ORIGINAL ARTICLE

CONTENTS

In Ovo evaluation effects of normal saline on cardiovascular system development

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ABSTRACT

Aim: To estimate the effect of normal saline on early development stage of embryo during pregnancy after LD50 dose determination and to record of heart electrocardiogram ECG for chick embryo and make histopathological sections for the heart to find if it suitable for pregnant women.

Materials and Methods: Novel data- acquisition record for embryo ECG, vision findings as well as histopathological study for 42h of development can support the exact harm and sudden death causes for embryo during first months of pregnancy.

Results: LD50 results show significant variation ($p<0.05$) between dose 2µl and other lethal doses and ECG show same variation according to recording in amplitude, QRS, and heart rate on the other hand the histopathological slides show clear teratology signs of veins malformation specially in dose 6, 8, and 10 µl and heart muscle rupture with sign of edema and pathological apoptosis.

Conclusions: Due to harmful effect of normal saline on chick embryo development especially heart and veins which lead to sudden death, they must not use for pregnant women during first 4 months or before pregnancy.

KEY WORDS: *In Ovo* evaluation, embryo cardiovascular system, saline

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INTRODUCTION

Experiments have employed the chick experimental model as a xenograft host for decades. Developmental biology's last uncharted territory is the study of early human development, which can now be done with the chick system [1, 2]. Comparative gene expression profiling between humans and chickens has been done, and the results show that cDC subsets are homologous [3]. Human and chicken dendritic cells link innate and adaptive immune responses, yet these diverse cell types are known to differ significantly. For example, the bursal secretory DCs in the bursa of Fabricius contain a particular subgroup of DCs found in birds but not mammals [4]. The chorioallantoic membrane of the chicken embryo has resurfaced for various uses. Compared to classical models, this one is more advantageous due to its cost-effectiveness, time efficiency, and ease of use. Because the chicken embryo eventually develops an embryonic immune system that is functionally similar to the human immune system, this review illustrates how the chicken embryo can be utilized as a model for immunological-based studies [5]. The chorioallantoic membrane assay for LD50 in chick embryos might provide a quick and low-cost substitute for rodents. It also has minimal bureaucratic barriers and is simple to execute. In addition, this model makes it possible to use a wide range of analytical techniques in Nano toxicological studies, from other side, chick embryo model was the pathogenic and immunization evaluation for a lot of drugs [6, 7]. Numerous studies have shown that the formation of coronary arteries starts when PE mesothelial cells migrate from the liver primordium to the surface of the heart, where they differentiate into a range of cell lineages that constitute different parts of the heart [8]. Thus, the technique that can simultaneously characterize non-invasively the cardiac and body movements (heartbeats) in terms of both strength and frequency could be applied to improve the development of precision poultry production systems and monitor embryos in physiological studies by using ballistocardiography methodology to investigate the non-invasive behavioral pattern of the cardiac and body movements of embryos during the incubation period that relies on an eggshell that has electric charges on it, or a single capacitor plate [9, 10]. A few embryos experienced cardiac failure and passed away in a matter of hours; intriguingly, these embryos have a cardiac failure pattern resembling that of end-stage heart failure in humans [11]. The primary mechanism of electrophysiology and ionic currents in the fertilized egg (embryonic

chicken) is similar to those of mammalian hearts. On the other hand, little is understood about the heart repolarization mechanism during development [12]. By developing ischemic data, the chick embryo model can further our understanding of pathophysiology and help us explore the fundamental mechanisms or possible therapeutic approaches in ischemia-associated disorders. This will increase our ability to treat ischemic stroke in humans [13].

AIM

The aim is to estimate the effect of normal saline on early development stage of embryo during pregnancy after LD50 dose determination and to record of heart electrocardiogram ECG for chick embryo and make histopathological sections for the heart to find if it suitable for pregnant women.

MATERIALS AND METHODS

STUDY DESIGN

Two separate experiments were conducted in two parts: first, for the LD50 study as shown in Fig. 1, and second, the same groups were selected for the cardiovascular toxicology study with control and saline as shown in Fig. 2.

Fertile eggs (*Gallus domesticus*) were selected for this project by incubate 75 eggs after air sac injection with normal saline (locally produced i/v 0.9% normal saline solution) to determine the LD50 dose (by use Hamilton syringe), and all embryo were monitored by non-invasion electrocardiography (ECG), vision test for blood vessels and heartbeat.

