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ORIGINAL ARTICLE

BIOCHEMICAL STUDIES ON AVIAN INFLUENZA VIRUS (H5N1) INFECTINGBALADI CHECKEN AND DUCK EGYPTIAN BIRDS

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Abstract:

Avian influenza viruses is the most important and dangerous disease in birds. In Egypt we have witnessed a dramatic increase in the number of highly pathogenic avian influenza (HPAI) outbreaks.Since2006 HPAI H5N1 infected the commercial poultry production sectors and backyards in Egypt. The outbreak caused great economic losses in poultry industry and still considered a renewable problem. Ducks and chickens are the most important hosts of AIV with distinctive response to infection. Measuring of some biochemical laboratory parameters in the clinical practice can estimate the degree of injury of different organs which reflect the viral complications inside the body. Thus,this study included 30 healthy and 30 infected duck and chicken serum samples. Infected birds were obtained from outbreaks of house breeding and backyards from 2009-2011, were showed clinical findings in the form of general respiratory signs,nervous manifestation with greenish watery diarrhea in addition high mortality rate and the rapid test kits approved the infection with avian influenza. The biochemical parameters measurements were carried out by a spectrophotometer, some parameters as; ALT, AST, Glucose, Uric acid, Creatinine and LDH showed an elevation in their serum levels, while others as Calcium and proteins revealed a decreaselevels for both an infected Chicken and Duck Baladi groupsas in a comparison with healthy control groups.

KEYWORDS:

Biochemical parameter, High pathogenic avian influenza, Chicken and Duck Baladi, H5N1 .

INTRODUCTION

Influenza A viruses genus of the Orthomyxoviridae family are roughly spherical with glycoprotein spikes on the surface and genome consisting of 8 RNA fragments that encode 10 proteins. Haemagglutinin (HA), neuraminidase (NA) and matrix (M2) proteins are embedded in the envelope lipid bilayer derived from the host cell. The M1 protein underlying the envelope is the major determinant of virion morphology.The nucleoprotein (NP) associates with each RNA segment to form the ribonucleoprotein (RNP) complex, which also contains small amounts of the three polymerase subunits. The nonstructural proteins NS1 and NS2 are found only in infected cells(Noda et al., 2006).Type A viruses are further subdivided into subtypes based on the antigenic differences in the HA and NA molecules. At present, there are 16 HA (H1–H16) and NA (N1–N9) subtypes .Each virus has one H and one N subtypes(Fouchieret al., 2005).

All Egyptian strains are very closely related and belonging to subclade 2.2 of the H5N1virus of Eurasian origin, the same one circulating in middle east region and introduced into Africa at the beginning of 2006 (Alyet al.,2008).

Migratory birds and waterfowl(wild ducks, geese & swans) are the main reservoirs carry H5N1 often without becoming sick,directly spread the highly pathogenic strain to chickens, crows, pigeons, and other birds, and the virus was increasing its ability to infect mammals as well(Brstilo, 2006).

During replication of influenza virus, antigenic changes can occur. Minor changes, called antigenic drift, are caused by the accumulation of point mutations during transcription of the viral genes, owing to the lack of proof-reading ability of the viral polymerase. Such changes, mainly those occurring in the genes coding for HA and NA, can generate new strains(Ghedinet al.,2005).

More significant changes, called antigenic shift, result from the acquisition of entirely new gene segments, through genetic re-assortment between two virus strains simultaneously infecting the same host. This may result in the emergence of novel influenza virus subtypes that may have increased virulence, a fact that can be aggravated by the lack of prior significant protective immunity in the new hosts(Ghedinet al.,2005).

Avian influenza A viruses strains are further classified as low pathogenic avian influenza viruses (LPAI) or highly pathogenic avian influenza (HPAI) on the basis of specific molecular genetics and pathogenic criteria. Most avian influenza A viruses are LPAI viruses that cause mild disease in poultry. In contrast, HPAI viruses can cause severe illness and high mortality in poultry. Some HPAI viruses (e.g., H5N1) have been found to cause no illness in some poultry, such as ducks. LPAI viruses have the potential to evolve into HPA1 viruses (Swayne and Halvoson, 2003).

The present study was conducted to investigate the effect of HPAI through the assessment of some biochemical parameters,in order to estimate the viral complications and outcomes on the clinical of the body among some Egyptian birds.

