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ORIGINAL ARTICLE

The Teratogenic Effect of Cyclophosphamide on the Embryos of Albino Rats and the Protective Effect of Folic Acid: Morphologic Study

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ABSTRACT

Background: Folic acid can protect cells and tissues against the side effects of cytotoxic drugs by removing free radicals and prevention of oxidative damage. The aim of the present work is to demonstrate the teratogenic effects of cyclophosphamide on rat embryos and the possible protective effect of folic acid.

Methods: 28 adult pregnant female albino rats were divided into groups as follows: **Group I: (control group)** 12 rats were further subdivided into two equal subgroups: **Subgroup IA: (negative control group)** received saline. **Subgroup IB: (positive control group)** received folic acid (2.4mg/kg/day). **Group II: (experimental group)** 10 rats received Cyclophosphamide (15 mg/kg) Intraperitoneally as a single dose on the 9th day of gestation. **Group III: (protected group)** 6 rats received folic acid (2.4 mg/kg/day) by orogastric tube from 1st to 19th gestational day. and Cyclophosphamide (15mg/kg) similar to the treated group. The skeletons were double stained by Alizarin red and Alcian blue stains and examined by Olympus SZ dissecting stereomicroscope.

Results: various eye, ear, tongue and fusion defects were recorded**.** Skeletal anomalies were in the form of incomplete and un-ossified skull bones, open arch of atlas, incomplete ossification of sacral vertebrae, supernumerary sacral vertebra, supernumerary rib, and incomplete ossification of ribs, wavy ribs, incomplete ossification of sternum, and incomplete ossification of metacarpus, metatarsus and phalanges. Their incidence increased sign ificantly in the experimental group compared to the control group while it decreased significantly in the protected groups for all reported anomalies except for incomplete ossification of skull bones and vertebrae and supernumerary sacral vertebrae and ribs.

Conclusion: Folic acid has a protective role from Cyclophosphamide induced-teratogenicity in albino rats.

Key words: Cyclophosphamide teratogenicity; Folic acid.

INTRODUCTION

Congenital malformations or birth defects are prevalent among all races, cultures, and socioeconomic levels and may be presented as isolated or part of a syndrome. According to World Health Organization (WHO) report, around 3 million fetuses are born annually with significant congenital malformations, which in 1997 accounted for an estimated 495,000 fatalities globally.[1, 2]

The teratogenicity of drugs varies significantly. If administered during the period of organogenesis (e.g., thalidomide), they trigger serious developmental disturbance. [3]

The cytotoxic drugs used in chemotherapy are designed to rapidly dividing cells and commonly have toxic impacts on tumor cells and healthy tissues with rapidly proliferating cells. [4] Cyclophosphamide (CP) is one of the most efficient and commonly used chemotherapeutic drugs. [5]

During the first trimester of pregnancy, exposure to CP can cause fetotoxic effects such as leucopenia, anemia, and severe bone marrow hypoplasia. [6] It will also result in embryonic and fetal resorptions, intrauterine growth retardation, cleft lip /palate, neural tube defects (NTDs), limb and skeletal defects. [6,7] Biological compounds with

antioxidant properties such folic acid can protect cells and tissues against the side effects of free radicals generated by CP exposure. [8,9]

The aim of the present work is to demonstrate the teratogenic effects of cyclophosphamide on rat embryos and the possible protective effect of folic acid.

METHODS

Chemicals

Cyclophosphamide (Baxter Healthcare Corporation Deerfield, Germany)

Folic acid (mutual pharmaceutical company, Inc).

Stains

Alcian blue stain (Biodiagnostic Company, Cairo, Egypt).

Alizarin Red stain solution has been prepared as follow: 0.005 mg Alizarin red stain (Sigma chemical company), 100 ml of 1% potassium hydroxide (KOH) solution

Equipment

Olympus SZ dissecting stereo microscope. Olympus corporation- Japan through their agent Optoscient Company, Egypt. (Embryology lab, Anatomy and Embryology department, Faculty of Medicine, Alexandria University).

Animals

Twenty-eight Wister adult female albino rats, obtained from Animal House center of Anatomy Department, Faculty of Medicine, Alexandria University. Each of average body weight of 120-200 gm. and approximately 6-8 weeks of age. Female rats were introduced to cages containing a male rat for mating (one male/four females), Mating was confirmed by presence of a vaginal plug which was considered day 1 of gestation. [7]

The animals were housed in stainless steel cages under the following conditions: 12 hours dark/light cycle 22±2º C and 50±10% humidity. They were given standard diet and water throughout the study period. Guidelines for care and use of animals, approved by the Animal House Center, Faculty of Medicine, University of Alexandria, were followed.

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals.

Groups

Twenty-eight adult pregnant female albino rats were divided into:

Group I: (control group) 12 rats were further subdivided into two equal subgroups: ***Subgroup IA: (negative control group)*** received standard diet, free access to water with daily intake of saline by orogastric tube from the 1st to the 19th gestational day (GD) [7]. ***Subgroup IB: (positive control group)*** 6 rats received folic acid (2.4mg/kg/day) by orogastric tube from the 1st to the 19th day of gestation.

Group II: **(experimental group)** 10 rats received Cyclophosphamide (15 mg/kg) Intraperitoneally (IP) as a single dose on the 9th day of gestation. [7]

Group III: **(protected group)** 6 rats received folic acid (2.4 mg/kg/day) by orogastric tube from the 1st to the 19th day of gestation and Cyclophosphamide (15mg/kg) IP as a single dose on the 9th day of gestation. [7]

Pregnant rats were examined daily throughout an experimental period of 20 days starting from day 1 of pregnancy for appearance of any signs of toxicity as changes in behavior, weight loss, decrease in water and food intake and death. All pregnant females were sacrificed on gestational day 20 by being anaesthetized. After caesarean section of the pregnant rats on the 20th day of gestation, the uterine horns were carefully exteriorized, dissected, opened and examined for the number of implantation sites and fetuses (live or dead). For skeletal examination, double staining technique was done using both Alcian blue and Alizarin red stains solution for staining both cartilaginous and bony parts of the skeleton. All of the fetuses were processed according to the following steps as reported in earlier studies [10,11]:

Preservation and fixation: The first step was skinning then eviscerating were done by clearing the thoracic and abdominal cavities. The samples were left in 90% or absolute ethanol for at least seven days.

Cartilage staining: The specimen was completely immersed in a solution of 0.01% Alcian blue which was prepared in 70 cc pure ethanol and 30 cc glacial acetic acid (7:3). The fetuses were left in this solution until complete uptake of the dye; for about 3 days.

Rehydration: The specimen was placed in a bath of 95% ethyl alcohol for two hours. This was repeated for two other hours in a new bath. Each specimen was placed in baths of successively decreasing concentrations of 75%, 40%, and 15% ethyl alcohol, two hours for each concentration.

Clearing: The samples were left in 1% KOH until the skeletal system of the embryo was exposed.

Bone staining**:** The specimens were put in 0.001% aqueous Alizarin red for three days in order to stain bony parts.

Washing: To eliminate any excess Alizarin red color, the specimens were immersed and rinsed in 1% KOH three times, several hours each time.

Clearing and dehydration: The samples were treated with ascending series of glycerol in 1% KOH, “Sequential series: 1:3, 1:1, and 3:1”, 24

hours for each step. Finally each fetal skeleton was examined by stereomicroscope and photomicrographs documenting the results were taken.

Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0**.** **(**Armonk, NY: IBM Corp**)** [13]. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. P values lower than or equal 0.05 were considered as statistically significant. **Chi-square test (χ^2) was used** for categorical variables, to compare between different groups. **Monte Carlo correction (MC)** of Chi-square was used when more than 20% of the cells had expected count less than 5. **F-test (ANOVA) was utilized** for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) was used for pairwise comparisons.

Kruskal Wallis test was utilized for abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) was used for pairwise comparisons. [**12]**

RESULTS

A. Morphologic changes

I. Abnormalities in the head region

a. Eye abnormalities:

Eye abnormalities were observed in the form of:

Microphthalmia (small eye bulge): In the control groups (Fig.1a); no microphthalmia was reported, in the experimental group; it was in 7 out of 58 (12.1 %), while in the protected group III; it was in 2 out of 42 (4.8 %) (Fig.1b, 1c). There was a significant statistical difference between the control and experimental groups. (Table I, fig.1e).

Exophthalmos (large eye bulge): In the control groups (Fig.1a), no exophthalmos was reported. In the experimental group, it was in 8 out of 58 (13.8 %). In the protected group III, 2 out of 42 (4.8%) (Fig.1d). There was a significant statistical difference between the control and experimental groups. (Table I, fig.1e).

