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Hemato-biochemical Profile of Ostriches (*Struthio Camelus*) based on Gender and Age of Birds

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ABSTRACT: The present study was conducted to determine the hematobiochemical analysis of ostrich (*Struthio camelus*) with respect to gender and progressive age. A total of 40 apparently healthy ostriches of either sex were spitted into young (subgroups; upto 1 year, 1 to 2 year, 2 to 3 year) and adult groups (subgroups; 3 to 4 years and > 4 years). Blood samples were collected from the wing vein of the ostriches maintained in one of the ostrich farms near the Gogra in Pakistan. For hematobiochemistry analysis, the serum samples were obtained by centrifugation of collected blood samples and kept in 1ml aliquots. The hematobiochemical parameters included total protein, glucose, urea, uric acid, creatinine, aspartate amino transferase, alanine aminotransferase, gamma Glutamyl transferase, lactic dehydrogenase, alkaline phosphatase, cholesterol, triglycerides, sodium, potassium, calcium, phosphorus and magnesium. The results showed a significant age-related difference in all serum biochemical values, however, the non-significant difference were observed in males and females within the same age group. The mean values of cholesterol, HDL-C, uric acid and creatinine were non-significant ($P>0.05$) between two age groups from young to adult age and between the sexes of the same age group. The statistical analysis explained that sodium (Na) and potassium (K) values were significantly ($P<0.05$) increased in young than the adult ostriches. Keeping in view these findings, we may conclude that this preliminary study embarked on establishing a set of reference values in serum biochemistry of ostrich in Pakistan.

Keywords: kidney function test, liver function test, minerals, Serum biochemistry

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INTRODUCTION

Poultry production systems have been integrated with human livelihoods for thousands of years, enhancing diet, income, and food and nutrition security (Shoaib et al., 2020; Yasmin et al., 2020; Farooq et al., 2021). Ostrich (*Struthio camelus*) belongs to a family of flightless birds (Ratites) and lives in deserts and savannahs of Africa and the Arabian Peninsula. The ostrich is called a camel bird due to its similarities with the dromedaries. It was denominated by Linnaeus in 1758, like *Struthio camelus* (Deeming, 2001). An adult ostrich, the largest non-flying bird in the world, weighs about 150 kg and is mainly raised for meat production because of its low cholesterol and fat content (Mancevica and Mugurevics, 2013; Elhashmi et al., 2021). Ostrich farming is a developing industry all over the world. This expansion in the ostrich farming over the globe is observed more pronounced in the past two decades (Al-Sobyail et al., 2011; Zaima et al., 2021a). Current survey indicates that about 0.55 Million ostriches are slaughtered per annum globally. Initially, in Pakistan, ostrich farms started as a part of the agro-tourism industry, but since then, ostrich meat (volais) is gaining wide acceptance (Zaima et al., 2021b).

In ostrich farming, the birds are more susceptible to health issues that results from unappropriated management of the breeders that results in environmental and nutritional stress (Miranda et al., 2008). To assess the health status of birds, hematobiochemical and specific plasma metabolites provide valuable information that can be helpful for the disease evaluation. These parameters are not only influenced by nutrition, body scoring, gender, age, captivity and circadian rhythm but can also be taken as physiological indicator that act as relevant diagnostic tool in veterinary medicine (Durgun et al., 2005; de Almeida et al., 2018; Moryani et al., 2021). These blood standards have specific values for sex, age and species. Another advantage is that they reflect diet and management practices, which explains why they are essential in the evaluation of clinical and nutritional disorders that affect birds (Blue-McLendon and Green, 2010)

For successful ostrich farming, diagnosis and monitoring of ostrich health need to be done routinely. Thus, clinical hematology and serum biochemistry are widely used as a diagnostic tool. No serum biochemistry parameters have been published or compiled for an ostrich in the Pakistani environment. Hence, this preliminary study was embarked on to establish a set

of reference values in serum biochemistry of ostrich in Pakistan.

MATERIALS AND METHODS

Ethical Concern

Ethical clearance for this research was granted by the Animal Care and Use Committee of University of Agriculture, Faisalabad-Pakistan (Ethical clearance number:962). No bird was harmed otherwise and no health hazard to the handlers of such type of slaughtering.

Experimental Groups

A total of 40 clinically healthy ostriches of either sex (20 males, 20 females) comprising five age groups of equal size (n=8) viz; young one (Up to 1 year, 1-2 year and 2-3 year) and adult (3-4 year and above 4 years) were used in this study (Table 1).

