Effect of In Ovo Injection with Ascorbic Acid on Hatchability, Hatching Weight and Muscular Gain in Broiler Chickens.

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With 12 figures and 7 tables. **Received October 2023, accepted January 2024**

Abstract

The present study was conducted on a fertile eggs of total number 200 of broiler Ross 308 from 31weeks laying flock, their weight ranged from (49.2-58.9gm). The eggs were inserted to an automatic incubator according to standard procedures (37.5 of temperature and 60% of RH). Eggs were candled and un fertilized eggs were removed at day 6 of incubation. At the same day of injection, the remaining were randomly subdivided into 3 groups at incubator each set contain 60 egg. Solutions for injection were freshly prepared at the same day of injection; ascorbic acid was dissolved at physiological saline 0.9 % then fertilized with 0.22Mm filter paper and subsequently placed in incubator 2 hours before injection.

The three groups were: non-injected control group (negative control), saline group injected with 0.6 ml of physiologic al isotonic saline (0.9%), and Ascorbic acid injected group
(3 mg of pure ascorbic acid with 0.6ml of physiological saline). Histological examination of Ascorbic acid group showed highlighting typical structure of hepatic tissue with intact central vein endothelial lining, highlighting obvious regular organization of muscle fibers with multinucleated light vesicular & elongated peripheral nuclei, highlighting obvious improvement along intestinal tissue structure with increasing in intestinal villi length with significant difference than control negative group as well as control positive group. Hatchability percentage noted better in ascorbic group than other groups. Also, platelet count, White blood cells count, monocyte level was better in ascorbic group.

Keywords: In Ovo Injection, Vitamin C, Broiler Chickens, Ross 308, Hatchability, Antioxidant, Liver, intestine, muscles.

Introduction

In contrast to mammals, bird species have a limited amount of nutrient and energy reserves in the viable egg to support embryonic growth and growth in neonates. As the primary energy sources for the nourishment of avian embryos during the pre-hatch period, glucose and glycogen are preferentially used, in ovo injection of high levels of L-ascorbic acid (12mg and 25mg) at day 18 of incubation in amnion may not be detrimental to hatchling quality but may promote embryonic livability (Mousstaaid et al., 2022) . In ovo vitamin C injection can be a tool to improve the carcass weight, production efficiency, and mortality of chickens (Yenilmez*.,* 2022). In ovo feeding of vitamin C at E11 at yolk 3 mg improves hatchability post-hatch perfor-mance, immune status and the antioxidant capacity of broiler chickens (Zhu *et al*., 2020). In ovo injection of L ascorbic acid in amnion (3 to 12 mg per egg) have a positive effect on the post hatch growth, leg muscle development and systemic antioxidant capacity of broilers. Higher injected dosages of ascorbic acid (36 mg per egg) may have the potential to improve broiler meat quality (Zhang *et al*., 2019). In ovo injection of 6 mg ascorbic acid/egg on 15th d of incubation in amnion could have a positive impact on hatchability, intestinal morphology and bone characteristics in broiler chickens, (Soltani *et al.,* 2019). In Ovo feeding of 3 mg vitamin C in yolk at E15 could improve the antioxidant activity and immune function in plasma, corresponding with the lower expression of pro-inflam-matory cytokines in spleen (Zhu *et al*., 2019). in ovo injection of ascorbic acid at 3 mg/egg at 18-day in air cell enhance antioxidant defense system and immune system for newly hatched chicks (El-Senousey *et al*., 2018). In-ovo injection ascorbic acid at albumin at level of 6 µg AA/100 µL water of eggs incubated at high temperature did not minimize the Negative effects of high rearing temperature on the performance and bone development of broiler chickens (Sgavioli *et al*., 2016). Injection of 4.5 mg GSE/egg on 18th d of incubation at air cell may increase hatchability of broiler chickens and it had no adverse effect on broiler chicken performance during starter period. Furthermore, its effect was almost similar to the effect of ascorbic acid (3mg) (Hosna *et al*., 2014). Vitamin C injected into air cell of chicken eggs failed to influence hatchability. But In duck eggs injection on the 20th day of incubation with selected doses of vitamin C (4 and 8 mg/egg) improved hatchability by decreasing the proportions of dead and unhatched embryos (Nowacze wski *et al*., 2012). The highest hatchability was obtained from the

group treated with ascorbic acid at 3 mg concentration in air cell injection at 13-day of incubation (Ipek *et al*., 2004).