Open eye: In the control groups (Fig.1a), open eye was reported in 1 out of 141 (0.7 %). In the experimental group, it was in 10 out of 58 (17.2 %). In the protected group III, it was in 1 out of 42 (2.4 %) (Fig.2a). There was a significant statistical difference between the control and experimental groups as well as between experimental and protected groups. (Table I, fig.1e).

b. ***Mouth abnormalities*****:** In normal rat fetus the mouth is closed with normal size of the tongue (Fig.1a).

Open mouth and protruded tongue: In the control groups, open mouth was reported in 1 out of 141 (0.7%). In the experimental group, it was in 14 out of 58 (24.1 %). In the protected group III, it was in 2 out of 42 (4.8%) (Fig.2b). There was a significant statistical difference between the control and experimental groups as well as between experimental and protected groups. (Table I).

c. Ear abnormalities:

Anotia (absence of ear): In the control groups and in the protected groups (Fig.1a), no anotia was reported. In the experimental group, it was in 3 out of 58 (5.2 %) (Fig1c), There was a significant statistical difference between the control and experimental groups. (Table I).

Low set ears: In the control groups (Fig.1a), no low set ears were reported. In the experimental group, it was in 7 out of 58 (12.1 %) (Fig.1b, 1d.) There was a significant statistical difference between the control and experimental groups as well as between experimental and protected group III. (Table I, fig.1f).

d. Neural tube defects:

Encephalocele**:** In the control groups (Fig.1a), no encephalocele was reported. In the experimental group, it was in 9 out of 58 (15.5 %). In the protected group, it was found in 1 out of 42 (2.4 %) (Figs.1d, 2a). There were significant statistical differences between the control and experimental groups as well as between experimental and protected group. (Table I, fig, 3c).

Meningeocele: In the control groups (Fig.1a), no meningeocele was reported. In the experimental group, it was present in 7 out of 58 (12.1 %) (Figs.3a, 3b). There were significant statistical differences between the control and experimental groups as well as between experimental and protected group. (Table I, Fig.3c).

II. Limb abnormalities: The normal position of forelimbs and hind limbs in normal rat fetuses is flexed in mid prone position (Fig.1a)

Hyperextension of forelimb: In the control groups, hyperextended forelimb was reported in 3 out of 141 (2.1 %). In the experimental group, it was found in 15 out of 58 (25.9 %). In the protected group III, it was present in 2 out of 42(4.8 %) (Fig.1b). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups. (Table I, Fig.3c)

Internal rotation of forelimb: In the control groups, it was reported in 2out of 141 (1.4%). In the experimental group, it was in 12 out of 58 (20.7

%). In the protected group, it was found in 2 out of 42(4.8 %) (Fig.2c). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups.(Table I, Fig.3c)

III. Hemorrhages:

a. Subcutaneous hemorrhage: It included hemorrhage in the back (Fig.4a), hemorrhage in the head (Fig.4b), hemorrhage in the neck (Fig. 4C) and hemorrhage in the forelimb (Figs.2a, 4d). In the control groups, it was reported in 4 out of 141(2.8 %). In the experimental group, it was in 20 out of 58 (34.5 %). In the protected group III, it was present in 4 out of 2 out of 42 (4.8 %). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups. (Table I, fig.4e)

b. Intra- abdominal hemorrhage: In the control groups, no intra-abdominal hemorrhage was reported. In the experimental group, it was in 11 out of 58 (19 %). In the protected group III, it was in 1 out of 42 (2.4 %) (Fig.2d). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups. (Table I, fig.4e).

B. Skeletal changes:

I. Skull abnormalities: Fig. (5a) shows normal ossification of skull bones and closed arch of atlas.

a. Incomplete ossification of skull bones: In the control groups, Incomplete ossification was reported in 1 out of 141 (0.7 %). In the experimental group, it was in 8 out of 58 (13.8 %). In the protected group, it was found in 2 out of 42(4.8 %). The incomplete ossified bones were parietal, squamosal and exoccipital bones (Figs.5b-d). There was a significant statistical difference between the control and experimental groups. (Table II**, Fig. 5e**).

b. *Unossified skull bones*: In the control groups, normal ossification was reported. In the experimental group, no ossification was present in 6 out of 58 (10.3 %). In the protected group, it was in 1out of 45 (2.2 %) in group III. Unossified skull bones were inter-parietal, supra-occipital and hyoid bones (Figs. 5b, 5c, 5d). There were significant statistical differences between the control and experimental groups as well as between experimental and protected group. (Table II, Fig. 5e).

II. Vertebral anomalies:

a. Open arch of atlas: In the control groups and in the protected groups (Fig.5a), no open arch of atlas was reported while in the experimental group, it was in 5 out of 58 (8.6 %) (Fig.6a). There were statistical significant differences between the control and experimental groups as well as between experimental and protected group III. (Table II, Fig. 6d).

b. *Incomplete ossification of sacral vertebrae:* In the control groups, normal ossification of vertebrae was reported (Fig.6b). In the experimental group, incomplete ossification was in 8 out of 58 (13.8 %), while in the protected group III, it was 2 out of 42(4.8 %) (Fig.6c). There were statistical significant differences between the control and experimental groups as well as between experimental and protected group III. (Table II, Fig. 6d)

c. Supernumerary sacral vertebra: In the control groups and in the protected groups, number of sacral vertebra was normal (Fig. 6b) while in the experimental group, it was reported in 3 out of 58 (5.2 %) (Fig.6c). There was a statistical significant difference between the control and experimental groups. (Table II, Fig. 6 d).

III. Anomalies of the ribs: The normal number of ribs is thirteen with normal antero-posterior curve and normal ossification (Fig. 7a).

a. *Supernumerary rib:* In the control groups, no supernumerary rib was reported. In the experimental group, it was in 8 out of 58 (13.8 %) while in the protected group, it was in 1 out of 42(2.4 %) (Figs.7b, 7c).There was a significant statistical difference between the control and experimental groups. (Table II, Fig. 7e).

b. Incomplete ossification of ribs: In the control groups, it was reported in 3out of 141 (2.1 %). In the experimental group, it was in 10 out of 58 (17.2 %) while in the protected group, it was in 1 out of 42(2.4 %) (Figs.7c, 7d). There were statistical significant differences between the control and experimental groups as well as between experimental and protected groups. (Table II, Fig. 7e).

c. *Wavy ribs:* In the control groups, no wavy ribs were reported. In the experimental group, it was in 9 out of 58 (15.5 %) while in the protected group, it was in 1out of 42(2.4%) (Figs.7b, 7 c). There were statistical significant differences between the control and experimental groups as well as between experimental and protected groups. (Table II, Fig. 7e).

IV. *Forelimb anomalies:* In normal forelimbs, the metacarpus and phalanges are ossified (Fig. S1 a).

Incomplete ossification of metacarpus and phalanges: In the control groups, incomplete ossification was reported in 2 out of 141 (1.4 %). In the experimental group, it was in 12 out of 58

(20.7 %) while in the protected group, it was 4 out of 42(9.4 %) (Fig.S1 b). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups. (Table II**,** Fig. S1 c).

V. ***Hind limb anomalies:*** In normal hind limbs, the metatarsus and phalanges are ossified (Fig. S2 a).

Incomplete ossification of metatarsus and phalanges: In the control groups, incomplete ossification was reported in 1 out of 141 (0.7 %). In the experimental group, it was in 14 out of 58 (24.1 %) while in the protected groups it was 2 out of 42(4.8 %) (Fig.S2 b). There were significant statistical differences between the control and experimental groups as well as between experimental and protected groups. (Table II**,** Fig. S1 c).

Table (1): Incidence of morphologic abnormalities in rat fetuses among the study groups

	Group I (control) (Ia, Ib)	Group II (Experimental)	Group III (Protected)		χ^2	^{MC}p
Number of examined Fetuses	141	58	42			
Eye						
Microphthalmia	0 (0.0%)	7 (12.1%)	2(4.8%)		16.164	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.052 ,p_3=0.296					
Exophthalmos	0 (0.0%)	8(13.8%)	2(4.8%)		18.641	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.052 ,p_3=0.185					
Open eye	1(0.7%)	10(17.2%)	1(2.4%)		20.057	<0.001*
Sig. bet. grps.	p_1<0.001* p_2=0.407 *,p_3=0.023*					
Mouth						
Open mouth & protruded tongue	1(0.7%)	14(24.1%)	2(4.8%)		29.566	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.132, *,p_3=0.012*					
Ear						
Anotia	0(0.0%)	3(5.2%)		0(0.0%)	6.950	0.015*
Sig. bet. grps.	p_1<0.001* ,p_2=–, p_3=–					
Low set ears	0(0.0%)	7(12.1%)		0(0.0%)	16.836	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=– ,p_3=0.020*					
Neural tube defects						
Encephalocele	0(0.0%)	9(15.5%)		1(2.4%)	21.252	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.230 *,p_3=0.042*					
Meningeocele	0(0.0%)	7(12.1%)		0(0.0%)	16.836	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=– ,p_3=0.020*					
Forelimbs						
Hyperextension	3(2.1%)	15(25.9%)		2(4.8%)	25.796	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.324,p_3=0.006					
Internal rotation	2(1.4%)	12(20.7%)		2(4.8%)	21.074 *	<0.001*
Sig. bet. grps.	p_1<0.001* p_2=0.226 *,p_3=0.038*					
Hemorrhages						
Subcutaneous Hge	4(2.8%)	20(34.5%)		2(4.8%)	36.784	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.622, *,p_3<0.001*					
Intra-abdominal Hge	0(0.0%)	11(19.0%)		1(2.4%)	26.556	<0.001*
Sig. bet. grps.	p_1<0.001* ,p_2=0.230 *,p_3=0.013*					
Number of total anomalies	11	123		15		

MC: Monte Carlo, p: p value for comparing between the studied groups, p_1: p value for comparing between control and experimental groups, p_2: p value for comparing between control and Protected groups, p_3: p value for comparing between experimental and protected groups, χ^2: Chi square test, *: Statistically significant at $p \leq 0.05$

Table (II): Incidence of skeletal anomalies in rat fetuses among the study groups.