The same strain was selected for second experiment (about 105 fertilized eggs) with selected dose and after 42h ECG monitoring, vision test and make histopathological slides to diagnosis of the lesions.

The following measurements, instruments and methods were used:

• Incubation: using a Cimuka PD30 SH incubator with well-calibrated heater and humidity for both experiments;

• ECG: using a IWX214 working physiological system with three electrodes for non-invasive testing 0.3-35 Hz, millivolts;

• Vision testing: after recording the ECG, the same eggs are examined to diagnose changes in blood vessels and heartbeats and compare with the ECG results.

HISTOPATHOLOGICAL STUDY

Embryos from the same tested eggs were dissected using a microtome (3 microns) after formalin fixation and drying to create a block. Whole body eosin/hematoxylin staining was prepared, and cardiac tissue and cell analysis was performed.

RESULTS

In examining the ECG of chick embryo we focused particularly on the relationship between amplitude, QRS segment, between QRS, and heart rate [14], (Fig.3). As indicated in Table 1, a Kruskil-Wallis nonparametric one-way ANOVA test was performed to see if the LD50 differed significantly ($p<0.05$).

Table 1 illustrates how the increased mortality associated with the higher doses, 8µl and 10µl, reduced the doses utilized in the lethal dose in subsequent studies. In addition, not fertile eggs add to the results for accuracy [15].

Table 2 obviously show the ECG reading parameters with significant differences (*P* value <0.05) between control and doses 2µl ,4µl ,6µl respectively for all selective embryo except in two doses 8 µl and 10 µl due to death in early stage (before 42h). On the other hand, clear significant differences between all parameters in amplitude, QRS segment, between QRS, and heart rate.

The mean rank by group interaction (Fig. 4) revealed significant differences (P value < 0.05) between control and 4 µL dose (mean range 23 and 53) in magnitude, with the same p value (< 0.05) between 2 µL, 4 µL and 6 µL doses (mean range 38, 53 and 8, respectively).On the other hand, the QRS segment values show a significant difference between ($p<0.05$) between all doses and control (average rate 28.83, 32.17, 8 and 53) with one exception between control and dose 2µl. Similar rhythm can be clarified for both parameters: between QRS segments and heart rate with significant differences ($p<0.05$) between control and both doses 2µl and 6µl, also p value (<0.05) between 4µl and 6µl doses.

After recording the ECG, a visual inspection of control and chicken embryos injected after 42 hours was carried out. However, the embryo injected with 2 µl showed some malformations compared to the control, while at doses of 4 µl, 6 µl, 8 µl and 10 µl, signs of teratology could be clearly diagnosed (Fig. 5), with a small ligation-like shape in the right vitelline artery. Image (Fig.5B) illustrates the precise method to stop blood flow to the specified area of the chicken embryo vascular bed, thereby blocking blood flow in the surrounding vascular bed. While, in image (Fig.5C) showed the ischemic area of the vascular bed with complete growth retardation. On the other hand, image (Fig.5D) clarified bleeding in the area vascular and surround with clotting was seen at the edge of area pellucid and area vascular. Curvature in the neural tube was seen in some embryos. Image

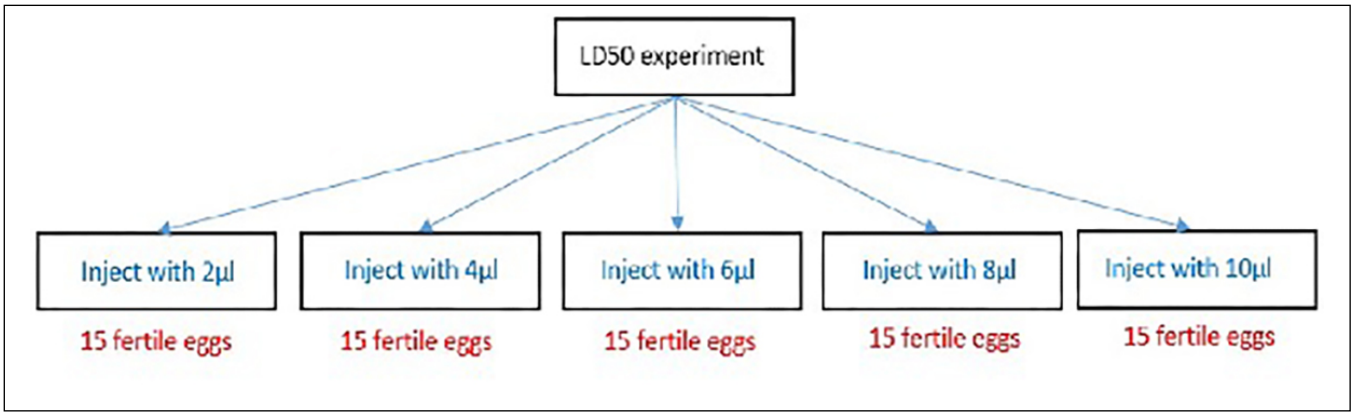

Fig. 1. LD50 study design.