Materials and Methods

All steps related to animals were approved by the institutional animal care and use committee (**Vet Cu 08072023740).** Fertile eggs of total number 200 of broiler Ross 308 from 31weeks laying flock, their weight ranged from 49.2-58.9gm were used. The eggs were inserted to an automatic incubator according to standard procedures (37.5 of temperature and 60% of RH). Eggs were candled and unfertilized eggs were removed at day 6 of incubation. At the same day of injection at 17th day of incubation, the remaining were randomly subdivided into 3 groups at incubator each set contain 60 egg. Solutions for injection were freshly prepared at the same day of injection, 3 mg of pure ascorbic acid from (DSM) was dissolved in physiological saline 0.9 % then, solution filtered with 0.22Mm filter paper and subsequently placed in incubator 2 hours before injection. The three groups were; Non-injected negative control, Saline group injected with 0.6 ml of physiological isotonic saline 0.9% and Ascorbic acid group (3 mg of pure ascorbic acid injected with 0.6ml of physiological saline) injected in the amnion that identified by candling, the surface of egg was disinfected with 70% ethanol, and the solution injected using 21-gauge needles. Immediately after injection the holes sealed with paraffin wax.

Animal husbandry

The number of hatchings in each treatment was counted as they emerged. The formula used to determine hatchability percent was (number of hatchlings / number of fertile eggs) x 100. The average weight of each group was calculated, the chicks were divided in three-layer cages with free access to food and water, the chicken was kept in a temperature-controlled space where the temperature was first set at 32°C to 34°C. Histological findings of intestine, liver and pectoral muscles were proved and raised for 2 days and comparing weight to the manual of Ross.

Chemical analysis

I: Homogenization method which we take for CK, MDA, GSH.

1. Prior to dissection, perfuse tissue with a PBS (phosphate buffered saline) solution, pH 7.4.cotaining 0.16 mg / ml heparin to remove any red blood cells and clots.
2. Homogenize the tissue in 5 – 10 ml cold buffer (i.e. 50 mM potassium phosphate, pH7.5.1 mM EDTA) per gram tissue, using tissue homogenizer.
3. Centrifuge at 4,000 rpm for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice

II: Blood Glucose Level

1. Blood collected from 4 days old chicks by cervical dislocation.
2. The collected blood inserted in sodium fluoride tube and plasma separated by centrifugation
3. The plasma is preserved at 4 degree Celsius up to 72 hrs.

General histological examination

The procedure for histological preparations is as described by Bancroft and Stevens (2016). Briefly, intestine, pectoral muscles, and liver tissues were sliced to 3-4 mm thick, fixed in 10% neutral buffered formalin (10% NBF), dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin. The paraffin blocks were sectioned with a microtome at (4-6µm) thickness and dyed with Hematoxylin and Eosin stain to study the general tissue structure. H&E-stained sections were examined via using Leica microscope (CH9435 Hee56r brugg) (Leica Microsystems, Switzerland). Histopathological quantitative scoring was evaluated in intestinal tissue for length of intestinal villi, crypt depth, and villi/crypt ratio per cross-sectional area using the image analysis system Leica QWin DW3000 (LEICA Imaging Systems Ltd., Cambridge, England). The most representative six fields were assessed for each section in all groups using 200x magnification via light microscopy transferred to the screen. Records were statistically described in terms of mean and standard deviation (mean ± SD).

Results

In Ovo Injection of Ascorbic acid at day 17th of Incubation have a significant effect on Hatchability percent, time of hatching, weight of hatched chick, platelet count, monocyte count, CK, blood glucose level, MDA, GSH, Liver, intestine, muscles and Antioxidant status of Broiler Chicken. There was a significant increase in hatchability percent in ascorbic group reached about 90%, while it was about 83% in control –ve group and about 86% in saline group. Weight of hatched chick (Table 1) was about 41gm in ascorbic group while was about 40 gm in control –ve and about 40.6 gm in saline group. The group injected with ascorbic acid hatched about 24 hours earlier than control –ve and saline group. Significant increase in platelet count (Fig.1), Monocyte count (Fig.2), WBCs count (Fig.3) in ascorbic group. The injected eggs with ascorbic acid showed a significant increase in ck activity (Fig.4), (Table 2) in pectoral muscle in comparison with control negative and saline groups ($P \leq 0.05$).

The eggs injected with ascorbic acid blood glucose levels of their chicks are significantly higher than both control -ve and saline groups (Fig.5), (Table 3). As shown in (Fig.6) and (Table 4) the eggs injected with ascorbic MDA levels of their chicks are significantly lower than both control -ve and saline groups. The injected eggs with ascorbic GSH Reduced levels of their chicks are significantly higher than both control -ve and saline groups (Fig.7), (Table 5).

