بالمقارنة بالارانب التى لم تعطى فيتامينات واحدث بها عدوى بالفيروس وفى غضون ذلك كانت الحالة الصحية لهذه المجموعة جيدة كما هو واضح من نتائج قراءات قياسات وظائف الكبد (وانزيمات اسبرتيت امينوترانسفيريز و الالانين امينوترانسفيريز وجاما جلوتاميل ترانسفيريز والبيليروبين) و وظائف الكلى (اليوريا والكرياتينين) والبروتين الكلى والالبيومين و الجلوكوز.

وقد اظهر الفحص المجهرى للكبد وجود تنكسات مختلفة فى خلايا الكبد ونخر وفرط فى عدد الخلايا الالتهابية. كما لوحظ نزف وارتشاح وفرط فى الخلايا وحيدة النواة فى الرئة. نقص وتنكرزللخلايا الليمفاوية فى الطحال.كما وجد جلطات صغيرةداخل الاوعية الدموية مما ادى الى وجود تنكرز فى بعض الانبيبات الكلوية. وقد اظهر الفحص المجهرى تحسن ملحوظ فى المجموعات التى تم اعطائها الفيتامينات وكذلك تحسن فى معظم القياسات المختلفة. بينما المجموعة الضابطة للتجربة والمجموعةالتى تم اعطائها الفيتامينات لم يظهر بها اى تغيرات باثولجية.

مما سبق نستنتج ان استخدام الفيتامينات له تأثير ملطف ووقائى ومنشط للمناعةعلى التغيرات المرضية الملحوظة نتيجة لاحداث عدوى صناعية بمرض النزف الدموي الفيروسى.