	Group I (control) (Ia, Ib) (n=141)	Group II (Experimental) (n=58)	Group III (Protected) (n=42)	χ^2	^{MC}p
Skull					
Incomplete ossification	1(0.7%)	8(13.8%)	2(4.8%)	14.442	0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.132, p_3=0.185				
Unossified skull bone	0(0.0%)	6(10.3%)	0(0.0%)	14.133	0.001*
Sig. bet. grps.	p_1=0.001*, p_2=–, p_2=0.038*				
Vertebrae					
Open arch of atlas	0(0.0%)	5(8.6%)	0(0.0%)	12.021	0.001*
Sig. bet. grps.	p_1=0.002*, p_2=–, p_2=0.061				
Incomplete ossification	0(0.0%)	8(13.8%)	2(4.8%)	18.641	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.052, p_3=0.185				
Supernumerary sacral vertebra	0(0.0%)	3(5.2%)	0(0.0%)	6.950	0.014*
Sig. bet. grps.	p_1=0.024*, p_2=–, p_3=0.268				
Ribs					
Supernumerary rib	0(0.0%)	8(13.8%)	1(2.4%)	18.512	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.230, p_3=0.075				
Incomplete ossification	3(2.1%)	10(17.2%)	1(2.4%)	14.512	0.001*
Sig. bet. grps.	p_1<0.001*, p_2=1.000, p_3=0.023*				
Wavy rib	0(0.0%)	9(15.5%)	1(2.4%)	22.696	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.230, p_3=0.042*				
Sternum					
Incomplete ossification	0(0.0%)	8(13.8%)	2(4.8%)	18.641	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.052, p_2=0.185				
Forelimb					
Incomplete ossification	2(1.4%)	12(20.7%)	4(9.5%)	21.139	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.024*, p_3=0.145				
Hindlimb					
Incomplete ossification	1(0.7%)	14(24.1%)	2(4.8%)	29.806	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2=0.132, p_3=0.009*				
Total anomalies	7	91	15	15	

MC: Monte Carlo, p: p value for comparing between the studied groups, p_1: p value for comparing between control and experimental groups, p_2: p value for comparing between control and Protected groups, p_3: p value for comparing between experimental and protected groups, χ^2: Chi square test, *: Statistically significant at $p \leq 0.05$

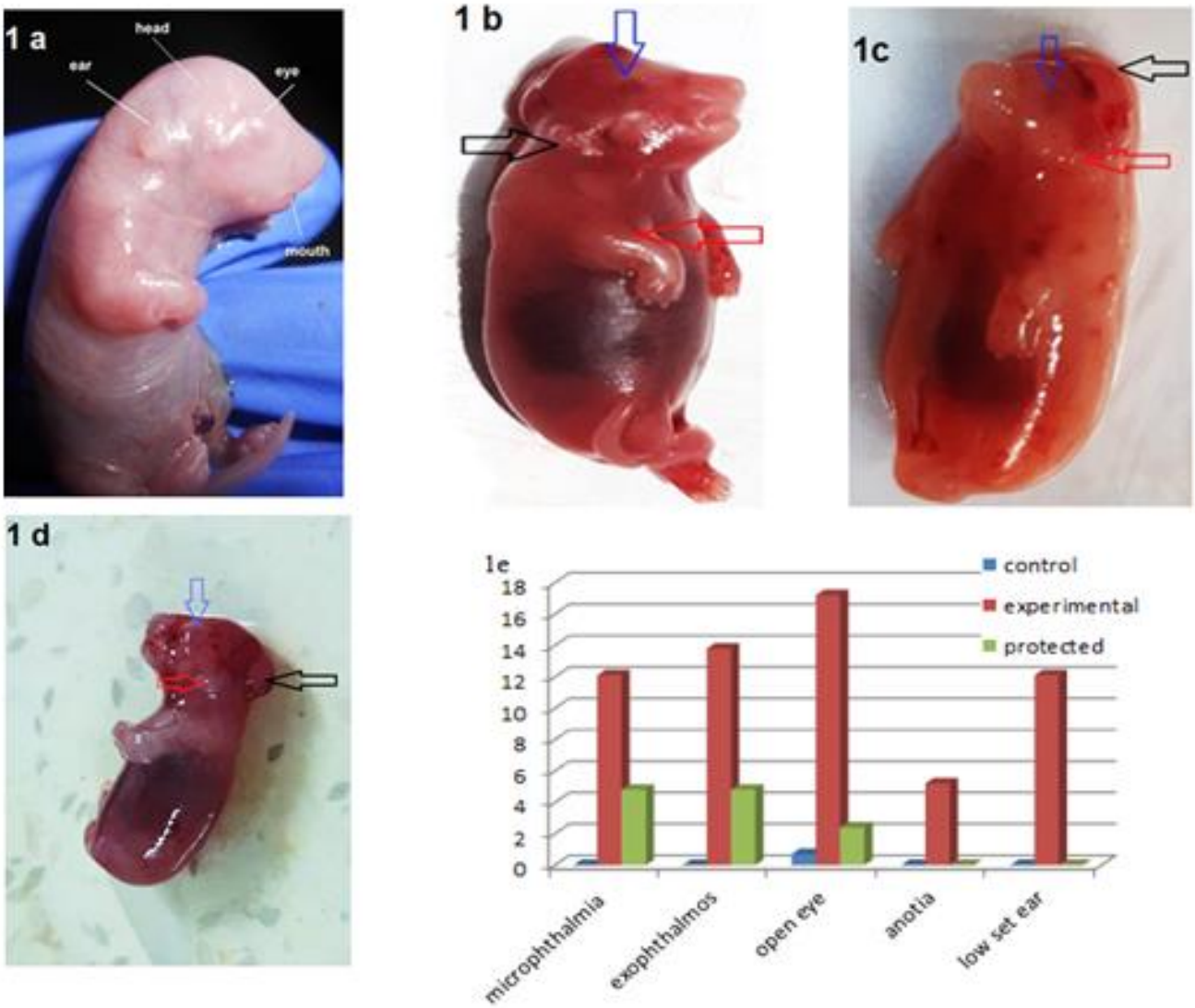

Figure 1:(a-d), A Photograph of a 20 GD rat fetus, lateral view showing:
1a: normal ear, eye bulge, contour of the head and mouth, 1b: microphthalmia (blue arrow), low set ear (black arrow), and hyperextended fore-limb (red arrow).
1c: microphthalmia (blue arrow), Anotia (red arrow), and encephalocele (black arrow).
1d: exophthalmos (blue arrow), low set ear (red arrow), and encephalocele (black arrow).
1e: Bar chart showing incidence of eye and ear anomalies among the study groups.