Histological findings revealed that negative control group displaying structure of intestinal tissue with normal intestinal villi, lined by simple columnar epithelium with goblet cells in between. Intestinal crypt marked by regular lining

epithelium with light & vesicular nuclei. Standard underlying C.T was also noticed. The muscular layer existed is in its normal appearance (Figs. 8a & 8b). Concerning, positive control group, it showed typical intestinal tissue structure with intact intestinal villi that graded a significant higher villi length than control negative group, lined by simple columnar epithelium, with few goblet cells in between. Typical intestinal crypt emerged the usual lining epithelium along with light & vesicular nuclei & the underlying C.T. is within its normal structure. The muscular layer appeared without any histopathological changes (Figs. 8c & 8d). Regarding ascorbic acid group, it highlighting obvious improvement along intestinal tissue structure with increasing in the intestinal villi length with significant difference than control negative group as well as control positive group, lined by its typical simple columnar epithelium, with goblet cells in between. Additionally, regular intestinal crypt lining epithelium with light & vesicular nuclei is seen, as well as normal underlying C.T. The muscular layer is also intact & with its normal structure (Figs. 8e & 8f). Histological records along liver tissue revealed that the negative control group exhibiting normal structure of hepatic tissue with intact central vein endothelial lining. Hepatic sheets lined with hepatocytes that mostly seemed with light & vesicular nuclei, some appeared with deep basophilic apoptotic nuclei, & others appeared with cytoplasmic vacuolation. Hepatic sinusoids existed in typical assembly between hepatic sheets (Fig. 9a). Positive control group showing intact central vein with normal endothelial lining. Hepatic sheets lined with moderate number of hepatocytes seemed with light and vesicular nuclei, some appeared with deep basophilic apoptotic nuclei, others appeared with cytoplasmic vacuolation. In between hepatic sheets hepatic sinusoids observed with slight dilatation (Fig. 9b). Ascorbic acid group highlighting typical structure of hepatic tissue with intact central vein endothelial lining. Mostly all hepatocytes seemed with light and vesicular nuclei, except scarce ones appeared with deep basophilic apoptotic nuclei, very few ones appeared with cytoplasmic vacuolation. Hepatic sinusoids presented with regular structure in between hepatic sheets (Fig. 9c). Histological sections of pectoral muscle tissue revealed that the negative control group exhibiting slight wavy appearance of muscle fibers, each muscle fiber is multinucleated, mostly with light, vesicular and elongated peripheral nuclei, while few ones appear with deep basophilic apoptotic nuclei. Notice vacuolation between muscle fibers (Fig. 10a). Positive control group showing slight regular muscle structure. Muscle fibers are in wavy arrangement, some of them are multinucleated with light, vesicular and elongated peripheral nuclei, others are with deep basophilic apoptotic nuclei, and also some vacuolation along muscle fibers is observed (Fig. 10b). Ascorbic acid group highlighting obvious regular organization of muscle fibers, mostly appear with mul-

tinucleated light, vesicular and elongated peripheral nuclei. In addition, decline in vacuolation along muscle fibers was noticed (Fig. 10c). Statistical analysis showed a great increase in villi lengths (Fig.11), (Table 6) and Crypt depth (Fig.12), (Table 7) in ascorbic group than control –ve and saline group.

Discussion

The aim of this study is to identify the chemical, histological and immunological effect of ascorbic acid on the hatched chick when injected in ovo at day 17th of incubation in the amnion. The group injected with ascorbic acid hatched about 24 hours earlier than control –ve and saline group, Weight of hatched chick was about 41gm. In ascorbic group while was about 40 gm. In control –ve and about 40.6 gm. In saline group, there was a significant increase in hatchability percent in ascorbic group reached about 90%, while it was about 83% in control –ve group and about 86% in saline group, this in accordance to (IPEK *et al*., 2004). Our investigations agree with Zakria and Al-Anezi (1996) that the best hatchability results for chicken when 3mg/egg of vitamin C were injected but on day 17th not on day 15th of incubation. In accordance with (Hajati *et al.*, 2014), in ovo injection of AA (3 mg/egg) increased the activity of GSH-PX in blood of broiler chickens. Similar to (Ayala *et al*., 2014). The current study found a reduction in the level of plasma MDA in AA group when compared with non-injected group or saline group which was an indication of reduced lipid peroxidation, which is due to a decrease in the oxidative stress. In a previous study, it was observed that the in ovo injection of AA (3 mg/egg) into fertile broiler hatching eggs at 17th day of injection did not negatively affect hatchability or embryo development, this in accordance with (Zhang *et al.,* 2018). Similar to (Tullett, 1990), During late incubation, the generation of excessive metabolic heat from the developing embryos is the primary cause for dead embryos or culling of chicks, reducing the hatchability and in agree with (Du *et al*., 2012), Vitamin C, as a co-factor of hydroxylase may promoting gluconeogenesis and enabling embryos due to higher nutrient content to adapt to the environment of incubation and pip of the eggshell. In agreement with (Yenilmez, 2022), in ovo vitamin C injection improved the post-hatch performance of chicks but we differ in that it increased the oxidative damage in broiler chicks.