Table 1. Grouping of young and adult birds.

Groups	Age	Male	Female	Total
Young Group	Up to 1 year	4	4	8
	1-2 year	4	4	8
	2-3 year	4	4	8
Adult Group	3-4 year	4	4	8
	Above 4 years	4	4	8
Total		20	20	40

Collection of Blood Samples

Blood samples were collected from the wing vein of the ostriches maintained in one of the ostrich farms near the Gogra in Pakistan. Two test tubes were used for the collection of blood samples from each ostrich. Serum was separated by centrifugation @ 500g for ten minutes of blood and collected in 1ml aliquots following by storage at -20°C. Then serum samples were used for respective analytical determinations.

Biochemical Analysis

Glucose

The level of glucose in serum was measured with the help of a commercially available diagnostic kit, which is known as Flutiest® GLU- Analyticon diagnostic kit.

Serum Lipid Profile

Total serum cholesterol and triglycerides (mg/dL) were measured with the help of a commercial reagent kit by Dia Sys Diagnostic Systems (Cat. No. 5760) USA while HDL-Cholesterol (mg/dL) in serum was

measured with the help of a commercially available reagent kit (Randox Laboratories Limited, UK). To calculate VLDL (Very low density lipids) and LDL (Low density lipids) were measured from following formulas

$$\text{VLDL} = \frac{\text{triglycerides}}{5}$$

$$\text{LDL} = \text{HDL} + \text{total cholesterol} - \text{Triglycerides}$$

Serum Proteins

The total proteins and albumin in serum were measured with the help of a commercially available Monoreagent Diagnostic Kit, K031, prepared by Bioclin®, laboratory, München, Germany.

Liver Function Markers

The concentration of ALT (Alanine transaminase) and AST (Aspartate aminotransferase) in serum were measured with the help of a commercially available diagnostic kit, which were prepared by Randox laboratories, UK and its reference number BT294QY.

Serum Enzymes

The concentration of ALP (Alkaline phosphatase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by ALP Fortress diagnostics, limited, Bioclin® München, Germany. The concentration of GGT (Gamma-glutamyl transferase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by Transferase GGT kinetic diagnostic kit. The concentration of LDH (Lactate dehydrogenase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by LDH Fortress diagnostics, limited, Bioclin®.

Renal function test

The concentration of creatinine in serum was measured with the help of a commercially available reagent kit prepared by the Creatinine Ecoline Merck diagnostic kit while Breuer and Breuer diagnostic kit was used for urea and uric acid determination in serum.

Mineral Profile

Some macro minerals like calcium (Ca), magnesium (Mg), phosphorus (P), sodium (Na) and potassium (K) were determined in serum. Ca, Mg and P concentration in serum was determined through the wet

digestion process and its absorbance was determined by the atomic absorbance spectrophotometer. Sodium and potassium concentration in serum was measured through a flame photometer.

Statistical analysis

Factorial one-way analysis of variance (ANOVA) was used to compare the means of parameters. Tukey's test was used to compare the group's mean at a 5% level of significance

RESULTS

Serum Glucose

Analysis of variance of glucose in young and adult ostriches of both sexes are presented in Table 2. The values of the glucose showed increasing trend ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and maximum value was observed in above 4 years group (Fig 2). Statistical analysis described the mean values of glucose were significantly ($P < 0.05$) different among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and adults group (3-4 year and above 4 years), but these values showed a non-significant ($P > 0.05$) difference between the sexes of the same age group and between the adults group. A rapid increase in the parameters of serum glucose were observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Serum Lipid Profile

Statistical results described that the mean values of cholesterol and HDL-C were non-significant ($P > 0.05$) among the five age groups from young to adult age and between the sexes of the same age group. However, significant ($P < 0.05$) increasing trend was seen in the mean values of triglycerides, LDL-C and VLDL-C from young to adult age (Fig 2). The maximum ($P < 0.05$) value of these parameters was measured in the adult ostriches regardless of gender as shown in table 2 and Fig 2.