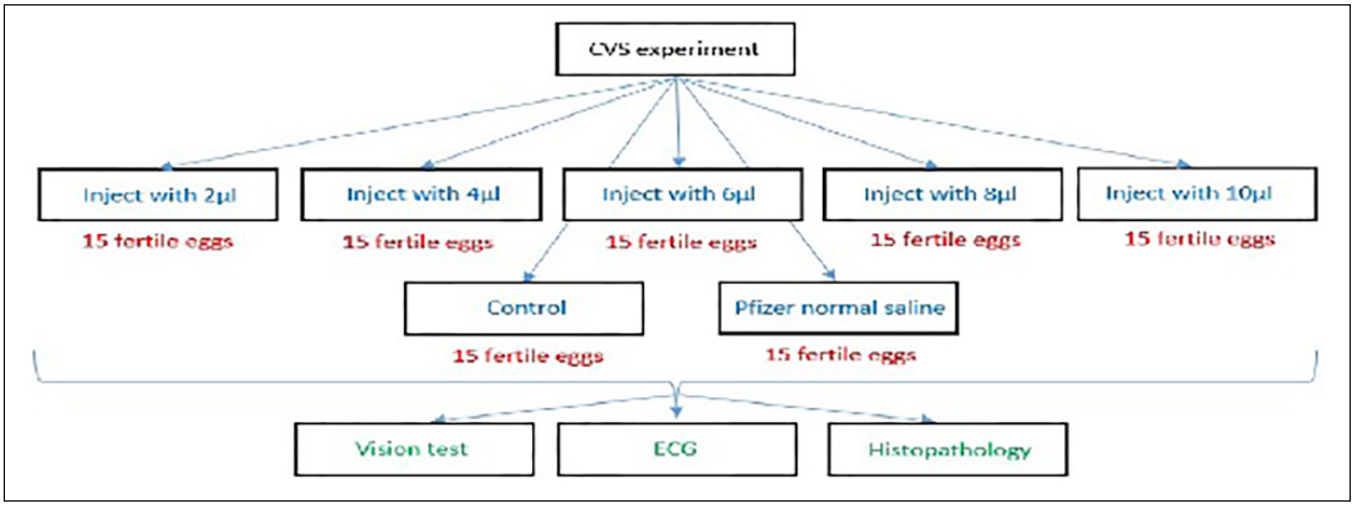

Fig. 2. CVS toxicology study design.