Conclusions

Injection of A.A. at dose 3 mg\egg in amnion at day 17th of incubation increased hatchability, increased weight of hatched chick, gave earlier hatching about 24 hours, improve platelet, WBCs, and Monocyte counts, improve intestine, villi length and crypt depth, improve liver function and muscular status and finally decreased oxidative stress on the hatched chick.

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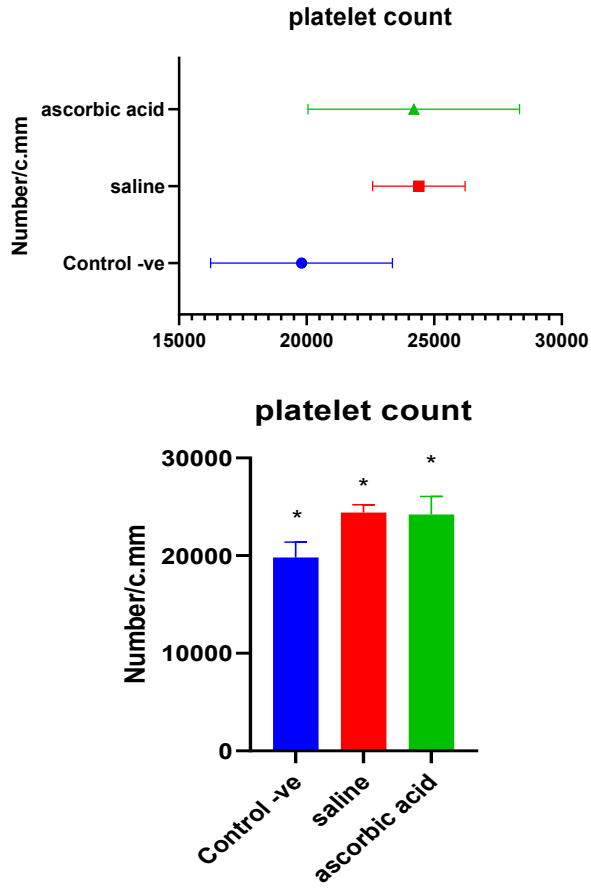


Fig. (1): showing that in ovo injection of ascorbic acid at day 17 of incubation resulted in increase in the number of platelets/c.mm.

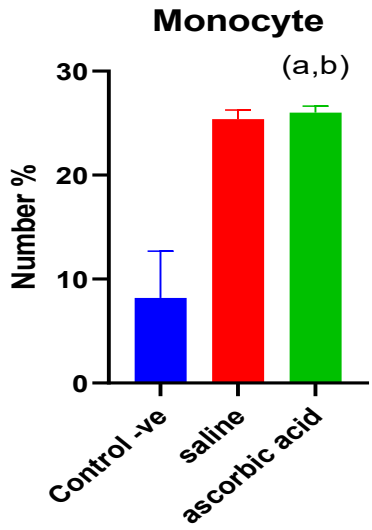


Fig. (2): showing increase in the level of monocytes in both injected groups (saline and ascorbic) in different groups (n=5); data are represented as Mean ± SEM.
(a) Significantly different from the control group at P ≤ 0.05.
(b) Significantly different from the saline group at P ≤ 0.05.

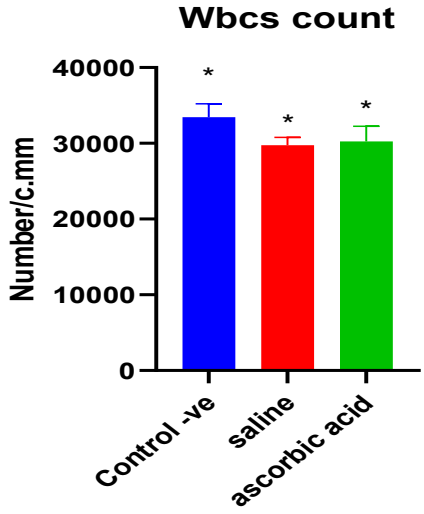


Fig. 3: demonstrated that there is a decrease in the number of wbcs count in both saline and ascorbic acid injected groups in different groups (n=5); data are represented as Mean ± SEM.