Serum Proteins

Analysis of variance of total serum proteins and albumin young's and adult ostriches of both sexes is presented in Table 2. The statistical analysis showed that the age is directly related to serum proteins values (Fig 2). The mean values of the total serum proteins and albumin increased ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years). Mean values of total serum protein and albumin were

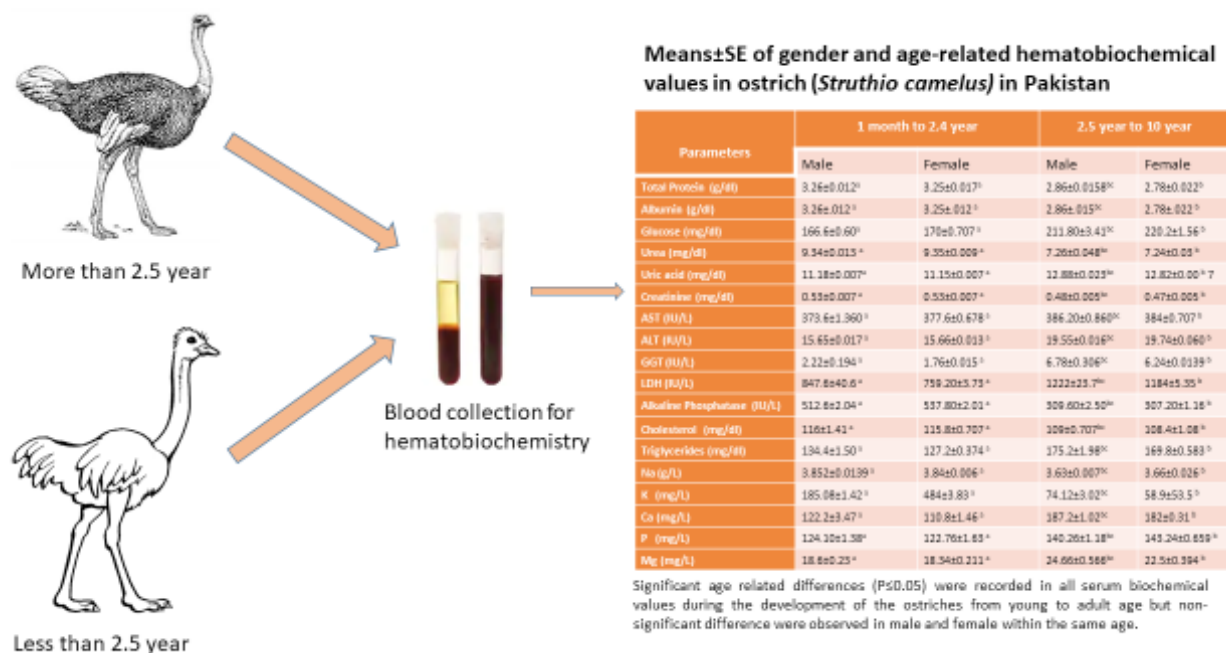


Figure 1. Schematic Representation of Hematobiochemistry

Table 2. Means ± SE values of different age-related hematobiochemical values in ostriches (*Struthio camelus*).