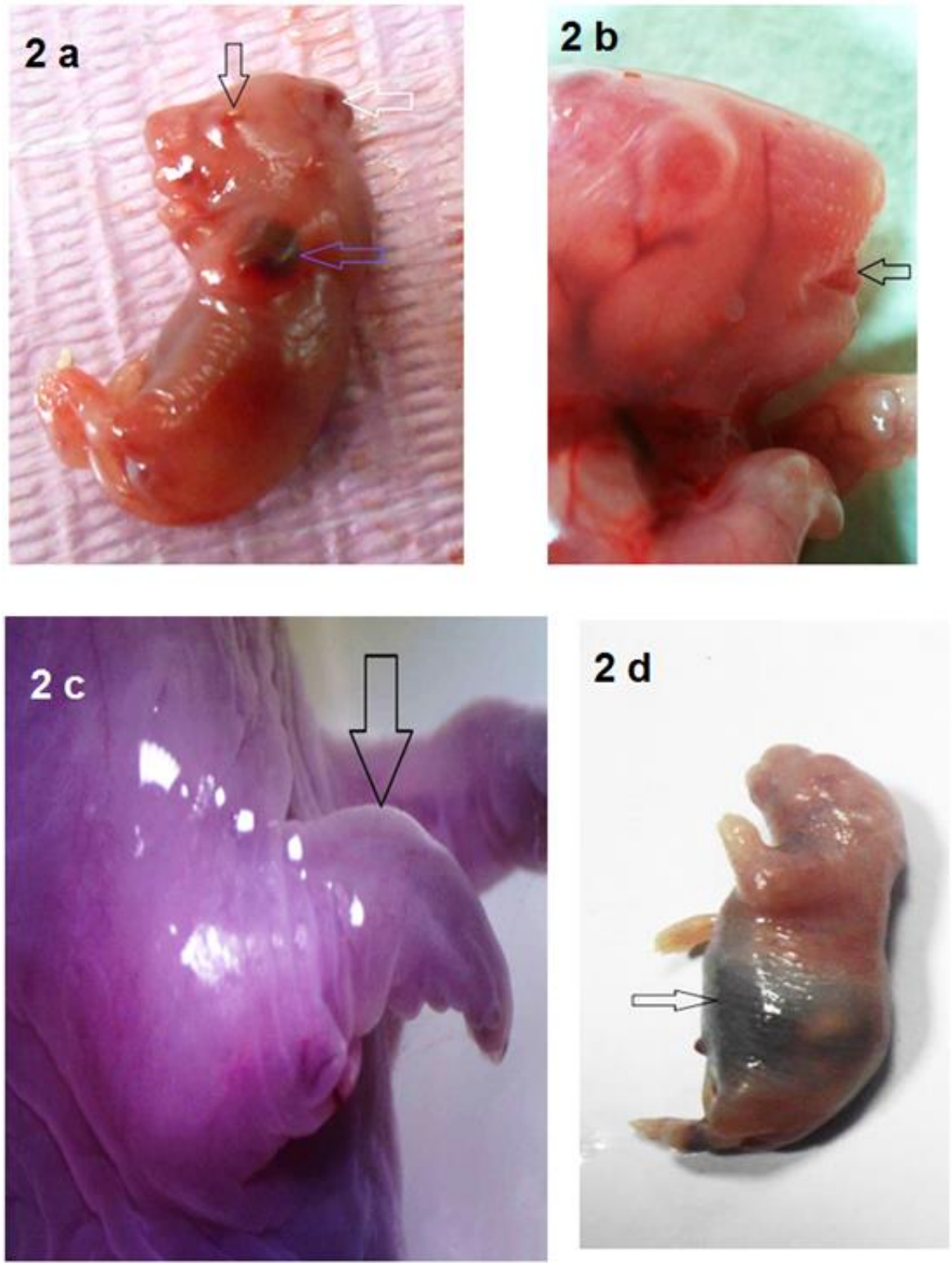

Figure 2: (a, b, c and d), A Photograph of a 20 GD rat fetus, lateral view showing:
2 a: open eye (black arrow), encephalocele (white arrow),
and subcutaneous hemorrhage in forelimb (blue arrow)
2 b: open mouth with protruded tongue (Arrow).
2 c: internal rotation of forelimb (Arrow).
2 d: intra-abdominal hemorrhage (Arrow).

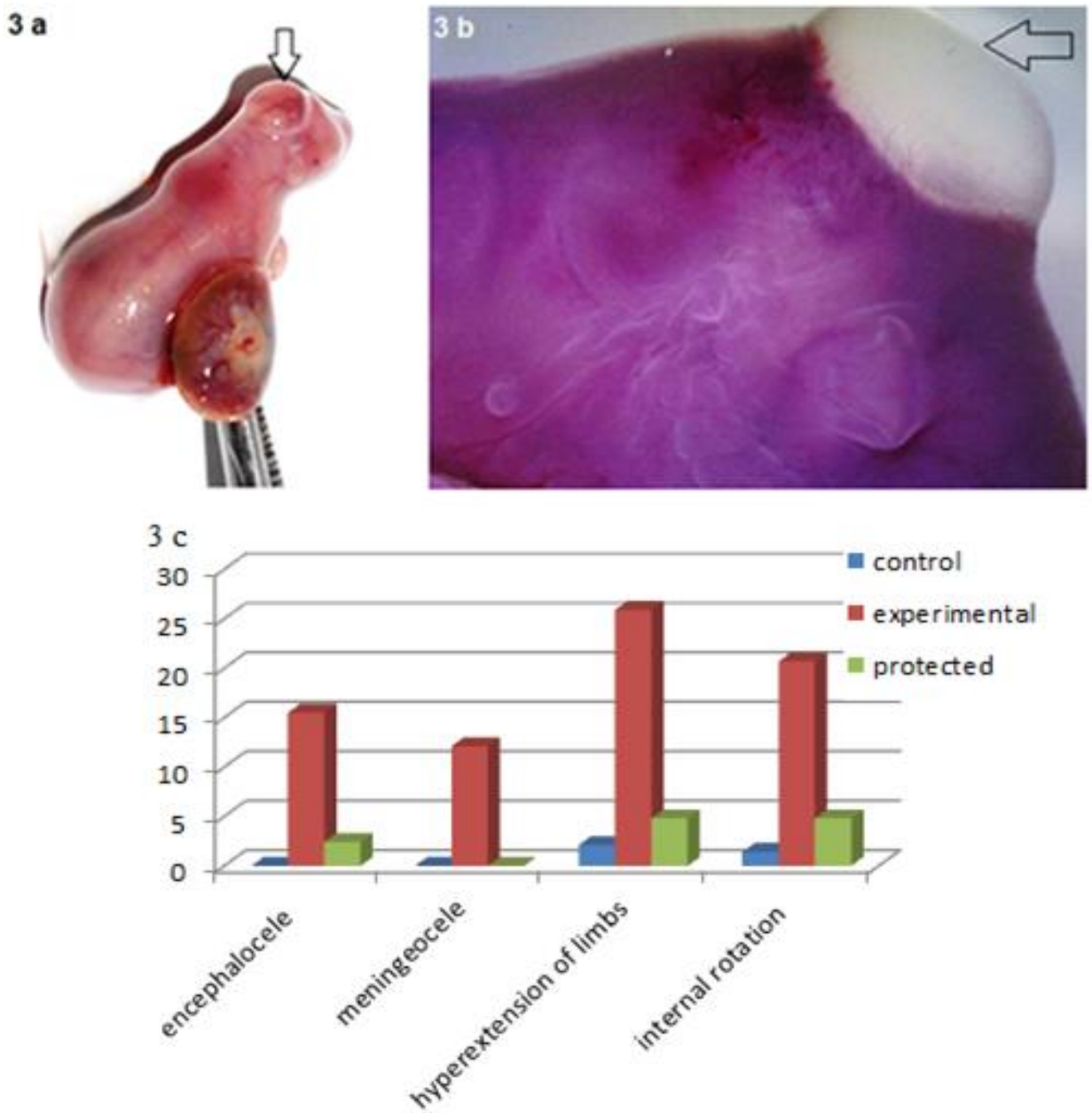

Figure 3: (a and b), A Photograph of a 20 GD rat fetus, lateral view showing: 3a: Meningocele (Arrow). 3b: A larger magnification of the head showing meningeocele (Arrow). 3c: A bar chart showing incidence of encephalocele, meningeocele, hyperextension and internal rotation of forelimb.