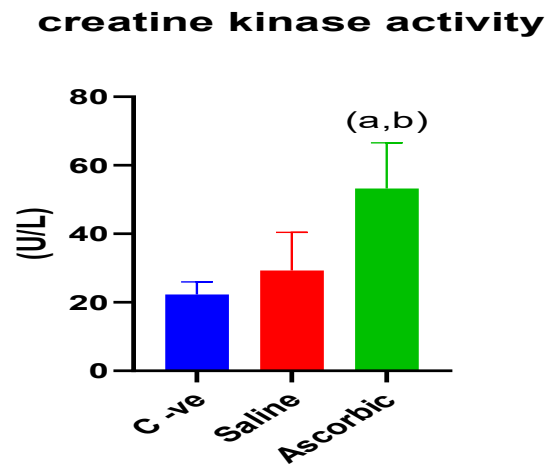


Fig. (4): Creatine kinase activity (U/L) in different groups (n=5); data are represented as Mean ± SEM.
(a) Significantly different from the control group at P ≤ 0.05.
(b) Significantly different from the saline group at P ≤ 0.05.

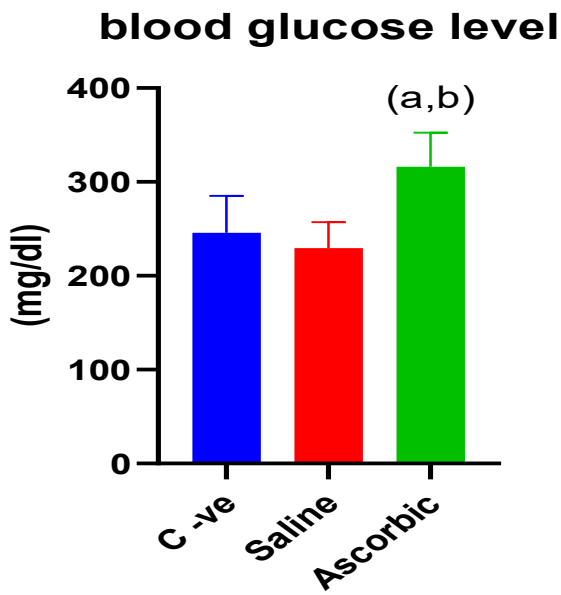


Fig. (5) blood glucose level (mg/dl) in different groups (n=5); data are represented as Mean ± SEM.
(a) Significantly different from the control group at P ≤ 0.05.
(b) Significantly different from the saline group at P ≤ 0.05.

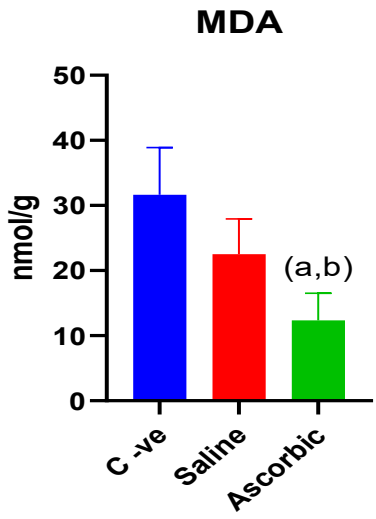


Fig. (6): MDA (nmol/g. tissue) in different groups (n=5); data are represented as Mean ± SEM.
(a) Significantly different from the control group at P ≤ 0.05.
(b) Significantly different from the saline group at P ≤ 0.05.

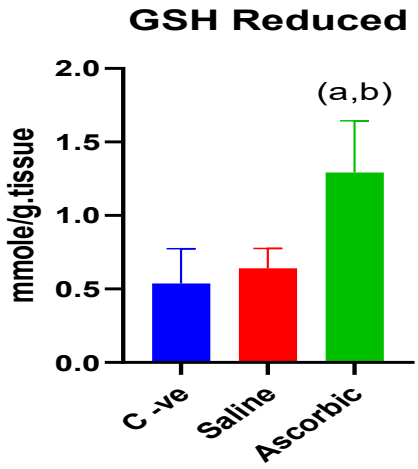


Fig. (7) GSH REDUCED (mmole/g.tissue) in different groups (n=5); data are represented as Mean ± SEM.
(a) Significantly different from the control group at P ≤ 0.05.
(b) Significantly different from the saline group at P ≤ 0.05.