Parameters	Means±SE (n=40)	Male (n=20)	Female (n=20)	Young Group			Mature Group		
				Upto 1 year (n=8)	1-2 year (n=8)	2-3 year (n=8)	3-4 year (n=8)	Above 4 year (n=8)	
Serum Glucose (mg/dl)	196.8±10.2	199.6±8.32 ^a	197.8±8.21 ^a	166.6±0.60 ^a	180±0.707 ^b	207±1.88 ^c	211.80±3.41 ^d	220.2±1.56 ^d	
Serum Proteins (g/dl)	Total Protein	3.81±0.47	3.84±0.55 ^a	3.71±0.44 ^a	2.56±0.01 ^a	2.96±0.02 ^b	3.88±0.05 ^c	4.86±0.08 ^d	4.78±0.08 ^d
	Albumin	2.98±0.34	3.28±0.46 ^a	3.11±0.49 ^a	1.98±0.01 ^a	2.26±0.02 ^b	2.91±0.06 ^c	3.76±0.04 ^d	3.58±0.05 ^d
Serum Lipid Profile (mg/dl)	Cholesterol	110.38±0.91	113.53±1.12 ^a	111.25±1.01 ^a	110.1±0.21 ^a	111.8±0.71 ^a	113±1.41 ^a	109±0.71 ^a	108.4±1.08 ^a
	Triglycerides	152.96±9.52	156.79±12.92 ^a	153.36±11.52 ^a	127.2±0.37 ^a	134.4±1.50 ^b	158.2±1.56 ^c	175.2±1.98 ^d	169.8±0.58 ^d
	HDL	29.01±9.80	32.01±6.68 ^a	331.12±5.98 ^a	27.24±0.18 ^a	28.40±0.38 ^a	28.40±0.36 ^a	30.74±0.59 ^a	30.30±0.07 ^a
	LDL-C	65.24±9.80	68.34±7.80 ^a	66.41±7.65 ^a	39.80±1.31 ^a	46.04±0.82 ^a	69.80±11.31 ^a	78.9±17.3 ^b	91.64±0.96 ^b
	VLDL	31.22±1.86	34.22±2.61 ^a	33.21±2.53 ^a	25.44±0.07 ^a	29.88±0.31 ^b	30.24±0.09 ^c	35.04±0.39 ^d	35.52±1.62 ^d
Serum Enzymes (μkat/l)	GGT	4.37±1.02	4.92±2.10 ^a	4.97±2.02 ^a	1.76±0.02 ^a	2.22±0.19 ^a	4.86±0.04 ^a	6.78±0.31 ^b	6.24±0.01 ^b
	LDH	1030.6±94.7	1030.6±89.62 ^a	1080.4±88.7 ^a	759.20±3.73 ^a	847.6±40.6 ^b	1140.10±15.56 ^c	1222±23.7 ^d	1184±5.35 ^d
	ALP	431.1±50.7	470.1±57.87 ^a	467.1±56.45 ^a	488.7±1.16 ^a	512.6±2.04 ^b	537.80±2.01 ^c	309.60±2.50 ^d	307.20±1.16 ^d
Liver Function Test (μkat/l)	AST	391.02±5.67	397.23±6.77 ^a	395.12±6.71 ^a	373.6±1.360 ^a	381.6±1.21 ^b	397.6±0.74 ^c	402.20±0.86 ^d	400±0.71 ^d
	ALT	16.92±1.44	17.72±2.54 ^a	17.62±2.54 ^a	11.98±0.01 ^a	15.65±0.02 ^b	17.66±0.04 ^c	19.55±0.06 ^d	19.74±0.06 ^d
Kidney Function Test (mg/dl)	Urea	8.53±0.57	9.45±0.68 ^a	9.43±0.67 ^a	8.66±0.05 ^a	9.34±0.03 ^b	10.13±0.05 ^c	7.26±0.05 ^d	7.24±0.05 ^d
	Uric acid	11.64±0.53	12.76±0.65 ^a	12.56±0.69 ^a	10.18±0.008 ^a	11.18±0.01 ^a	11.15±0.01 ^a	12.88±0.02 ^a	12.82±0.01 ^a
	Creatinine	0.49±0.02	0.48±0.03 ^a	0.47±0.03 ^a	0.44±0.006 ^a	0.53±0.01 ^b	0.53±0.01 ^c	0.48±0.01 ^d	0.47±0.01 ^d
Serum Mineral Profile (mg/L)	Na	4.24±0.33	4.88±0.68 ^a	4.74±0.65 ^a	3.85±0.01 ^a	4.84±0.04 ^b	5.21±0.06 ^c	3.66±0.026 ^d	3.63±0.007 ^d
	K	130.2±5.29	133.2±6.72 ^a	131.2±6.29 ^a	150.08±1.42 ^a	164±2.83 ^b	184±3.83 ^c	78.9±3.53 ^d	74.12±3.02 ^d
	Ca	148.4±15.5	151.42±17.85 ^a	150.4±16.85 ^a	110.8±1.46 ^a	122.2±3.47 ^b	140±1.37 ^c	182±0.31 ^d	187.2±1.02 ^d
	P	130.82±5.29	133.82±7.29 ^a	131.61±6.82 ^a	115.10±1.38 ^a	122.76±1.63 ^b	132.76±1.88 ^c	143.24±0.65 ^d	140.26±1.18 ^d
	Mg	20.69±1.44	21.99±1.97 ^a	21.69±1.84 ^a	16.6±0.23 ^a	18.34±0.211 ^b	21.34±0.51 ^c	22.5±0.394 ^d	24.66±0.566 ^d

abcd: mean values having different alphabet differ significantly from one another (P<0.05).