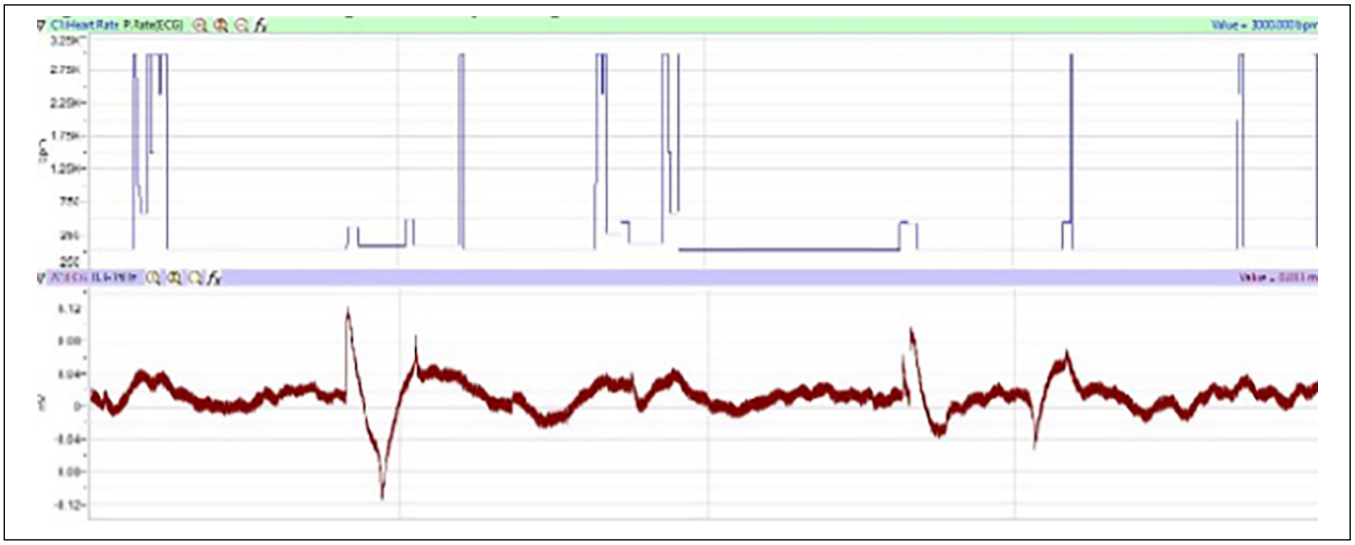

Fig. 3. Chart's surface displays online-calculated heart rate (HR) in BPM below and recordings of ECG signals above in µV.

(Fig.5E) of a completely dead embryo. The control group's hearts were seen to be developing typically. On the other hand, the regular saline-injected group had poorly grown hearts, as seen by the shorter transverse cardiac diameter (Fig. 6A). A histopathological analysis identified certain variations in the morphology of cardiomyocytes and ventricular structures. The embryos in the injected group showed a disordered arrangement of trabecular muscles in the left and right ventricles (Fig. 6B), along with some modifications in the tubular development of the heart. Cardiomyopathy in ventral aorta with primitive cardiac tube enlargement with partial ischemic sign as it shown in figure 7B. These findings suggested that normal saline impeded early embryos'

Table 1. Lethal dose (LD50) for saline solution on chicken embryo

Dose	Numbers	Dead	Live	Not fertile
Control	15	0	15	0
Normal saline	15	2	13	0
2 µl	15	5	5	5
4 µl	15	10	1	5
6 µl	15	10	5	0
8 µl	15	15	0	0
10 µl	15	15	0	0

Table 2. One-way ANOVA test for ECG recordings on chicken embryos

Dose	Parameters							
	Amplitude		QRS segment		Between QRS		Heart rate	
	Mean	SDs	Mean	SDs	Mean	SDs	Mean	SDs
Control	0.042	0.003	0.008	0.033	16.73	0.047	139.2	5.185
2 µl	0.145	0.001	1.888	0.023	5.069	0.017	218.2	1.334
4 µl	0.232	0.019	2.323	0.017	7.143	0.018	179.4	1.454
6 µl	0.017	0.001	1.424	0.015	1.042	0.012	299.6	2.063

*The mean difference is significant at the 0.05 level.

ability to build their hearts typically, which may be a crucial factor in embryonic demise.

DISCUSSIONS

An attempt is made to determine each test group's LD50 based on the data on embryo mortality (Table 1). Since mortality below and above 50% was occurred in various strains on different days, the LD50 values shown in Table 3 are derived on different dosages and days, it's obviously show the increasing of mortality rate start from normal saline group until completely lethal calculation for dose 10µl group compare with control group [16]. The estimated doses at LD50 for the inoculation of chicken embryos with the normal saline was 2µl, 4µl, 6µl, 8µl and 10µl respectively. This result is the first to report the appropriate dose inoculate these chicken embryos lines [17]. It should be taken into account that the embryo mortality on the dose. Therefore, determining the proper infectious dose is crucial; it should not be too high to prevent significant embryonic mortality [18]. Almost the whole incubation period was spent with the chicken embryos moving, but their pattern, pace, and type of movement varied during that time. The data in Fig. 3 show that two distinct forms of embryonic activity were observed during the incubation period: body and heart movements. Since the embryos were still small and immature during the early stages of incubation, body motions had little effect on the signal, which had a frequency between 0.3 and 0.8 Hz and 19–50 beats per minute [19]. The heart activity of chicken embryos causes micro-movements throughout the egg, which alters the lengths between the plates and, consequently, the potential difference between the receiving signal and the shell. At 42 hours after incubation, the first cardiac work single signals were recorded. The signal amplitude climbed non-steadily from 0.042 to 0.232 mV/s, and then abruptly dropped to 0.017 mV/s as the heart rate increased from 139 to 299 beats per minute. They were seen in the early hours before the high-dose group's embryonic fatalities [20]. The QRS segment during embryonic development in fertilized chicken eggs observed a gradual increase in QRS from 0.008 to 2.323mV/s; these results may be related to the expression during embryonic development of the genes encoding the cardiac potassium channels affected in repolarization [21]. On the other hand, visual test shows venous constriction, and after 48 hours of incubation, bleeding and clotting segments obtained from shell-free culture were obtained by transplantation of embryos at the early stage of cardiac loop formation using time-lapse analysis of chicken embryos with normal (left) and defective (right) vasculogenesis. Image series shown starts with embryos show a normal development of the extraembryonic vasculature except for groups 4µl and more after incubation. Nevertheless, the embryo on the right, which received a dose of 4 µl, died from end-stage heart failure with decompensated cardiac function for the group that received a 6 µl dose. A few hours later, the embryo showed signs of cardiac