SUBJECTS AND METHODS

Subjects:

The present study was carried out on 30 birds have different ages from both sexes suffering from avian influenza disease symptoms, out breaks from house breeding birds in some villages in El-Sharkya; and 30 healthy bird from bird selling markets in Zagazig. The subjects of the study were dividedinto the following groups:Group I- Comprised of 15 healthy individuals acting as reference (control chicken Baladi group); Group II- comprised of 15 infected individuals (infected chicken Baladi group); Group III- comprised of 15 healthy individuals (control duck Baladi group); Group VI- comprised of 15 infected individuals (infected duck Baladi group).

Blood sampling:

Under the clinical supervision, blood specimens (3-7 ml) from each Bird were drawn from the sub-wing vein or sub-jugular vein into vacutainer collecting tubes (Paget &Thosmon, 1979). The non-heparinized blood specimens were left 20 minutes to clot, then centrifuged at 3000 rpm for 10 minutes using cooling centrifuge (IEC centra-4R, International Equipment Co., USA). The sera were separated at once by micro pipette, divided into aliquots and stored at -70°C, until biochemical measurements could be completed as soon as possible.

Determination of serum biochemical parameters:

All the biochemical measurements were carried out using Pretest exp. Biochemistry analyzer (spectrophotometer).

A. Determination of serum aminotransferase enzymes activity:

I. Serum alanine aminotransferase (ALT) activity (IU/L):

Serum alanine aminotransferase (ALT) activities were determined according to the kinetic method described by Schumann and Klauke, 2003. The assay was performed according to the instruction manual of Human reagent kits purchased from Human Gesell Schaft fur Biochemical und DiagnosticambH, Germany.

The activity of serum ALT (IU/L) was calculated according to the following equation:

ALT activity (IU/L) = ΔA_{340}/min x1746

II. Serum aspartate aminotransferase (AST) activity (IU/L):

Serum alanine aminotransferase (AST) activities were determined according to the kinetic method described by Schumann and Klauke (2003). The assay was performed according to the instruction manual of Human reagent kits purchased from Human Gesell Schaft fur Biochemical und DiagnosticambH, Germany.

AST activity (IU/L) = ΔA_{340}/min x1745

B.Determination of total proteins level (g/dl):

Serum proteins were determined according to Biuret colorimetric end point method according to the instruction manual of Vitro scient reagent kits.

Calculation:

Total protein (g/dl) = [A_{Sample}/ $A_{Standard}$] × (concentration of standard 6.0 g/l).

C.Determination of albumin level (g/dl):

Serum albumin concentration was determined according to the photometric systems described by **Johnson et al. (1999).** The assay was performed according to the instruction manual of Diasys reagent kits purchased from Diasys Diagnostic systems GmbH, Germany.

Serum albumin (g/dl) = [A_{Sample} /$A_{Standard}$] ×(concentration of standard 4.0 g/l).

D.Calculation of globulin level (g/dl):

Serum globulin level was calculated by subtracting the albumin value from the total proteins value according to the following formula:

Serum globulin (g/dl) = total proteins (g/dl) – albumin (g/dl)

E.Albumin/globulin (A/G) ratio:

Another protein index is the A/G ratio. This ratio was calculated by dividing serum albumin value over the serum globulin value.

F.Determination of serum Glucose levels (mg/dl):

Serum Glucose level was determined according to the quantitative enzymatic, colorimetric method (GOD/PAP). The assay was performed according to the instruction manual of Vitro scient reagent kits purchased from Medical Devices Safety Services MDSS GmbH Burckhardtstr 30163.1 Hannover, Germany.

Serum glucose (mg/dl) = [A_{Sample} / $A_{Standard}$] × concentration of standard.

G.Determination of serum creatininelevel (mg/dl):

Serum creatinine level was determined according to the Kinetic colorimetric (Fixed rate) Jaffe reaction quantitative method. The assay was performed according to the instruction manual of Vitro scient reagent kits purchased from Medical Devices Safety Services MDSS GmbH Burckhardtstr 30163.1Hanover, Germany.

Conc. of serum creatinine (mg/dl) = $[A_{Sample} / A_{Standard}] \times$ conc. of standard.

H.Determination of serum Uric acid levels (mg/dl):

Serum Uric acid level was determined according to the quantitative enzymatic, colorimetric method (Uricase/PAP)(Tietz, 1995& 2005). The assay was performed according to the instruction manual of Vitro scient reagent kits purchased from Medical Devices Safety Services MDSS GmbH Burckhardtstr 30163 .1 Hanover, Germany.

Serum uric acid (mg/dl) = $[A_{Sample} / A_{Standard}] \times$ conc. of standard.

J. Determination of serum Calcium levels (mg/dl):

Serum Calcium level was determined according to the quantitative colorimetric endpoint method based on the cresolphthalein determination of calcium in serum complexone reaction on manual or automated systems. The assay was performed according to the instruction manual of Vitro scient reagent kits purchased from Medical Devices Safety Services MDSS GmbH Burckhardtstr 30163.1 Hanover, Germany.