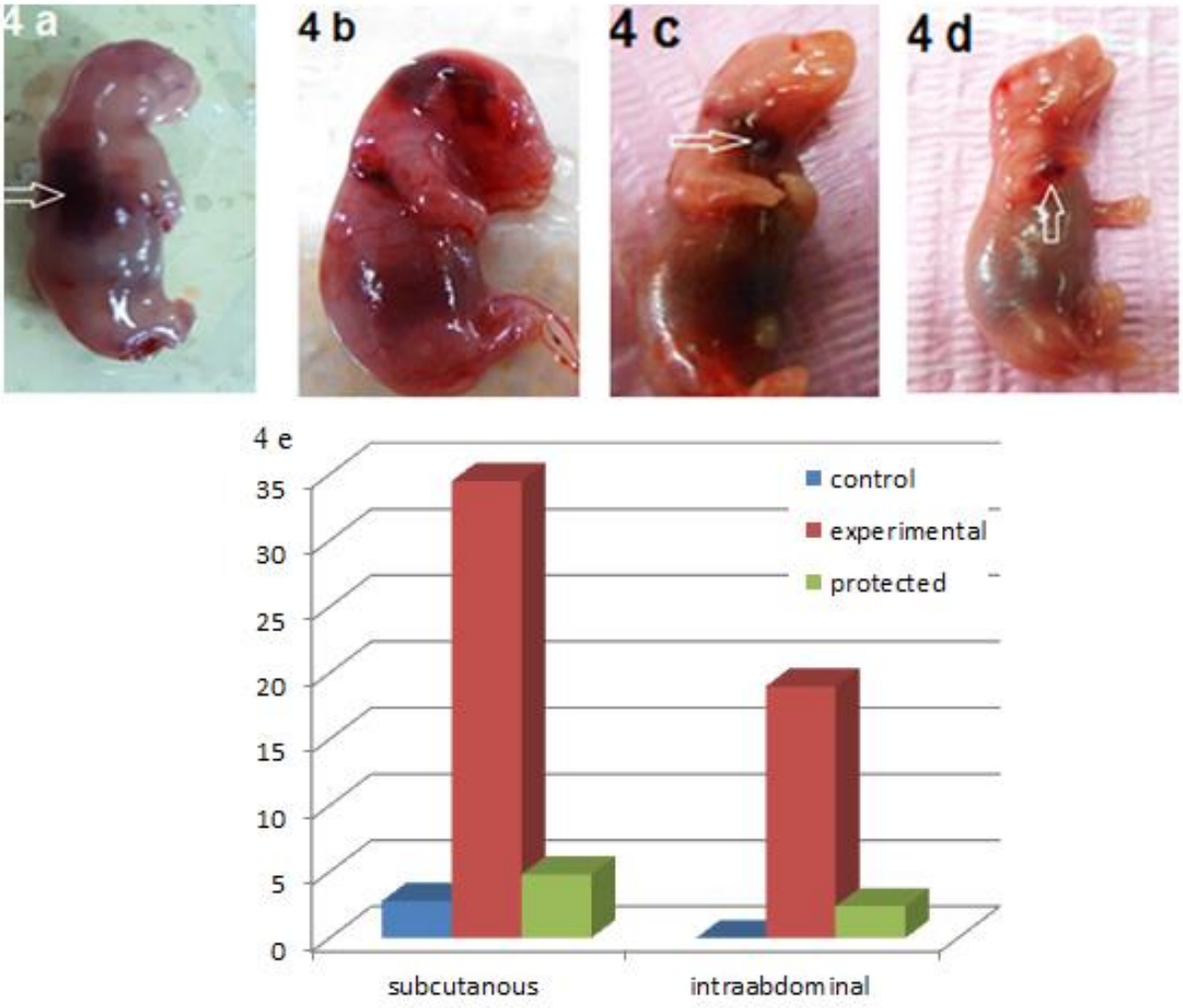

Figure 4: (a-d), A Photograph of a 20 GD experimental rat fetuses showing subcutaneous hemorrhages in different regions (white arrows)
4 a: in the back, 4 b: in the head, 4 c: in the neck, 4 d: in the forelimb
4 e: A bar chart showing the incidence of hemorrhages (subcutaneous and intra-abdominal) among the study group.

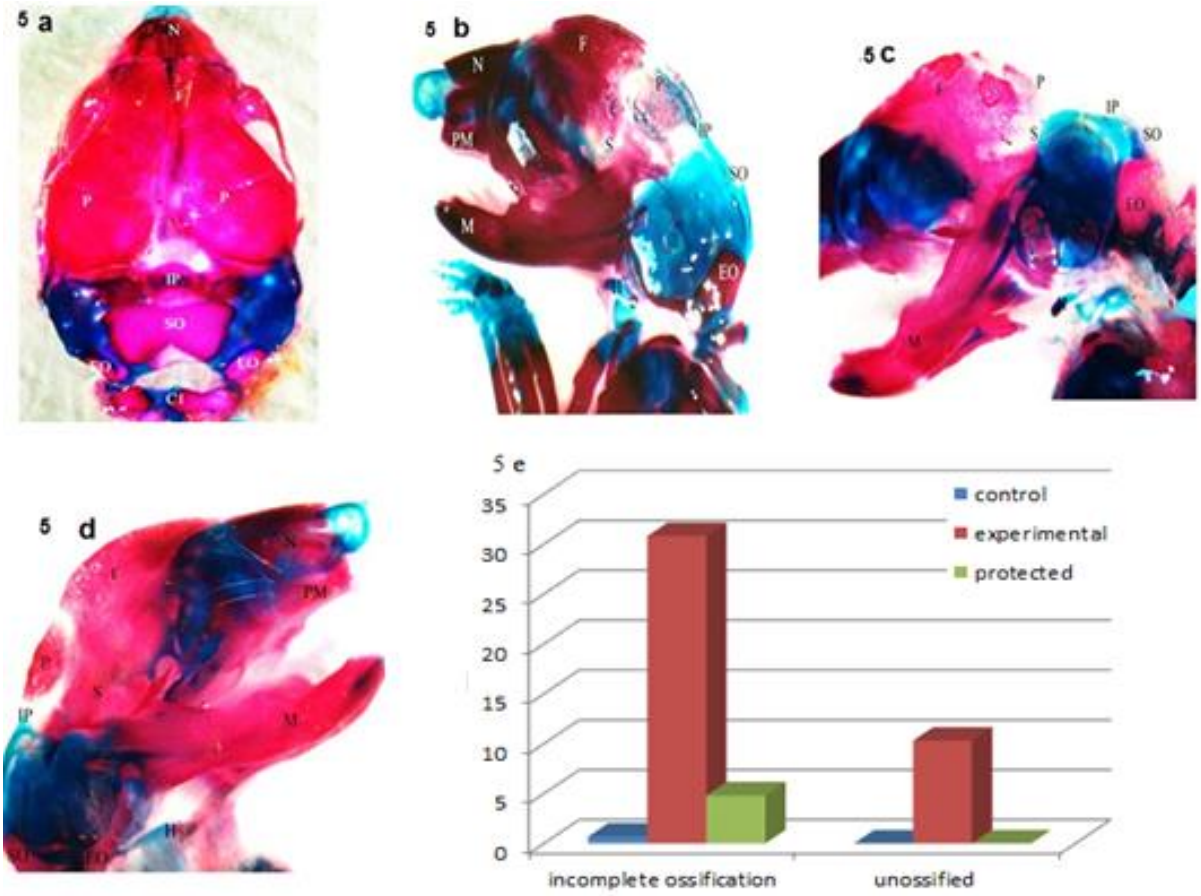

Figure 5: (a-d); A Photograph of a 20 GD fetal skull showing: 5a: A normal fetal skull, lateral view showing: N (Nasal), F (Frontal), P (Parietal), IP (Inter-parietal), SO (Supraoccipital), EO (Exoccipital) bones and normal closed arch of C1 (Atlas). 5 b: Incomplete ossification of P (Parietal) and S (Squamosal) bones, unossified IP (Inter-parietal), SO (Supraoccipital) and H (Hyoid) bones. Normal ossification of PM (Premaxilla), M (Mandible). 5 c: Incomplete ossification of S (Squamosal) bone, unossified P (Parietal), IP (Inter-parietal) and SO (Supraoccipital) bones. 5 d: Incomplete ossification of P (Parietal) bone, unossified IP (Inter-parietal) bone. 5 e: A bar chart showing the incidence of skull anomalies (incomplete and unossified skull) among the study groups.