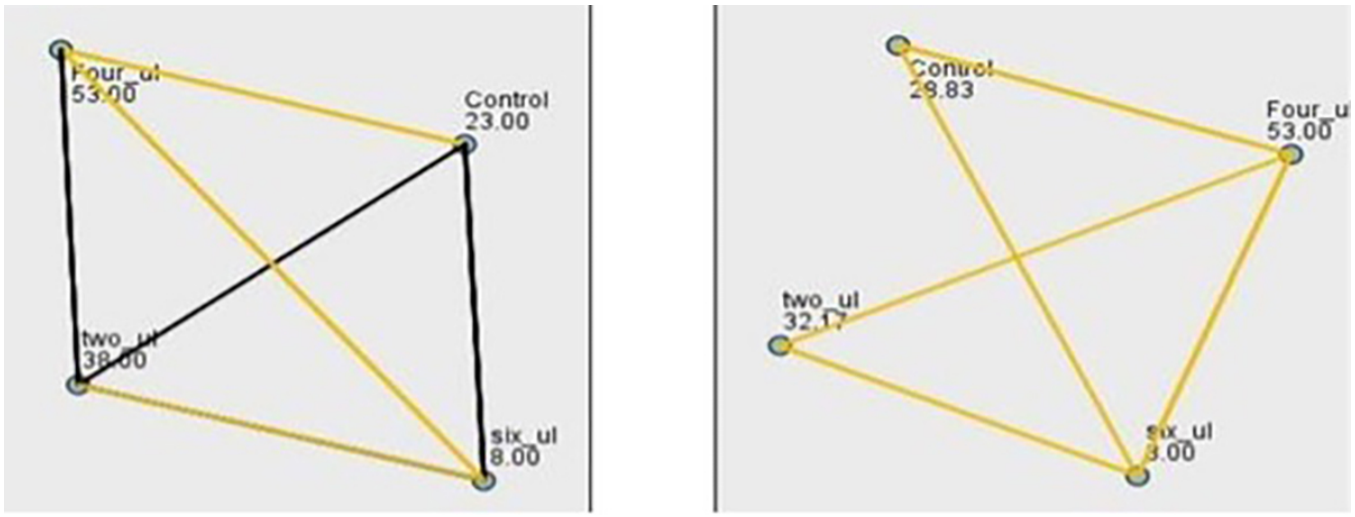

Fig. 4. Pairwise comparison between doses for both amplitude and QRS in chicken embryo.

