Effects of Dietary Soybean Phytoestrogens Usage on the Skeleton of Albino Rats During In-Utero Development

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Abstract: Soybean is recognized as the major dietary source of phytoestrogens, which is commonly found in the diets of domesticated and experimental animals. No data existed in the literature about the potential interactive effects of isoflavone mixture present in soybean on the embryonic skeletal growth and development. Therefore, this work aimed to investigate the possible skeletal anomalies resulting from usage of dietary soybean phytoestrogens when given to the pregnant dams of albino rats during the critical period of in- utero development. A total of 12 pregnant albino rats were divided into three groups (4 rats for each): A control group fed on casein- based diet free from soybeans, the second group received low phytoestrogenic diet containing 20% soybeans and the third group was fed on high phytoestrogenic diet containing 30% soybeans. All groups were treated from gestation day (GD) zero through GD 20. Dams were sacrificed on GD 20 and the obtained foeti were examined grossly and then stained by the double staining method using alizarin red and alcian blue. Samples from the foetal metacarpi and metatarsi were collected for histological and histochemical examination. Our findings suggested dose dependent effects of the dietary soy phytoestrogen treatment on the in- utero development of the albino rat foeti skeleton which varied from delayed ossification in some bones of the low dose treatment to delayed ossification of most bones together with cleft plate and incomplete closure of the sagittal suture between the frontal and parietal bones of the skull in the high dose treated group. Our entire results confirmed that exposure to a mixture of phytoestrogens present in soybean during the critical periods of development especially the prenatal period possessed a high risk not only on the animal but also on the human.

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Introduction

Phytoestrogens are naturally occurring compounds present in plants and have been found to possess estrogenic properties. Soy is recognized as the major dietary source of phytoestrogens **(Bingham et al., 1998; USDA-Iowa State University Isoflavone Database, 2002).** Soy-based products have been shown to contain significant quantities of total isoflavones with soybeans and soy flour containing the highest quantities as the concentrations of phytoestrogens in soybean **(FSA, 2003).** Due to the proposed benefits of isoflavones, a wide variety of newly marketed soy-based milks, coffee lighteners, yogurts, ice creams, and other dairy products have emerged, and these products are consumed by both adolescents and adults especially by pregnant women **(Klein and King, 2007)**. Moreover, soy formula is being fed to increasingly more infants as their sole source of nutrition.

Phytoestrogens display estrogen-like activity as they are structurally similar to human estrogens and can bind to the estrogen receptor. Therefore, like any hormone, too much or too little can alter hormone-dependent tissue functions **(Guillette et al., 1995).**

Dennery (2007); Palis (2008) and Balkrishnan et al. (2010) stated that flavonoids (one of the principal groups of phytoestrogens) transfer across the placenta to accumulate in the fetus.

During embryo development, teratogenic agents can lead to significant congenital anomalies (**Stanley et al., 1986 and Vickers et al., 2002**). In this connection, **Lemmen et al. (1999)** reported that most organ systems in the rat fetus are highly sensitive to phytoestrogens and also recorded the expression of estrogen receptor in the atrial wall, brain, kidney, urethra, bladder neck, mammary gland primordium, midgut, cartilage primordia and perichondria and added that these specific expression patterns observed

for both ERa and ERb suggesting specific functions for these receptors during development and they suggested that these tissues expressing estrogen receptors may be targets for hormonal disruption through exposure to exogenous estrogens.

Spongy and incompletely or poorly ossified bones as well as unossified ossification centers were considered as important indicators of delayed ossification of the rat fetal skeleton (**Aliverti et al., 1979). Chahoud and Paumgartten(2005)** also reported that retardation implies both a smaller fetal size and a lower body weight, as well as a less developed skeleton Prior to this study, no data existed in the literature about the potential interactive effects of isoflavone mixture present in soybean on embryonic skeletal growth and development. Therefore, this work aimed to investigate the possible skeletal anomalies resulting from usage of dietary soybean phytoestrogens when given to the pregnant dams of albino rats during the critical period of in utero development from GD 0 to GD 20 (just before birth).

Material and Methods

All protocols were approved by the Animal Care and Committee of Faculty of Veterinary Medicine, Suez Canal University.