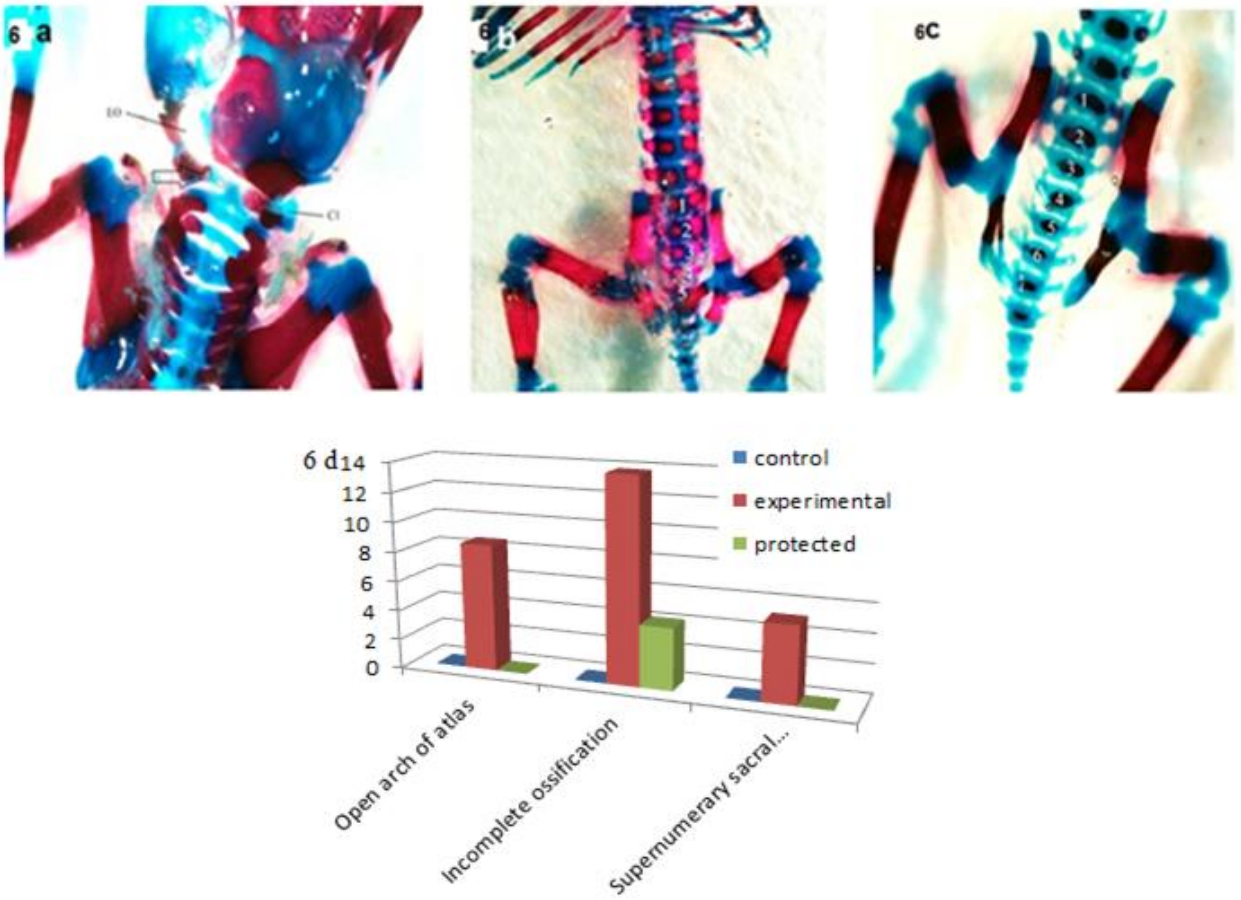

Figure 6: (a-c), A photomicrograph of a 20 GD fetal skeleton showing:
6a: Fetal skull and cervical vertebrae of experimental group, posterior view, showing incomplete ossification of EO (Exoccipital) bone and open arch of C1 (Atlas) (black arrow)
6b: Normal fetal vertebrae, ventral view, showing normal number (5 sacral vertebrae) and normal ossification of sacral vertebrae
6c: Fetal sacral vertebrae of experimental group, ventral view incomplete ossification of sacral vertebrae and supernumerary sacral vertebra.
6d: A bar chart showing the incidence of vertebral anomalies (open arch of atlas, incomplete ossification and supernumerary sacral vertebra)

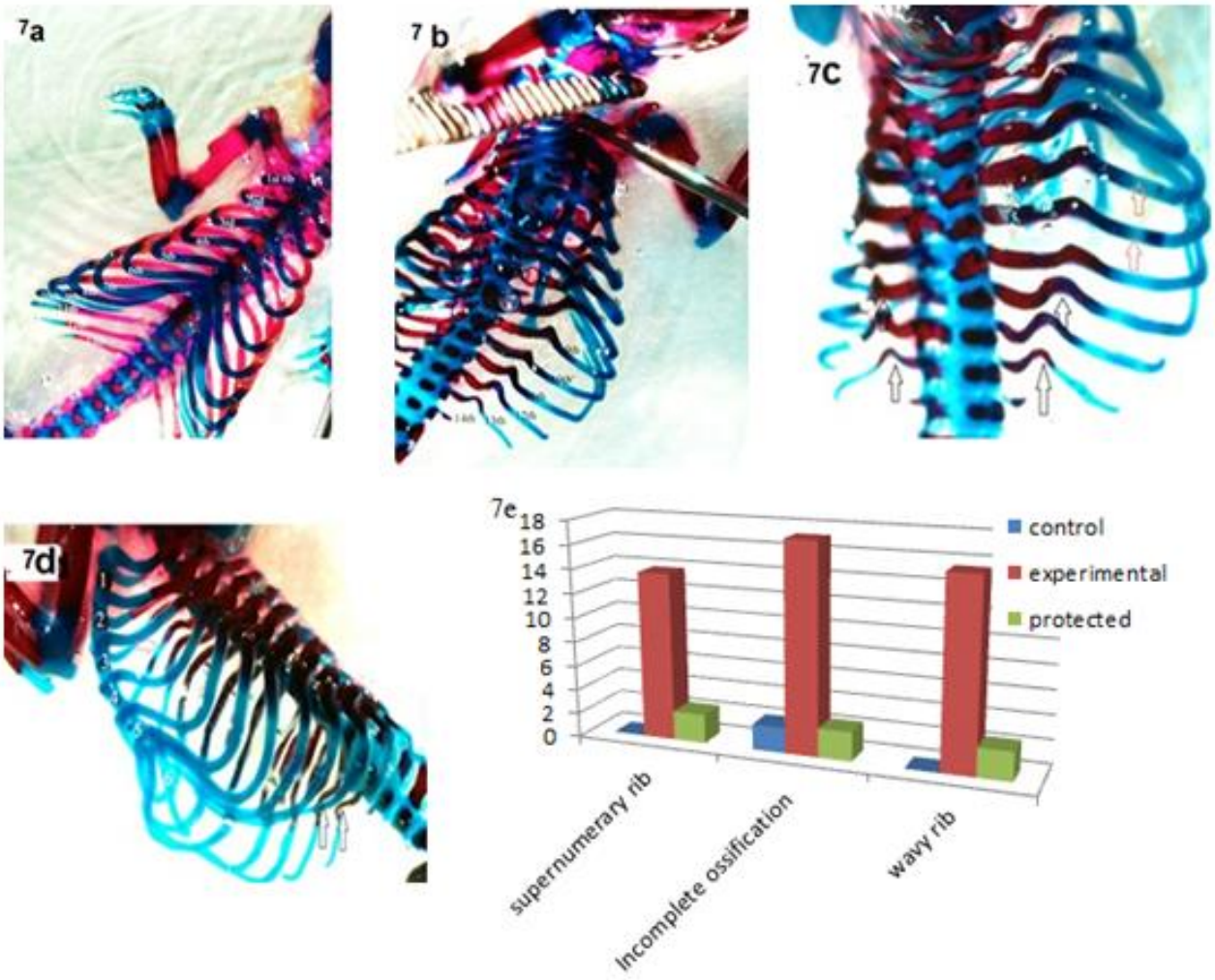

Figure 7: (a-d), A photomicrograph of a 20 GD fetal thoracic cage, ventral view, showing
7a: Normal number, curve, and ossification of ribs
7b: Supernumerary rib and abnormal curve of ribs (wavy ribs)
7c: Abnormal curve of ribs (wavy ribs) (black arrows) and incomplete ossification of ribs (red arrows)
7d: Unossified lower three sternebrae, incomplete ossification of ribs (red arrow) and abnormal curve of ribs (wavy ribs) (black arrows).
7e: A bar chart showing the incidence of rib anomalies (supernumerary, incomplete ossification and wavy ribs) among the study group.

DISCUSSION

Programmed cell death (apoptosis) is a part of normal development of an individual during intra-uterine life. Altered rate of programmed cell death at a critical period of development may lead to serious structural defects. Also, altered rate of cell proliferation may induce malformations. Thus agents that interfere with the cell proliferation or differentiation cause congenital malformations. Cyclophosphamide is one of such agents. [14]

The present study deals with determining the efficiency of folic acid in preventing the teratogenicity of Cyclophosphamide on albino rat embryos.

Albino rats wistar strain as experimental animals were chosen in the present study because they are larger in size which makes handling, sampling and performing procedures easier. And due to their anatomical, physiological, and genetic similarity to humans and they are resistant to infections.[15,16]

Early embryogenesis is marked by extensive differentiation of all organ systems developing during early (7th to 11th) and late (12th to 14th) day post coitum. It is characterized by the formation of some essential new fetal structures and by the regression of others. [17]

In the present study, Cyclophosphamide was injected intraperitoneally (IP) to the albino rats avoid the common gastrointestinal complications associated with oral administration of a cytotoxic drug. Folic acid was supplied orally to minimize the amount of IP injected materials that may be hazardous to the rat causing intraperitoneal hemorrhage.