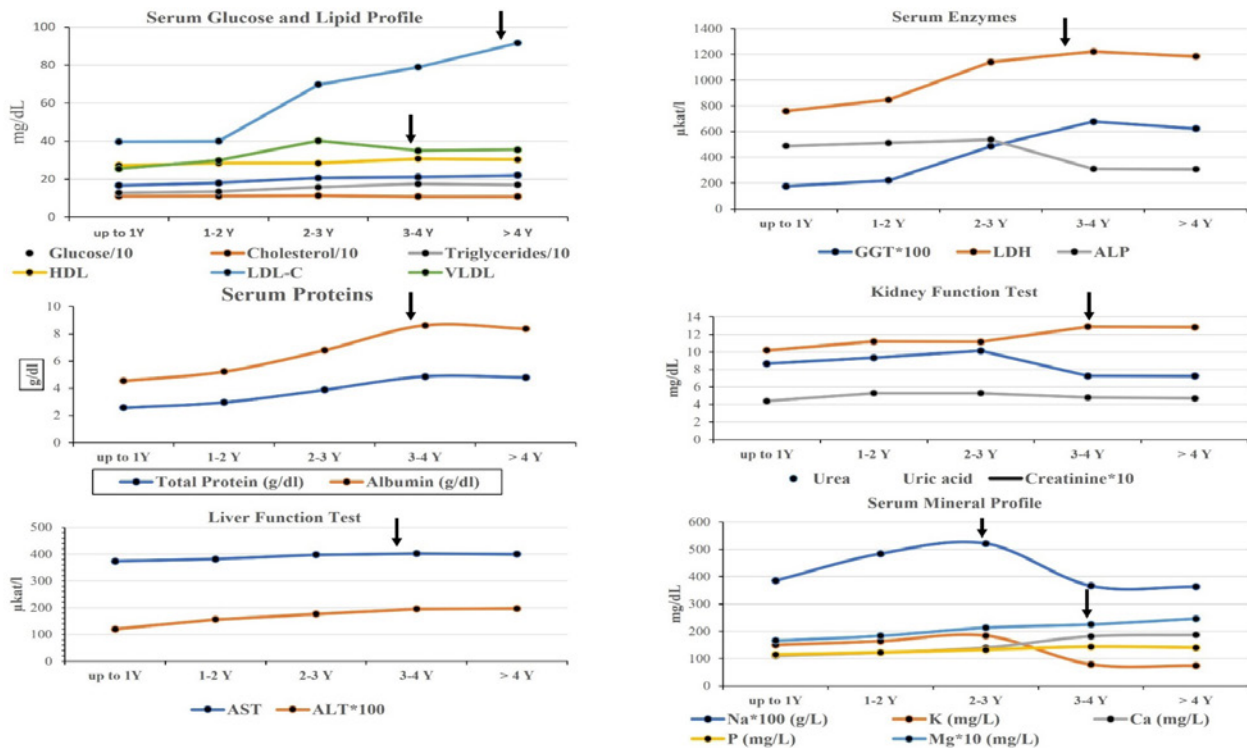


Figure 2. The pattern of different hematobiochemical values in the ostriches in relation with the progressive age. The black arrow showed the plateau of specific value at specific age where it maintained at particular point

significantly ($P < 0.05$) greater in adults as compared to young groups. These values were statistically non-significant ($P > 0.05$) among the sexes.

Liver Function Markers

The mean values of the liver function test, including serum Aspartate transaminase (AST) and serum Alanine transaminase (ALT) levels were presented in Table 2 and fig 3. The statistical analysis showed that the age is directly related to Liver Function values with the highest value in above 4 years birds. The mean values of AST and ALT increased ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years). Mean values of AST and ALT were significantly ($P < 0.05$) greater in adults as compared to young groups. These values were statistically non-significant ($P > 0.05$) among the sexes but significant ($P < 0.05$) differences were observed among the mean values of the young and adult age groups between the adults group. (Table 2 and Fig 2). A rapid increase in the parameters of liver function markers were observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Serum Enzymes

Analysis of variance of Gamma Glutamyl Transferase (GGT), Lactic Dehydrogenase (LDH) and

Alkaline Phosphatase (ALP) in young and adult ostriches of both sexes are presented in table 2. The statistical results of serum enzymes value, GGT and LDH were significantly ($P < 0.05$) greater in the adult ostriches' group as compared with the young ostrich group but the mean values of ALP were significantly ($P < 0.05$) greater in young's as compared adult ostriches (Fig 2). These values showed a non-significant ($P > 0.05$) difference between the sexes of the same age group and between the adult age grouped. Gamma Glutamyl Transferase GGT ($\mu\text{kat/l}$) value was significantly ($P < 0.05$) affected by the age of birds as greater values were observed in adult groups (Table 2). A rapid increase in the parameters of serum enzymes we observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Renal function test