Experimental animals

The current study was performed using 12 sexually mature female albino rats and 4 males of the same species obtained from the laboratory animal house, Faculty of Veterinary Medicine, Suez Canal University. All animals were kept under a constant day/night cycle (12 h Light/12 h Dark) for 20 days (pregnancy period) in a climate controlled condition with temperature adjusted to 22 ± 2^{o} C. The female rats were mated with males overnight on a 3 to 1 basis. The female rats were examined for sperm in the vaginal smear next morning, and sperm positive ones were considered as day zero pregnancy.

Treatment

The pregnant dams were then randomly assigned to three experimental groups (4 rats for each) as the first control group was fed on a casein-based diet (free from soy), while the second and third treated groups were fed on a diet containing low and high level of soybean respectively as shown in table (1). The pregnant dams were treated from gestation day (GD) zero to GD 20.

On the 20th day of gestation, after complete anesthetization by ethyl ether inhalation, the dams from the three examined groups were placed on an experimental table and caesarian sections were done to collect the foeti.

Fetus preparation

The intact uteri with the fetuses and placentas were removed and longitudinally incised. All the obtained fetuses were weighed using a 1/ 100 g sensitivity scale and their CVRL, head lengths as well as head circumferences were measured with 1/10 cm sensibility caliber. They were examined grossly and sacrificed with ether.

Fetus staining

A number of 40, 30 and 20 foeti form casein based (control), low phytoestrogenic and high phytoestrogenic groups, respectively were divided into two halves, the first half for double staining with alizarin red and Alcian blue and the second half for the histochemical examination.

1- Double staining of the skeleton with alizarin red and Alcian blue

The foeti were firstly skinned and eviscerated; the cervical and dorsal muscles were gently removed. Also, skin of feet and tail was removed carefully to achieve better staining results. Then, they were put in 95% ethyl alcohol for 7 days and subsequently put in pure acetone for degreasing for 3 days. The fetuses were lastly stained by the double staining method proposed by **Kimmel and Trammel (1981)** to examine the bony and cartilaginous elements of the foetal skeletons**.** The bones were stained red with alizarin red while the cartilaginous components (epiphyseal and joint cartilages, intervertebral discs, as well as nasal and costal cartilages) were stained blue with alcian blue.

After that, they were undergone a transparency process to visualize the skeletal system of all fetuses using a dissecting microscope. Anomalies such as absence, abnormal form and length of the bones were recorded. Examination included bones of skull, ribs, sternum, as well as bones of the thoracic and pelvic limbs. Finally, they were transferred gradually into pure glycerin for further usage. Photographs were taken using a Nikon D100 camera (60 mm, micro objective).

1.a. Staining Solutions

The acidic staining solution consisted of 0.14% Alcian blue GX (Sigma) dissolved in 70% ethanol, filtered, (5 parts), 0.12% Alizarin red S (Fluka) dissolved in 96% ethanol, filtered, (1 part) glacial acetic acid, (8 parts) and 70% ethanol, (50 parts). The basic staining solution consisted of 0.7% KOH dissolved in distilled water, (250 parts), 0.5% Alizarin red S (Fluka) dissolved in distilled water, filtered, (1 part). The clearing solution consisted of 70% ethanol, (2parts), glycerin, (2 parts), benzyl alcohol, (1 part).

***1.b. Staining* procedures**

Fixed specimens were immediately placed for at least 24 hr. in the acid staining solution at room temperature, then in ethanol 96% for at least 6 hr. Placing specimens in the basic staining solution for 30 hr. at room temperature, while renewing the solution at least three times. Clearing and hardening

was performed by placing specimens in the cleaning solution for at least 8 hr. Conservation of double-stained fetuses was performed in a 1:1 ethanol 70% and glycerin mixture. Store in pure glycerol.

2-Histochemical examination

Safranin-O and toluidine blue were an indicator of cell chondrogenesis, is a cationic dye that stains acidic proteoglycans present in cartilage tissues (**Mackay, 1998).** Safranin-O stained the cartilage cells and matrix with dark to light red while toluidine blue stained the cartilage by dark to light purple.

Metatarsal bones were collected and fixed in 10% neutral buffered formalin solution for 48 hours. Paraffin sections of these bones were then stained with Safranin-O or toluidine blue stain **(Kahveci Z et al., 2000)** to detect the ossification centers as following:

Deparaffinize and hydrate slides to distilled water. Stain with Weigert's iron heamatoxylin working solution for 10 minutes. Wash in running tap water for 10 minutes. Add 200 µl of 1% acetic acid to each tissue section; allow reacting for 10 - 15 seconds and then removing acetic acid. Add a 200-µl aliquot of Stain solution (Safranin-O or toluidine blue) to each tissue section and let stand at room temperature for 5 minutes to stain the tissue then remove stain solution. Add absolute ethanol for destaining. Perform dehydration, clearing and mounting.