Serum calcium (mg/dl) = $[A_{Sample} / A_{Standard}] \times$ concentration of standard.

k.Determination of Lactate dehydrogenase enzyme (LDH) level (U/l):

Serum lactate dehydrogenase level was determined by Kinetic method according to the recommended quantitative determination of lactate dehydrogenase reference method of DGKC (1987). The assay was performed according to the instruction manual of Vitro scient reagent kits purchased from Medical Devices Safety Services MDSS GmbH Burckhardtstr 30163.1 Hanover, Germany.

CALCULATION:

Determine the change in absorbance per minute (ΔA/min) from the linearportion of the reaction curve and calculate the LDH activity by using the following formula:

U/l = 8095 × ΔA 340 nm/min

One international unit U: International unit(i.e. amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified condition).

The general formula for converting ΔA/min into U/l is:

$$U/l = \frac{\Delta A/min \times Vt \times 1000}{LP \times Vs \times \Sigma}$$

Where:
V_t: total reaction volume in ml
V_s: sample volume in ml
Σ: millimolarabsrpitivity of NADH
LP: cuvette pathlength in cm
1000: convertion of U/ml to U/l, Millimolarabsobativity of NADH
At 334 nm = 6.18,
At 340 nm= 6.3 and
At 365 nm = 6.4

STATISTICALANALYSIS

Results were expressed as the mean ± SD. Differences between healthy control and infected groups were tested for significance using a T-test where; * = significant & ** = highly significant. Differences were considered significant at $P < 0.05$ level using SPSS version 14 computer program and

Microsoft Excel 2013.

RESULTS

Clinical findings:

The common clinical signs observed in examined birds were cyanosis in of comb and wattles, facial swelling, edematous wattles and necrosis of comb, conjunctivitis, congestion of the shank and sometimes hemorrhagic spots on shank with elevated body temperature., greenish diarrhea, nervous signs (torticollis), sneezing, off food followed by, death.

Biochemical parameters results:

All the biochemical tests results in this study were statistically analyzed by computerized program named SPSS 14.0 for windows evaluation version and Microsoft Excel 2013 to make paired T-test where (* = significant, ** = highly significant) as was seen in Tables(1& 2).

Table 1:Serum biochemical parameters mean values± SD. of an infected chicken Baladi group in a comparison with a healthy control group. P-Value< 0.05 indicates a highly significant difference between the two groups.

Group / Parameter	chicken		P-Value
	Control	Infected	
Serum ALT	28.4±1.8	43.1±3.7	<0.05**
Serum AST	183±20.6	300.2±14.8	<0.05**
Serum Protein	4.9±0.23	3.3±0.2	<0.05**
Serum Albumin	2.5±0.07	1.85±0.15	<0.05**
Serum Globulin	2.4±0.24	1.4±0.12	<0.05**
Serum Glucose	231.4±28	305.7±57	<0.05**
Serum Creatinine	0.78±0.2	1.28±0.08	<0.05**
Serum Uric acid	8.4±0.32	11.1±0.33	<0.05**
Serum Calcium	8.5±0.4	4.8±0.25	<0.05**
Serum LDH	618.4±37	1447.6±113	<0.05**

Table 2:Serum biochemical parameters mean values± SD. of an infected Duck Baladi group in a comparison with a healthy control group. P-Value< 0.05 indicates a highly significant difference between the two groups.

Group / Parameter	Duck		P-Value
	Control	Infected	
Serum ALT	29.1±1.6	57.8±6.1	<0.05**
Serum AST	148.9±7.2	272.8±9.0	<0.05**
Serum Total Protein	5.6±1.3	3.7±0.2	<0.05**
Serum Albumin	2.5±0.07	1.8±0.17	<0.05**
Serum Globulin	3.1±0.18	1.9±0.2	<0.05**
Serum Glucose	194±10.9	276±44	<0.05**
Serum Creatinine	0.59±0.04	0.96±0.09	<0.05**
Serum Uric acid	5.27±0.3	6.92±0.23	<0.05**
Serum Calcium	6.7±0.3	4.4±0.37	<0.05**
Serum LDH	633.4±62	1204.8±103	<0.05**

The results showed a highly significant increasing in the s.ALT level values of an infected chicken Baladi group, when compared with its healthy control group (Table 1). Similarly our present study showed a highly significant increasing in the s.ALT level values of an infected Duck Baladi group in a comparison with its healthy control group (Table 2).The present study revealed a highly significant increasing in the

s.AST level values of both an infected Chicken Baladi group and an infected duck Baladi group in a comparison with their healthy control groups, as seen in Tables(1) and (2) respectively.On the other hand, a highly significant decrease in serum total protein, albumin, and globulin concentration for an infected chicken and Duck Baladi groups when compared with their healthy control groups (Tables 1 and 2).