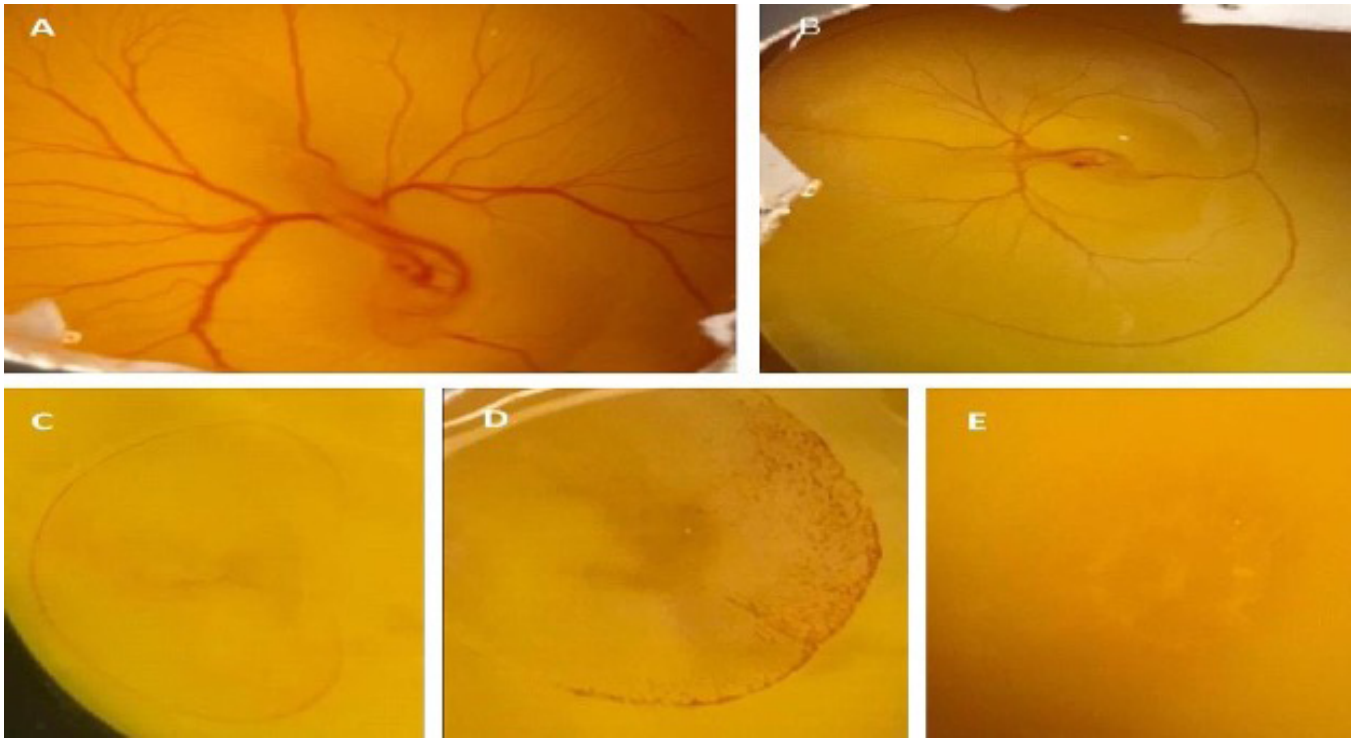

Fig. 5. Morphology (visual test) of chicken embryo at 42h: (A) Control embryo (2.0X); (B) Dose 2µl; (C) dose 4µl; (D) dose 6µl; (E) dose 8µl of normal saline solution.

failure, including peripheral vascular stasis and congested organs due to central pooling. According to the Histopathological investigation, the experimental groups (4µl and 6µl) experienced lesions in their heart tissue due to the doses utilized, which could potentially account for the high dose-related death rate of chicken embryos. The chicks probably died in the early hours, which resulted in histopathological lesions in the heart muscle chicken embryos. However, the other chicken embryos survived since the effect of normal saline was insufficient to cause death. Because the disturbed hemodynamics during the development of the heart valve and ventricle are so important, the results show an immediate decrease in the levels of the left atria ventricle (AV) canal, which leads to a further reduction in wall shell stress levels (WSS) in the left AV canal and relatively increased WSS levels in the right AV canal. According to Syamantak et al., a novel ex vivo ischemia model might be derived from the chick embryo partial ischemia paradigm. As a result,