Statistical analysis

All values were presented as mean ± standard error. The statistical differences between the groups were determined by analysis of variance (one way ANOVA) using SPSS® software (Statistical Package for Social science, version 17.01, IIIinois, USA). The statistical significance was set at $P < 0.05$ while considered highly significant at $P < 0.01$.

Table (1): Composition of the experimental diets*/kg of the diet.

Component	Casein-based diet (Control) (gm)	Low phytoestrogen diet (gm)	High phytoestrogen diet (gm)
Yellow Corn	72.5	35	28
Soybean seeds	----	20	30
Rice	----	27	30
Gluten	10	11	5
Casein	10	----	----
Soybean Oil	----	3	3.5
Corn Oil	3	----	----
DiCalcium Phosphate	1.4	1.3	1.1
Ground lime stone	1.5	1.3	1.1
Common Salt	0.4	0.4	0.4
Lysine	0.3	0.2	0.1
Methionine	0.3	0.3	0.3
Premix**	0.6	0.5	0.5
Total	100	100	100

*The control and two experimental diets were formulated to fulfill all the nutritional requirements of pregnant female rat according to (NRC, 1995) using different sources of protein like casein and corn for control diet while various amounts of soybean seeds with corn as a source of protein in case of low and high phytoestrogenic diets but finally all the diet ingredients in all the experimental diets were balanced.

**Premix produced by Muvco. Supplied per kilogram diet: 12.000 and 2.000 IU of vitamin A and D3و respectively; 10 g vitamin E, 1 g vitamin K, 0.005 g vitamin B2, 0.0015 vitamin B6, 10 g pantothenic acid, 0.02 niacin, 0.6 gm choline chloride, 0.03g iron, 0.06 g manganese, 0.004 g copper, 0.05 gm zinc, 1 mg vitamin B1, 0.001 mg vitamin B12, 1 mg folic acid, 0.05 mg biotin, 0.3 iodine , 0.1 mg cobalt and 0.01 mg selenium.

3. Results

Firstly, there were no mortalities among the mothers of the three examined groups throughout the entire period of the experiment.

In addition, calculation of the resorption rate revealed highly significant increase ($P<0.01$) among the mothers fed on high phytoestrogenic diet, where about 26.50% of total foeti were resorped. On the other side, a slightly non-significant increase of resorption rate (10.30%) in low phytoestrogenic group compared with that in case of the casein based group (4.95%) (Table 2).

Morphometric findings of the foeti:

As compared to the average lengths (CVRL) of the fetuses in the control group, which was 3.7±0.058cm, the lengths of those in the low phytoestrogenic group were slightly shorter (3.52±0.072 cm CVRL) with significant difference ($P<0.05$), while the shortest foeti

were observed in the high phytoestrogenic group (2.8±0.06 cm) with high significant difference (P<0.01).

Also, the foeti of the high phytoestrogenic group showed a clear highly significant decrease in the body weight (P<0.01), while there was a significant decrease in the low phytoestrogenic group foeti (P<0.05) when compared with the weights of the control group foeti (4.55±0.12, 3.9±0.06 and 2.3±0.06 gm. in control, low and high phytoestrogenic groups, respectively).

Moreover, it was recorded that the head parameter measurements such as head length and circumference were about 1.33±0.038 and 1.8±0.048 cm, respectively in the control group while 1.22±0.025 and 1.7±0.047 cm in the low phytoestrogenic treated group. Whereas, in case of high phytoestrogenic treated group, these measurements were 0.905±0.032 and 1.1±0.039 cm, respectively (table 2). So, a clear highly significant decrease in the head circumference and head length in the high phytoestrogenic group in comparison with the control group. While, there was non-significant decrease in the head circumference among the low phytoestrogenic group foeti**,** but showed a significant decrease in the head length.

Alizarin red and alcian blue findings:

1- Control (Casein-based diet) group

Many bones of the skull such as the frontal, parietal and interparietal bones manifested complete ossification and stained red in all foeti of this group. While, ossification was not yet completed in the temporal and some parts of occipital bones. Most of the dorsum of the skull showed ossification areas (Fig. 1-A).