The mean values of urea (mg/dl) showed increasing trend ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and were found significantly ($P < 0.05$) greater in young's as compared to adult groups. Although, these values were statistically non-significant ($P > 0.05$) between the sexes but significant ($P < 0.05$) differences were observed among the mean values of the young and adult age groups (Fig 2). Statistical results described the mean

values of uric acid and creatinine were non-significant ($P>0.05$) between two age groups from young to adult age and also between the sexes of the same age group (Table 2).

Mineral Profile

The mean values of the mineral profile, including Sodium (Na; g/L), potassium (K; mg/L), calcium (Ca; mg/L), phosphorus (P; mg/L) and magnesium (Mg; mg/L) are summarized in Table 2. The statistical analysis explained that Na and K values were significantly ($P<0.05$) increased in the young group than the adult group of ostriches (Fig 2). However, the values of Mg, Ca and P were significantly ($P<0.05$) increase in the adult group of ostriches as compared to the young group of ostriches. The values of all mineral profiles followed non-significant changes in males and females within the same age, as shown in Table 2.

DISCUSSION

The poultry birds are extensively studied in Pakistan other than the ostriches. The ostrich farming is flourishing in Pakistan under the umbrella of Punjab Livestock and Dairy Department. The hematobiochemical parameters are considered the physiological indicators that helps to assess the health status and act as relevant diagnostic tool in the veterinary medicine. Therefore, this study was focused to observe the changes in hematobiochemical parameters of the ostriches in relation to progressive age and to establish a set of reference values of ostrich in Pakistan.

Generally, blood glucose level was greater in birds as compared to mammals (200 to 500 mg/dl). Contrary to mammals, glucose homeostasis in birds was controlled by glucagon because of the abundance of alpha cells in the pancreas (Thrall et al., 2004). In this study, the glucose level of young ostriches in males and females was 170 ± 0.71 mg/dl and 166.6 ± 0.60 mg/dl, respectively, which was in accord with the Khazraiiinia et al. (2006) and Albokhadaim et al. (2012b). De Almeida et al., 2018 reported these values in emu which is lower than our findings of adult ostriches. Significantly ($P<0.05$), greater glucose in adult birds may be attributed to more consumption and type of diet. Moreover, oscillations in glucose levels may be associated with the management of animals, concerning aspects like stress.

Mean values of cholesterol level of different age groups were found no difference ($P>0.05$) and the same trend was described by Khazraiiinia et al. (2006)

and Samour et al. (2011). The same trend was observed in the emu (de Almeida et al., 2018). Serum cholesterol levels for most bird species range from 100 to 250 mg/dL (Lumeij 2008).

The mean values of triglycerides were significantly greater ($P<0.05$) in the adult group of ostriches as compared to the young and the same trend was described by Thrall et al. (2004) and Khazraiiinia et al. (2006), however, Khaki et al. (2012) reports the lower limits than this study. Cholesterol values were recorded a significant change during the end of the egg-laying period of ostriches (Hrabčáková et al., 2014). Despite many published reports showing lower levels of cholesterol and fat in ostrich meat and lower levels of fat in broiler chicken meat compared with cattle and sheep meat, this study showed lower serum lipids in birds.

The mean values of total serum proteins and Albumin were significantly ($P<0.05$) different between two age groups from young to adult age (Table 2) there is paucity of literature describing the age relating changes in ostriches. But De Almeida et al. (2018) reported the comparable values of these parameters in emus. Young birds have a high rate of metabolism due to fast muscle and feather growth that required a high protein diet.