The rats were injected with Cyclophosphamide as a single dose of 15 mg/kg on the 9th day of gestation. This dose was selected according to Khaksary Mahabady et al. [10] who found that administration of this dose on the 9th GD caused significant growth retardation and morphological alterations in rat fetuses. Slott et al. [18] observed that Cyclophosphamide can produce teratogenicity in 50 and 100% of fetuses at dose of 10 and 15 mg/kg respectively. The mechanism of action of folic acid was reported by Joshi et al ,as it is an effective free radical scavenger. If present in physiology, folic acid can protect bioconstituents from free radical damage at least by competition which otherwise can lead to oxidative stress. In spite of being water soluble molecule, folic acid can inhibit lipid peroxidation. The scavenging and repair of thiyl radicals by folic acid makes it a potential vitamin to be called as an antioxidant and to concer free radicals and protect against oxidative stress[19]. The dose of folic acid (2.4mg/kg/day) was chosen according to Hou J et al. [20] who studied the protective effects of enalapril maleate and folic acid tablets against contrast-induced nephropathy in diabetic rats.

Gerlinger et al [21] found that the subcutaneous administration of 50 mg of CP per rabbit on gestational days 10 to 13 induced primarily cleft palate with micrognathia (small jaw), resorptions and decreased fetal size. They added that injection of rabbit with 300 mg of CP was lethal to the dam after the second injection while injection with 100 mg induced 100% fetal resorptions

In the present work, eye anomalies were in the form of microphthalmia, exophthalmos and open eye. Microphthalmia and exophthalmos were significantly evident in the experimental group compared to the control group and their incidence decreased non significantly in the protected groups compared to the experimental group. Regarding open eye, there was a significant increase in its incidence in the experimental group as compared to the control group and it decreased significantly in the protected groups as compared to the experimental group.

Malformations of the eyes included absence of lids, blebs over eyes, exophthalmos, ectopia lentis and unilateral anophthalmia as reported by Gupta et al. [22] in their study of eye malformations induced by cyclophosphamide in chick embryos. They clarified that the active alkylating form of the drug is easily taken up by the rapidly growing cells in the embryo or neoplastic tissue. It leads to inhibition of DNA synthesis that probably leads to localized cell deaths which is sufficient to suppress their proliferative rates within the embryos resulting in malformations. Also Wendler[17] in the study of pathogenesis of cyclophosphamide-induced fetal anomalies in rats reported developmental arrest of the eyelids and flattening of the orbit caused exophthalmia to appear in the fetuses.

Open eye anomaly was reported by Porter et al. [23] in their study of the trans placental teratogenesis and mutagenesis in mouse fetuses treated with cyclophosphamide and this was in agreement with the present work.

Regarding mouth abnormalities; open mouth with protruded tongue were reported in the present study and there was a significant increase in their incidence in the experimental group as compared to the control group and their incidence significantly decreased in the protected groups as compared to the experimental group. This explains the teratogenic effect of cyclophosphamide and the protective role of folic acid.

In support of these results Wendler[17] found that nearly 80% of the fetuses of rats showed moderate to severe micrognathia as a result of which the tongue protruded out of the opened oral cavity due to the effect of cyclophosphamide.

In the present work, some ear anomalies were recorded which were anotia and low set ears. Regarding anotia, there was a significant increase in its incidence in the experimental group as compared to the control group which could be attributed to the toxic effect of cyclophosphamide. Regarding low set ears, there was a significant increase in its incidence in the experimental group as compared to the control group and it decreased significantly in protected group as compared to the experimental group which suggest the protective effect of folic acid against toxic effect of cyclophosphamide.

In consistent with the present results, Rengasamy et al. [24] and Kirshon et al [25] described many anomalies as multiple eye anomalies, low set ears and hearing defects in the first trimester which occur as a result of prenatal exposure to cyclophosphamide. Padmanabhan et al. [26] in their study of congenital anomalies of the ear resulting from cyclophosphamide treatment in the rat found ear anomalies as microtia in 97.5% of fetuses at term and the ears of affected fetuses were low set and dorsally placed. The present study differs in the variability of ear anomalies.

Ivnitsky et al. [27] found that mice treated with CP had brain and craniofacial abnormalities associated with increased level of TNF-α in fetal brain, and also maternal immune stimulation decreased the extent of malformation caused by CP in these mice and decreased the expression of TNF-α in fetal heads.

In a study of Folate status and the immune system done by Dhur et al. [28], it was reported that cell mediated and humoral immunity is affected by Folate deficiency as blastogenic response of T lymphocytes to certain mitogens and the antibody responses to several antigens are decreased in case of folic acid deficiency.

Regarding neural tube defects (NTDs) in this study; encephalocele and meningeocele were reported, their incidence increased significantly in the experimental group compared to the control groups. There was significant decrease in the incidence of encephalocele and meningeocele in protected group III as compared to the experimental group.

Wendler [17] in his study on Wister albino rats reported that there were impressive malformations in the region of the head in the form of occipital hemorrhagic encephalocele that was located particularly in the region of the parietal bones which were deficient in most cases.

Najafzadeh et al. [29] evaluated the effect of mesna and Echinacea purpurea on CP-induced teratogenicity. They used intraperitoneal cyclophosphamide at dose 15 mg/ kg in rats on 13th day of gestation. They determined fetal defects similar with our study including open eye and limb defects and others not reported in our study as exencephaly; brain outside skull. Those anomalies decreased by 75 mg/kg and 200 mg /kg quercetin, respectively. [30]

Preconception consumption of folic acid, or multivitamins containing folic acid, has been shown in intervention studies to reduce the risk for NTDs as Bailey et al. [31] reported in their study of folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage.

In addition, Beauden et al. [32] found that maternal Folate deficiency alone is sufficient to induce neural tube defects (NTDs) in embryos.

In the present study, some limb abnormalities were reported which included hyperextension and internal rotation of forelimbs. These anomalies were significantly increased in the experimental group as compared to the control group and there was a significant decrease of them in the protected groups in comparison with the experimental group.

In consistent with these results, Logsdon et al. [33] stated that cyclophosphamide at a dose of 20 mg/ kg in mice on 10th day of gestation could produce teratogenicity. They determined limb defects which is similar to our study. These anomalies were decreased by 75 mg/ kg and 200 mg/ kg quercetin.

The Hungarian randomized clinical trial and some observational studies indicate that folic acid may reduce the overall risk of congenital malformations or the risk of a specific group of them which are oral clefts, cardiac defects, anomalies of the urinary tract except hypospadias, limb reduction defects, omphalocele, and anal atresia. [34]

Regarding hemorrhages, there were subcutaneous and intra-abdominal hemorrhages which were reported in the present work. They were significantly evident in the experimental group as compared to the control group and there was a significant decrease in their incidence in the protected groups as compared to the experimental group.

In a study by Wendler[17] on Wister albino rats, it was found that the severe toxic effects of the cyclophosphamide were evident from the edema and often large fluid-filled skin blebs, often containing blood (hemorrhages). A typical localization of hemorrhages was subcutaneous and in the tissues surrounding both kidneys.

Hsieh et al. [35] found in their study that cyclophosphamide provoked malformations as limb defects, renal dysgenesis, cleft palate, everted viscera, exencephaly and intracranial hemorrhage. They explained that cyclophosphamide can induce Carnitine deficiency and energy starvation which may provoke intracranial hemorrhage as brain tissues always have relatively high energy demands. And they added that folic acid successfully ameliorated the intracranial hydrogen peroxide elevation and the reduced glutathione depletion which is effective in decreasing incidence of intracranial hemorrhage.

Regarding skeletal changes; multiple skeletal anomalies were reported in the present work as incomplete and failure of ossification of skull bones that were significantly evident in the experimental group and their incidence decreased in the protected groups. Vertebral and rib anomalies included incomplete and un-ossification of vertebrae, open arch of atlas, supernumerary vertebra, wavy and supernumerary ribs that were significantly evident in the experimental group and their incidence decreased in the protected groups. Incomplete ossification of sternum was significantly reported in the experimental group and significantly decreased in the protected group. Incomplete ossification of limb bones (metacarpus, metatarsus and phalanges) was also reported in the present study as its incidence increased significantly in the experimental group as compared to the control group and decreased significantly in the protected groups as compared to the experimental group.

In agreement with the present results, delayed ossification in forelimb and several anomalies in sternum as fused sternebrae were observed by Mahabady et al. [36] in their study of protective effect of quercetin on skeletal and neural tube teratogenicity induced by cyclophosphamide in rat fetuses.

Singh et al. [37] reported that the suppressive effect of the cyclophosphamide on the proliferating mesenchyme is likely to depend upon the stage of differentiation of the limb bud at the time of the administration of the drug. The skeletal elements which differentiate at earlier stages will be more resistant than those which differentiate later. They also found the presence of un-ossified bones in the hands of all malformed forelimbs.

Gibson et al. [38] reported that Cyclophosphamide (20 mg/kg) treatment of pregnant mice on days 10 and 11 of gestation induced a wide variety of skeletal abnormalities in offspring. Three types of anomalies were noted; absence or non-ossification of ribs, fusion of the long bones, and digital defects, findings which are similar to the findings in the present study.