The obtained results showed a highly significant increasing in the serum glucose concentration of an infected chicken Baladi group when compared with its control group (Table 1). As well as a highly significant increasing in the glucose concentration values of an infected duck Baladi group when compared with its control group (Table 2).

Our results indicated a highly significant increasing in serum creatinine concentration for an infected chicken Baladi group when compared with its control group (Table 1) as well as a highly significant increasing in creatinine concentration for an infected duck Baladi group when compared with its control group (Table 2).

The present study shows a highly significant increase in the serum uric acid concentration of an infected Chicken Baladi group when compared with its control group (Table 1). Similarity our results showed a highly significant increasing in the serum uric acid concentration of an infected duck Baladi group when compared with its control group (Table 2).

As seen in the illustrated results in Tables(1 and 2), a highly significant decreasing in the serum calcium levels for both an infected Chicken and Duck Baladi groups when compared with their healthy control groups. In contrast, the present study results cleared a highly significant increasing in the serum LDH level concentration for an infected Chicken Baladi group when compared with its control group as seen in Table (1). Besides a highly significant increasing in serum LDH concentration values for an infected duck Baladi group when compared with its control group as seen in Table (2).

DISCUSSION

Diagnostic of H5N1 among poultry and birds by different biochemical tests is a very important part or tool in the clinical examination for both healthy and sick birds. The continuing propagation of highly pathogenic H5N1 viruses among poultry and migratory birds, shows a continuing and potentially escalating threat to human (WHO, 2008 and Peiriset al., 2005). Preparedness for a possible H5N1 pandemic requires not only enhanced prevention efforts but also a heightened awareness of the clinical characteristics of H5N1 cases (Liuetal., 2005).Common laboratory findings have been leukopenia, particularly lymphopenia; mild to moderate thrombocytopenia; and slightly or moderately elevated aminotransferase levels. Marked hyperglycemia, and an elevated creatininelevels were also occurred(Sedyaningsihetal., 2007).

Some studies suggest that the lower respiratory tract is the major site for H5N1 viral replication, although initial infection may occur in either the upper or lower respiratory tract. H5N1 virus and viral RNA have been detected also in feces and intestine. While (88%) of cases required respiratory failure, Liver function impairment, renal dysfunction and cardiac failure occurred in (43%), (17%) and (50%) of cases respectively (Guet al., 2007). Virus infected cells are detected in the myocardium, adrenal glands and pancreas. Neurons & the brain also become infected. In highly pathogenic form, the illness in chickens and turkeys is characterised by a sudden onset of severe symptoms and a mortality that can approach 100 % within 48 hours (Swayne and Suarez, 2000).

Blood chemical parameters, including aminotransferases (sALT, sAST), total protein, Albumin, globuin, Glucose, creatinine, uric acid, lactate result obtained by other authors.

Serum alanine aminotransferase (ALT), also called serum glutamic pyruvic transaminase (sGPT), value has been widely used as sensitive laboratory parameter in clinical practice to evaluate the degree of liver injury. Otherwise aspartate transaminase (AST), also calledserum glutamic-oxaloacetictransminase (s GOT), is a nonspecific for the liver but is considered to be the "liver enzyme" in birds (Kaplan, 1979). Our results explained that both serum aminotransferases ALT and AST values were found to be increased in avian flu cases. Our present study showed an increasing in the mean ALT values (43.1±3.7U\L) in Group II of an infected chicken Baladi in comparison with healthy chicken control group. Similarly, group IV of an infected duck Baladi group showed an elevated ALT level (57.8±6.1U\L). Our results areon the same line with Sedyaningsihetal.(2007) who stated that H5N1 avian flu virus may cause slightly or moderately elevation in aminotransferases levels. Also our results in the present study revealedan elevation in the ASTmean values in both Group II of an infected chicken Baladi300.2±14.8(U\L) and group IV an infected duck Baladi272.8±9.0(U\L) which in accordance with Sedyaningsih*etal*.(2007).