Significantly ($P<0.05$), increasing trend of ALP and AST was seen with the increasing age in ostriches that could be related to the greater metabolic rate, which was directly related to the turnover characteristics of cell and tissue in the growing phase (Bovera, 2007). The values of ALP found in this study were in line with Samour et al. (2011), who reported the value of ALP as 531 ± 198 μ kat/l in young ostriches. Greater levels of ALP indicate elevated osteoblastic activity, trauma and disease condition (Harr, 2006). The AST (μ kat/l) value determined in this study were slightly greater than the values determined by Verstappen et al., (2002) in adult ostriches 321 ± 56 μ kat/l. AST was not necessarily sensitive to liver damage but also used in conjunction with Creatine Kinase (CK), a muscle-specific enzyme (Harr, 2006). The greater value of AST can be linked to the muscle stress and trauma associated with handling during blood sampling (Samour et al., 2011). Gamma Glutamyl Transferase GGT (μ kat/l) value was significantly ($P<0.05$) affected by the age of birds as greater values were observed in group B (Table 2). Verstappen et al. (2002) reported the value of GGT in adult ostriches as 0.25 ± 0.47 μ kat/l, which was not even close to the present study results. GGT value can be increased in inflammation, neoplasia and

biliary obstruction (Harr, 2006). This difference in the present study can be attributed to the different analytical methods and health status of the birds.

Lactic Dehydrogenase LDH values determined by (Samour et al., 2011) in one-years-old ostrich was $437.8 \pm 38 \mu\text{kat/l}$ that was lower than the value of the present study. Age was directly related to the LDH value as in adult birds. These results were comparable with Verstappen et al. (2002), who reported $1107 \pm 470 \mu\text{kat/l}$ level of LDH. These variations in the LDH value might be due to its close association with the skeleton muscles, handling and restraining of the ostrich. Moreover, discrepancies in LDH value may also be due to the difference in sampling time as its half-life is shorter than other enzymes.

The urea level was observed in the current study, falls under the normal ranges (0-10 mg/dl) in non-carnivores' birds but significantly ($P < 0.05$) changed with the progressing age. Urea was excreted by the renal corpuscles, which depends upon the hydration status of the birds and it was used diagnostic tool for the pre-renal azotemia in some birds (Thrall et al., 2004). The current study revealed the numeric value of uric acid showed a non-significant trend among different groups and gender. It was a nitrogenous waste. Its value in birds was generally greater than mammals (Khazraia et al., 2006; Bovera et al., 2007). In birds, uric acid is used as a health indicator because its sequential evaluation gives an idea about the progression and treatment of disease. The mean value of creatinine level in ostriches was similar to the previous studies as measured greater levels in this study (Thrall et al., 2004; Khazraia et al., 2006). The significantly greater levels of creatinine for group B are difficult to explain: the creatinine level is considered an index of muscle metabolism, and a creatinine increase normally occurs when the muscular tissue turnover accelerates. However, this result may be related to the greater levels of AST recorded between the sexes of the same age group. Same trend has been reported in eagles by Nazifi et al. (2008). In this study, the mean values of urea were non-significant ($P > 0.05$)

Na and K values were significantly ($P < 0.05$) increased in the young group than the adult group of ostriches. But the values of Mg, Ca and P were significantly ($P < 0.05$) increase in the adult group of ostriches as compared to the young group of ostriches. The values of all mineral profiles were not so much changed in males and females within the same age, as shown in table 2. In this study, the serum miner-

al values of calcium, phosphorous, and magnesium were significantly greater in adult birds, but sodium and potassium were more elevated in young bird and were in agreement with Bovera et al., (2007). Calcium and phosphorous were essential for laying birds and bone mineralization. Deficiencies in these minerals may lead to different abnormal egg formation and bone. Osteoid matrix deficiencies were also associated with the other micro-minerals like copper, zinc, magnesium, etc. Calcium and phosphorus values were recorded a significant change during the end of the egg-laying period of ostriches. Some variations were observed in the values obtained in the present study and the values mentioned in the relevant literature (Jaime et al., 2010). These differences in observations might be due to variations in management, environmental temperature, sample storage, and the time duration between processing and collection. The present study augments the existing diagnostic tools for health problems and routine monitoring of the health status of ostriches.

CONCLUSION

The information presented in this research paper provides the knowledge with important gender and age-related changes data that can be used for diagnosis of diseases and monitoring of ostrich health that needs to be done routinely. Close scrutiny of the data indicates that the serum biochemical parameters values is rapidly increased during the young age; however, it maintains a plateau with minor increments in the adult age. The sex of the birds had no significant effect on serum biochemical parameters in ostriches (*Struthio camelus*). These findings can be extremely helpful for diagnosis of pathological processes and routine monitoring of the health status of ostriches.

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CONFLICT OF INTERESTS

All the authors declared they have no conflict of interest.

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