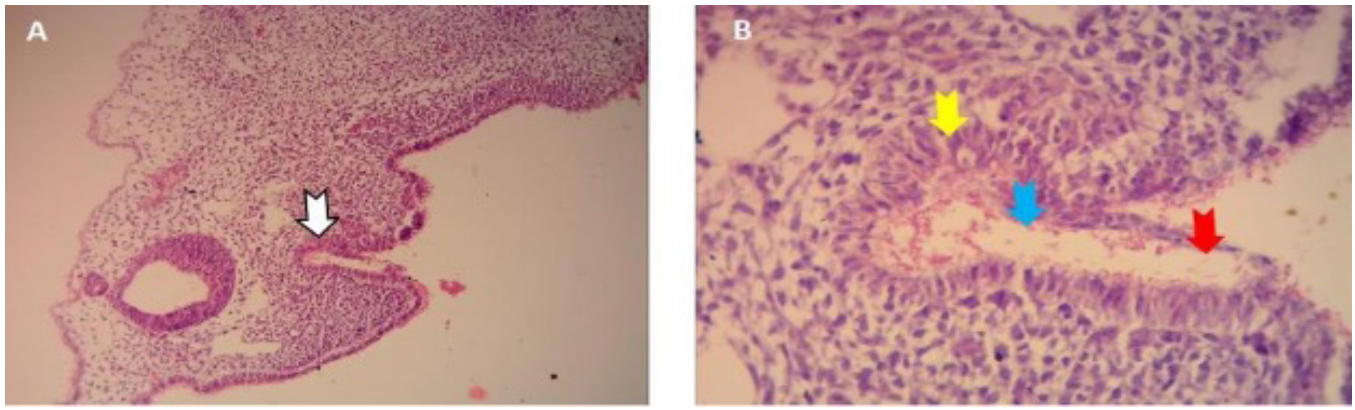

Fig. 6. Microtomes of control group of chicken embryos stained with eosin/hematoxylin: (A) control group 42h with normal tubular heart development (E&H); (B) ventral aorta (yellow), Primitive cardiac tube (Red), Vitelline vein development (E&H) (Blue).

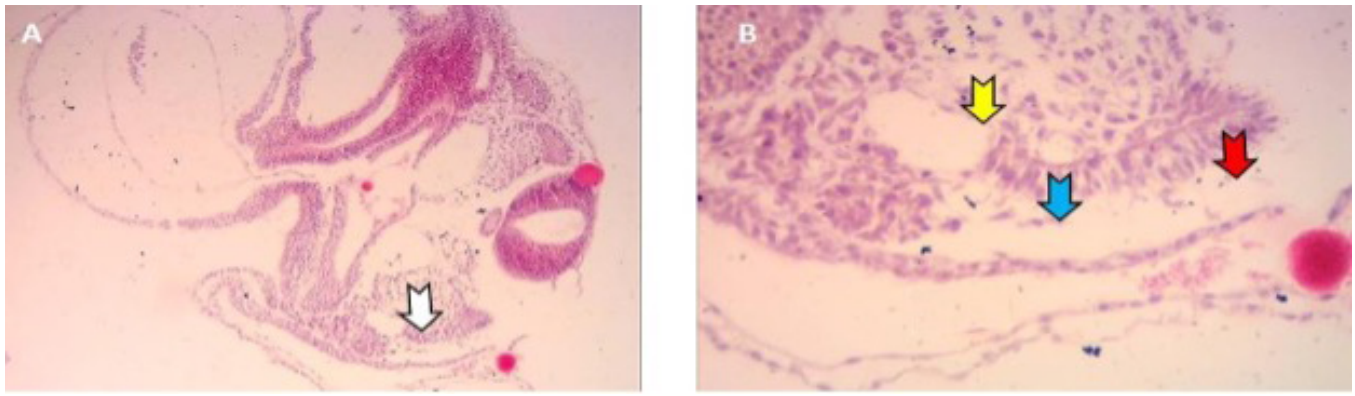

Fig. 7. Microtomes of 2 µl group of chicken embryos stained with eosin/hematoxylin: (A) dose 2, group 42h with moderate changes in tubular heart development (E&H) (Red), (B) ventral aorta cardiomyopathy (yellow), primitive cardiac enlargement (Blue), vitelline vein partial ischemia sign (E&H) 600x (Blue).

the current model can be utilized with established in vivo ischemia models to assess the effectiveness of anti-ischemic medications and gain a mechanistic understanding of the onset of ischemia.

CONCLUSIONS

Normal saline, especially 0.9% NaCl, contains more minerals than the solution itself. Some of these are related to the water source used to manufacture it, such as chlorine in tap water, or may be due to uncontrolled storage conditions that result in some toxic substances being released from the container itself. Therefore, for intravenous prophylaxis, the solution should be used with caution, especially during pregnancy due to its life-threatening effects on embryos in the early months of pregnancy, resulting in sudden embryonic death due to heart problems.

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This work done according to describe euthanasia procedures for chick embryos in various stages of development as well as to ensure euthanasia procedures and it will be our pleasure to introduce all documents and images to support above. The College of Pharmacy's Ethical Committee permitted the study to proceed in some health locations.

CONFLICT OF INTEREST
The Authors declare no conflict of interest

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