The obtained results explained that mean values of serum total protein for Group II of an infected chicken Baladi3.3±0.2(g\dl) and of group IV of an infected duck Baladi3.7±0.2(g\dl) showed a decreasing in a comparison with healthy control groups. This might be attributed to liver damage. Our results is in

agreement with (Oladele*et al*., 2005) who stated that the total protein concentration may decrease in birds having severe liver damage that the total protein is not synthesized in the right quantity, because the liver is responsible for protein synthesis, and any disease condition that interferes with the normal the function of the liver may lead to decrease in the concentration of total proteins.

Abdu *etal*.(2005) stated that decrease in the serum albumin concentration in clinically sick birds might be due to necrosis & degeneration of the liver and renal tubules & glomerular necrosis of the kidney. HPAI has been associated with liver and kidney pathology, and this lead to albumin loss.

In accordance with Abdu *etal*.(2005),theresults in the present study show that both group II of an infected chicken Baladi and group IV of duck Baladi infected had a decreased albumin levelswhich were 1.85±0.15(g\dl), and 1.8±0.17(g\dl) respectively. Furthermore the present study showed that, group II of an infected chicken Baladiand group IV of an infected duck Baladidemonstrated a decreased globulin levels which were1.4±0.12(g\dl) and 1.9±0.2(g\dl) respectively.

Although, it is expected that clinically sick bird should have higher globulin levels than apparently health birds during infections, because globulins (especially gamma globulin) essentially produce antibodies that act against the disease agents during infection, the decrease in globulin levels in serum of infected ducks and chickens may be caused by enteropathies and nephropathies associated with HPAI in which the levels of globulins may fall due to protein loss.Similarly, it was reported that AI virus isolates may induce the fore mentioned condition in form of severe lymphoid necrosis in the spleen, bursa of fabricius, intestine, liver and kidney that lead to destruction of lymphocytes which in turn results into depletion in globulins (Abdu *etal*., 2005).

Blood glucose levels in the bird are significantly greater than in the cat and dog and can go higher with stress. Diabetes mellitus is uncommon in birds. Our results revealed that group II of an infected chicken Baladihad an elevated serum glucose level 305.7±57(mg\dl). Furthermore, the present study showed an increased levelreached 276±44 (mg\dl) for an infected ducks in group IV, which in accordance with Sedyaningsihetal. (2007) who stated that marked hyperglycemia may occur due to infection with avian influenza virus. However, serum glucose does not elevated in ducks over the normal range references.

Serum creatinine is a high sensitive parameter to indicate the kidney function. The present study explained that serum creatinine levels showed an elevation in group II of an infected chicken Baladi1.28±0.08 (mg/dl), and in group IV of an infected ducks 0.96±0.09 (mg/dl). Our results in the present study agree with Abdu etal.(2005) and Sedyaningsih *etal*.(2007).

Uric acid is the main nitrogenous waste in avian species and an elevation occur following significant disease in the kidneys. It is the major end product of deamination of amino acids in avian species and is excreted by the kidney mainly by tubular excretion. Since the rate of secretion is largely independent of the state of hydration, the measure of uric acid is the most reliable method to assess renal function in birds (Amand, 1985). In this study group II of an infected chicken Baladi revealed serum uric acid level 11.1±0.33(mg/dl), this range is near to that found in control group (group I). That could be explained as the infection has not affected on uric acid concentration. While, in duck Baladi infected group IV showed slightly increasing in the serum uric acid 6.92±0.23(mg/dl) as compared with healthy control group.

Calcium is a mineral required by all animals and birds as an essential part of their diet, along with other elements such as phosphorus, magnesium, potassium, sodium, iron etc. Bone and shell structure depend on a combination of calcium and phosphorus in the right ratio; and the uptake and assimilation of calcium which is linked to Vitamin D3.Swayne and Suarez (2000)were found that one of avian influenza symptoms is soft-shelled eggs, cessation in egg production or weight loss due to low calcium concentration in the blood (hypocalcaemia). In this study, Group II of an infected chicken revealed a decreased serum calcium level 4.8±0.25(mg/dl), while group IV of an infected duck Baladi shows a decreased calcium level 4.4±0.37(mg/dl). These results were in agreement with (Swayne and Suarez, 2000).

Lactate dehydrogenase (LDH) is a non-specific enzyme in birds, similar to mammals. Liver disease, skeletal muscle damage, and cardiac disease most commonly contribute to an elevation. Sampling error resulting in damaged blood cells can also elevate LDH as the blood cells release this enzyme(Campbell, 1997).

The results within hand indicate that group II of an infected chicken Baladi revealed an elevated LDH level 1447.6±113(mg/dl),furthermore group IV of an infected duck Baladishowed that elevated LDH 1204.8±103 (mg/dl). This elevation may be due to the myocardium infarction in accordance with (Swayne and Suarez, 2000).

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