The nasal cartilages were stained blue on the facial area of the skull with complete ossification of the nasal bone (Fig. 1-D). Also, ossification areas were seen on the base of skull from the base of the occipital bone caudally through the palatine and incisive bones rostrally. However, in the lateral surface of the skull the ossification process was clear from the maxillary bone to the parietal bones with incomplete ossification of the temporal bone, which appeared blue-stained (Fig. 1-B&C)

Concerning the vertebral column, the thoracic spinous processes, most of cervical, sacral and caudal vertebrae were fully stained blue indicating their cartilaginous nature (Fig. 2). The cervical vertebrae were unossified except the arches of the atlas and axis in which ossification process started to appear (Fig. 2-A). The arches of cranial group of the thoracic vertebrae with their bodies showed clear ossification, while the caudal group of vertebrae was much less ossified (Fig. 2-B). The lumbar vertebrae spinous and transverse processes were stained blue while their bodies showed clear ossification (Fig. 2-C). The four sacral and first four caudal vertebrae possessed little ossification in the centrum of their bodies (Fig. 2-D).

The proximal two-thirds of all the thirteen ribs were completely ossified except their heads and tubercles, which were still cartilaginous in nature. On the other hand, the distal third of the ribs and their costal cartilages together with the costal arch were stained blue. In addition, the cartilage–ossification ratio of the body of the ribs increased as directing caudally (Fig. 3-A). Moreover, most of the examined foeti presented 5-6 ossified sternebrae (Fig. 3-B). The scapular spine was stained blue, whereas, the scapular dorsal margin, main body, glenoid cavity as well as the ventral angle were stained red (Fig. 4- A).

The ilium showed complete ossification except the iliac crest, which was still cartilaginous. Also, most of the posterior part (ischium) was stained red. While the rest of the pelvic bone including pubis (anterior part) and the acetabulum still possessed the cartilaginous nature (Fig. 5-A).

Diaphyseal ossification was observed in the long bones of both extremities (humerus, radius, ulna, femur, tibia and fibula) The measurements of the ossified and unossified portions of these long bones were recorded with calculation of the ossification percentage for each bone (Figs. 4&5) and (tables 3&4).

The carpal and tarsal bones resembled a cartilage draft. Partial ossification appeared in the diaphysis of the large four metacarpal as well as the metatarsal bones. It was also noted that the proximal and middle phalanges of the anterior and posterior paws were completely cartilaginous in nature (Fig.6 A&B). Also, red-stained areas in the distal phalanx diaphysis of the anterior paw were observed (Fig. 6.A), however, that of the posterior paw was still cartilaginous (Fig. 6.B).

2- Low phytoestrogenic group:

Foeti of the low treated group showed to a large extent a similar pattern of ossification to the control group. Complete ossification of most bones of skull except the temporal, supraoccipital, and pterygoid bones that stained blue indicating their cartilaginous nature (Fig. 1).

Focusing on the vertebral column, ossified regions were similar to that of the control group (Fig. 2-A,B,C) except the lumbar vertebrae where ossification appeared in their arches but the bodies were still cartilaginous. In addition, there was an abnormal fusion between the bodies of the sacral and the first four caudal vertebrae. Moreover, the 1st caudal vertebra appeared ossified, while the remainder caudal vertebrae were stained blue indicating that they were still cartilaginous (Fig.2-D)

The ribs showed the same number, shape and pattern of ossification as that of the control group with presence of small non-ossified areas on the distal end

of the ribs, which appeared blue-stained (Fig. 3-A). Moreover, only the 3rd and 4th segments of the sternum became ossified (Fig.3-B). .

Concerning examination of the bones of the thoracic and pelvic limbs, there was clear moderate decrease in size of the scapula, humerus, radius, ulna, femur, tibia and fibula. Moreover, all the long bones showed obvious decrease in the length of the ossified portion of their diaphyses and in turn increase in the unossified areas of both epiphyses in relation to the whole length of the bones indicating moderate delay in the ossification process. The scapulae of the foeti in this group had slightly shorter spines with clear cartilaginous glenoid cavity and ventral angle compared with those of the control group (Figs. 4&5) and (tables 3 &4). The carpal and tarsal bones were cartilaginous in nature similar to the control group. The metacarpal and metatarsal bones as well as distal phalanx of the anterior paw showed small red-stained areas in their shafts. Meanwhile, the middle and proximal phalanges of the anterior paws together with all the phalanges of the posterior paw were still cartilaginous (Figs. 6A &B).