In a study of prevention of congenital malformations in the offspring of diabetic mice by folic acid done by Oyama et al. [39], it was reported that folic acid prevented congenital malformations including skeletal malformations as well as NTDs in the offspring of diabetic mice. And they also suggested that the preventive effect of folic acid is independent from homocysteine metabolism and possibly mediated by decreasing the abnormal apoptosis during organogenesis.

The present findings confirmed the toxic and teratogenic effects of cyclophosphamide which could be ameliorated by the administration of folic acid before and during cyclophosphamide injection.

CONCLUSIONS

Cyclophosphamide is a teratogenic drug that causes evident morphological and skeletal anomalies. Its teratogenic effect is more evident during period of embryogenesis.

Folic acid is protective drugs against teratogenicity of Cyclophosphamide regarding morphological and skeletal anomalies.

CONFLITS OF INTEREST

None.

FINANCIAL DISCLOSURES
None

SUPPLEMENTARY FILE: Figures S1-S2

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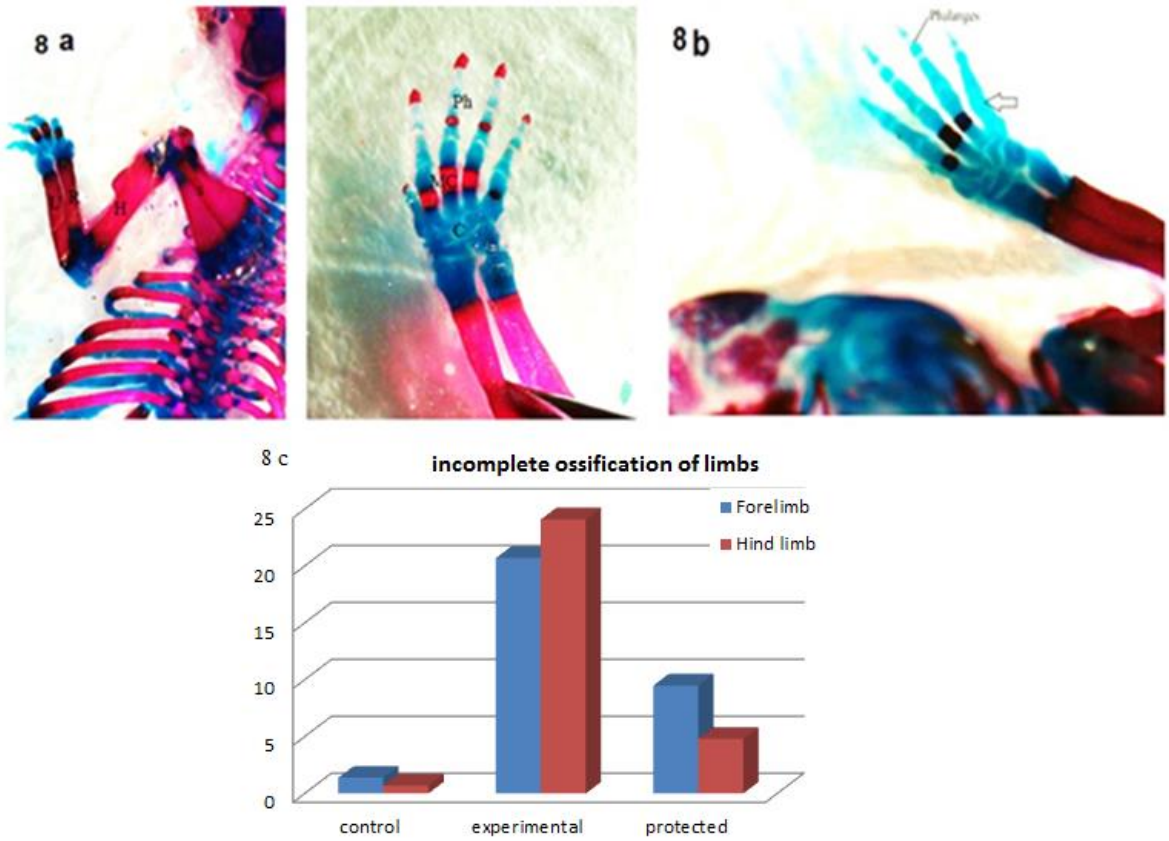

Figure

S1: (a,b), A photomicrograph of a 20 GD fetal skeleton showing:
8 a: normal fetal forelimb skeleton showing: S (Scapula), H (Humerus), R (Radius), U (Ulna), C (Carpus), MC (Metacarpus), Ph (Phalanges)
8 b: experimental rat, unossified fifth metacarpal bone (black arrow) and unossified phalanges
8 c: A bar chart showing the incidence of incomplete ossification of forelimb and hind limb bones among the study group.

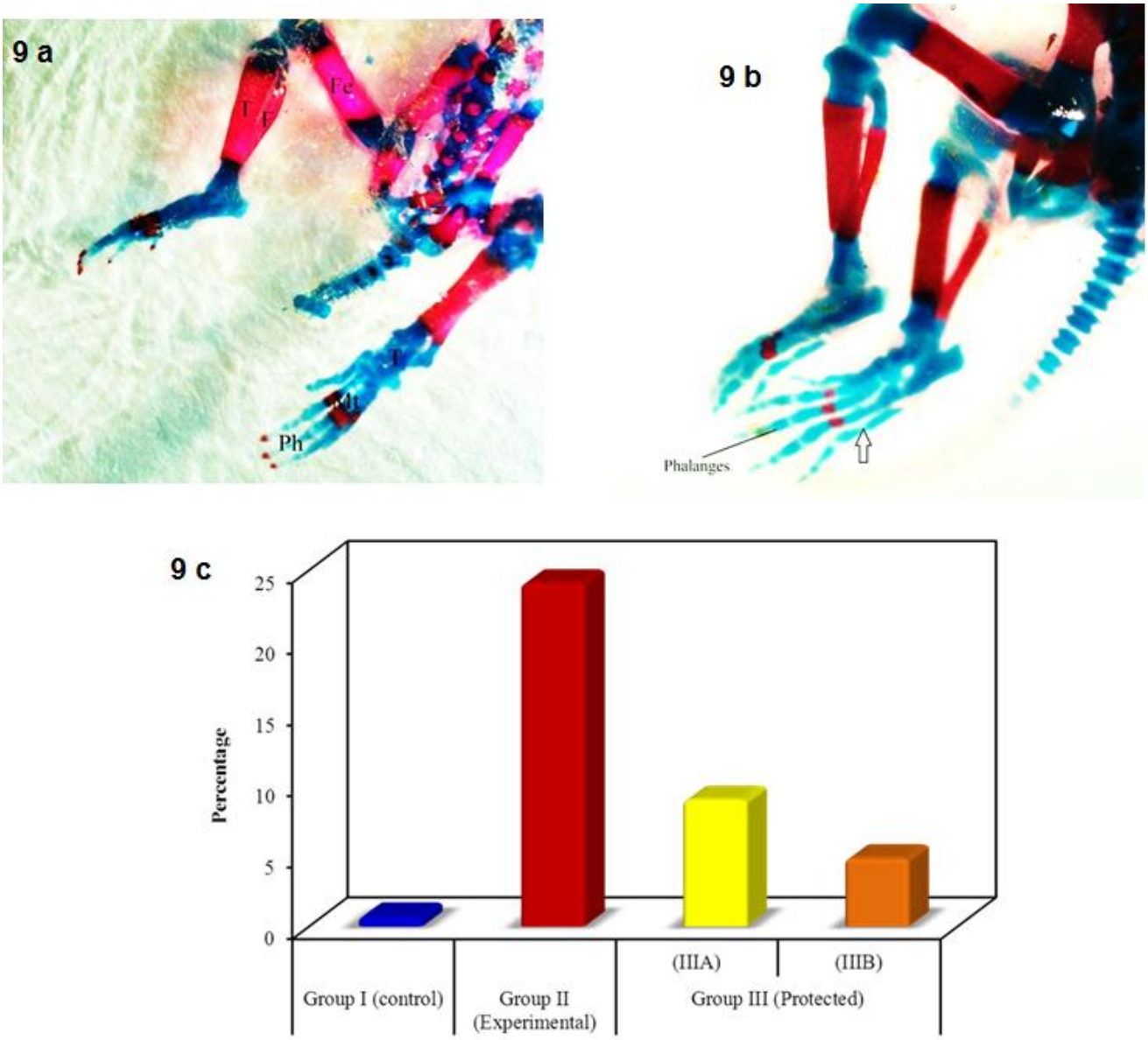

Figure S2: (a, b), A photomicrograph of a 20 GD fetal skeleton showing:
9 a: normal fetal hind limb skeleton showing: Fe (Femur), T (Tibia), F (Fibula), T (Tarsus), MT (Metatarsus), Ph (Phalanges)
9 b: experimental rat hind limb, unossified fifth metatarsal bone (black arrow) and unossified phalanges
9 c: Bar chart showing incidence of incomplete ossification of hind limb