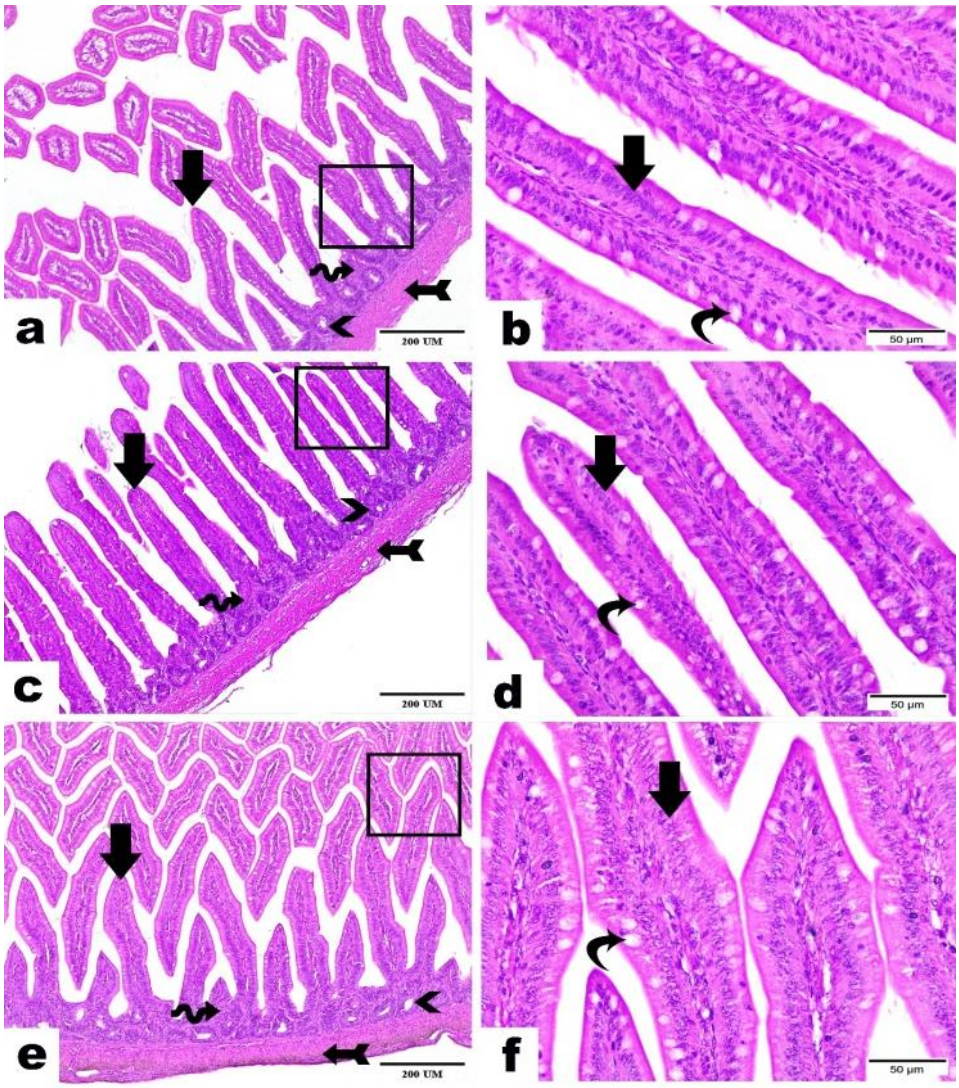


Fig. (8): Photomicrographs demonstrating the histopathological differences in intestinal tissue sections between poultry studied groups (hematoxylin & eosin stain, magnification power= x100, x400 & scale bar= 200µm, 50µm) as follows: Sections from negative control group (a) & (b), positive control group (c) & (d), and ascorbic acid group (e) & (f) exhibiting structure of intestinal tissue with intestinal villi (rectangles), simple columnar epithelium (arrows), goblet cells (curvy arrows), intestinal crypt (arrow heads), propria C.T. (wave arrows), and muscular layer (arrow with tails).

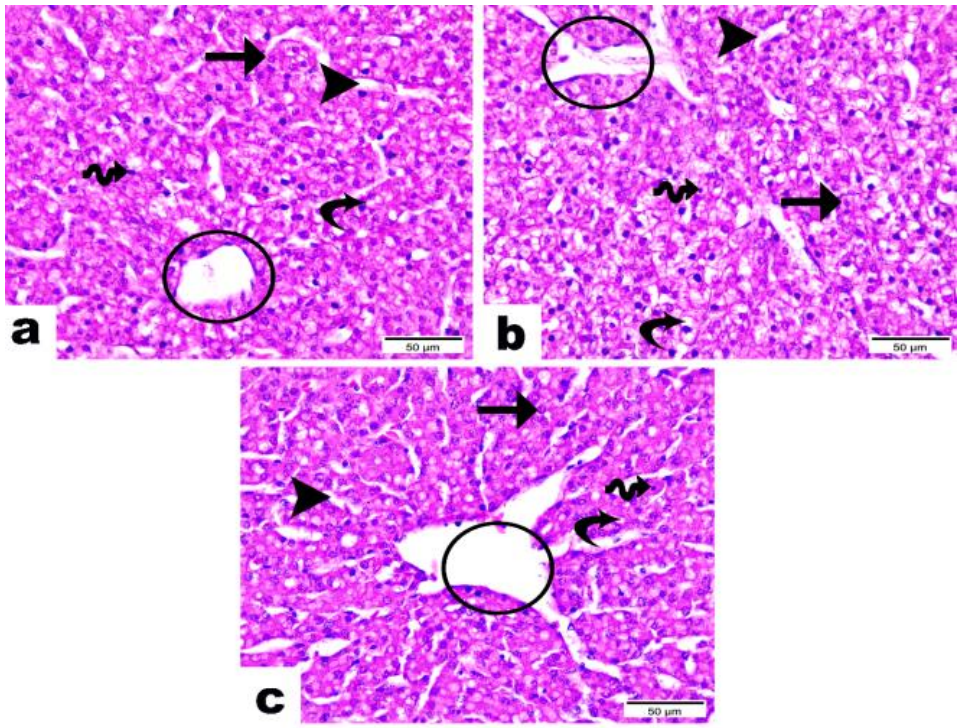


Fig. (9): Photomicrographs demonstrating the histopathological differences in liver tissue sections (central vein area) between poultry studied groups (hematoxylin & eosin stain, magnification power= x400 & scale bar= 50µm) as follows: Sections from negative control group (a), positive control group (b) & ascorbic acid group (c) demonstrating hepatic tissue with intact central vein endothelial lining (circles), hepatic sinusoids (arrow heads), normal hepatocytes (arrows), deep basophilic apoptotic nuclei (wave arrows), & hepatocytes with cytoplasmic vacuolation (curvy arrows).

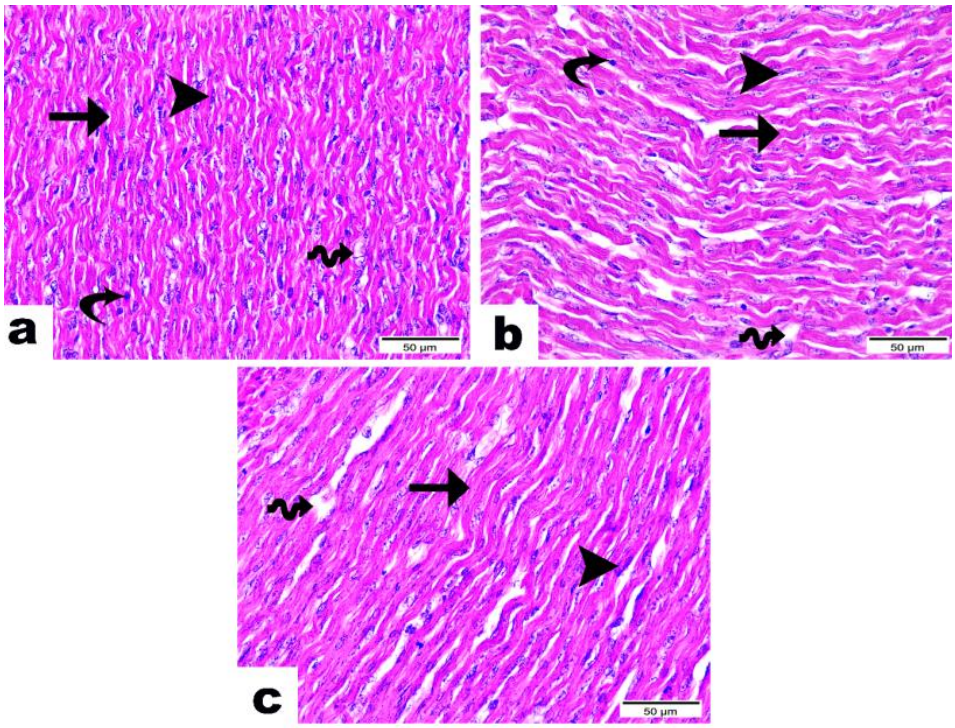


Fig. (10): Photomicrographs demonstrating the histopathological differences in pectoral muscle tissue sections between poultry studied groups (hematoxylin & eosin stain, magnification power= x400 & scale bar= 50µm) as follows: Sections from negative control group (a), positive control group (b) & ascorbic acid group (c) presenting muscle fibers (arrow), light, vesicular & elongated peripheral nuclei (arrow head), apoptotic nuclei (curvy arrow), and interstitial vacuolation (wave arrow).

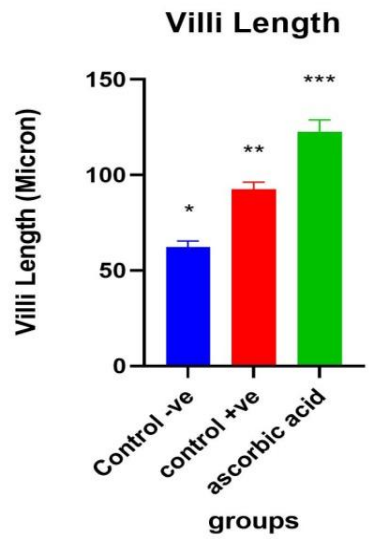
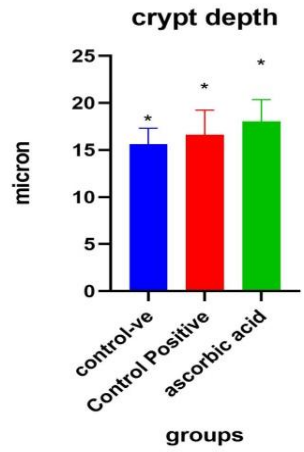


Fig. (11): showing increase villi length in ascorbic group than the other groups.



(Fig. 12): showing increase crypt depth in ascorbic group than the other groups.

Table (1): showing increase hatching weight of ascorbic group than the other groups.

Descriptives

Hatching weight

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum	Between-Component Variance
control _ve		36	40.0417	2.81246	.46874	42.0901	43.9933	37.80	48.90	
control +ve		54	40.6789	2.32010	.31573	40.2456	41.5122	35.16	45.90	
ascorbic		54	41.0067	2.75563	.37499	40.2545	41.7588	34.60	48.40	
Total		144	41.4675	2.75323	.22944	41.0140	41.9210	34.60	48.90	
Model	Fixed Effects			2.61555	.21796	41.0366	41.8984			
	Random Effects				.65727	38.6395	44.2955			1.11855

Table (2): Creatine kinase activity (U/L) in different groups (n=5); data are represented as Mean ± SEM.

C -ve	Saline	Ascorbic
22.28 ± 1.651	29.30±4.986	53.23±5.959

Table (3): blood glucose level (mg/dl) in different groups (n=5); data are represented as Mean ± SEM.

C -ve	Saline	Ascorbic
245.68±17.655	229.64± 12.240	316.04±16.240

Table (4): MDA (nmol/g.tissue) in different groups (n=5); data are represented as Mean ± SEM.

C -ve	Saline	Ascorbic
31.63±3.236	22.53±2.423	12.34±1.865

Table (5): GSH (nmol/g.tissue) in different groups (n=5); data are represented as Mean ± SEM

C -ve	Saline	Ascorbic
0.538±0.105	0.641±0.059	1.292±0.157

Table (6): showing increase villi length in ascorbic group than the other groups.

	Villi Length (Micron)	
Control Negative	**Control Positive**	**Ascorpic Group**
63.374	96.92	125.629
61.376	93.009	130.412
64.566	95.625	119.21
56.171	90.456	124.448
64.988	86.667	112.401
62.834	91.952	123.755

Table (7): showing increase crypt depth in ascorbic group than the other groups.

	Crypt depth (Micron)	
Control Negative	**Control Positive**	**Ascorpic Group**
13.16	**14.422**	**20.223**
15.84	**19.723**	**16.763**
14.299	**15.62**	**20.099**
16.97	**13.038**	**16.124**
15.556	**18.384**	**19.999**
17.83	**18.439**	**15.132**

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