3- High phytoestrogenic group:

High group foeti showed a clear defect in the ossification process in different parts of skeleton. In the Skull, there were no ossification signs in the interparietal, temporal, supraoccipital, exoccipital, pterygoid fossa, zygomatic, pterygoid and nasal bones as well as the tympanic bulla, the borders of the parietal bones and the palatine process of the maxillary bone (Fig.1). The presence of cleft in the palatine bone was characteristic to this group of foeti appeared in 75% of the affected foeti (Fig. 1c). Moreover, a wide non-stained area was observed at the interfrontal and interparietal junctions in about 90% of the treated foeti (Fig.1-A).

Concerning the vertebral column staining pattern, all the vertebrae of the cervical and thoracic regions stained blue (Fig. 2-A&B). In the lumbar region, red ossified areas appeared within arches of 2nd to 6th lumbar vertebrae, while the 1st lumbar vertebra was completely stained blue. In addition, blue cartilaginous fusion between the vertebral arches was present (Fig. 2-C). The sacral vertebrae showed a marked defect in the ossification process as they maintained the cartilaginous nature. Moreover, an incomplete formation of the vertebral bodies with absence of their centrum together with ventral fusion of the bodies of the sacral vertebrae and the first two coccygeal vertebrae as well as dorsal fusion of their laminae were observed (Fig. 2-D). The coccygeal vertebrae kept their cartilaginous template without any evidence of ossification (Fig. 2-D).

The ribs were complete in number and normal in form but there was delayed ossification of their distal ends, which appeared blue-stained (Fig.3A). The sternum of the high group did not show ossification process in any of the sternal segments (Fig. 3-B).

The long bones of the thoracic and pelvic limbs in the high treated group showed marked significant decrease in the morphometric measurements compared with those in the control and low treated groups with increase in the unossified areas on the expense of the ossified parts in relation to the whole length of the bone (Figs. 4&5) and (tables 3 &4).

The scapulae of foeti in this group was smallest in size among the examined groups and their cartilaginous areas represented by the ventral angles and glenoid cavities appeared larger in size relative to the whole scapular size. Also, the scapular spine of about 85% of the foeti appeared extremely short. In addition, there was a marked blue-stained line appeared close to the dorsal border of the scapula indicating incomplete ossification of the bone in about 60% of the treated group foeti (Fig.4).

On the other hand the coxal bone appeared completely maintaining the cartilaginous draft except part of the ilium and a small area of the ischium (Fig. 5-A).

Regarding the paws, it was clear that all bones of the paws including the carpal or tarsal, metacarpal or metatarsal as well as the phalanges were stained blue indicating that there was severe delay in their ossification process (Fig. 6).

Microscopical examination of the metatarsal bone:

Using the paraffin sections prepared from the metatarsal bones as a tool for confirmation of the ossification pattern occurring in our experimental groups, it was revealed that normal stages of ossification process occurred within the diaphyses of the metatarsal bones in the control and low groups including rest zone of non proliferative chondroblasts, and then proliferative chondroblasts zone directed toward the ossification center, then the zone of mature hypertrophic chondroblasts that were replaced by osteoblasts to form the primary ossification centers with formation of the developing periosteum covering the newly formed bone in addition of the already existed perichondrium which covered the cartilaginous template(Fig.7). The ossification centers in the control group appeared to be more developed and calcified than in the foeti of the low group (Fig.7).

On the other hand, in the high group only the resting zone and the proliferating chondroblasts zone were present indicating delayed ossification process confirming the result detected by the double staining technique of these bones previously mentioned and shown in (Fig. 6).

Histochemical staining of the cartilage using Safranin-O and toluidine blue matched our previous results as the different zones of bone formation were

obvious from the resting zone, which stained dark red or purple to the proliferating chondroblast zone, which stained pale red or purple and non- stained area of the newly formed primary ossification center in case of foeti of the control and low groups, however, the primary ossification center showed much more larger calcified area in the control group than that in the low group (Fig. 8).

On the other hand, the high treated group foeti showed only the resting zone and proliferating chondroblast zone with few number of scattered hypertrophic chondroblasts within the proliferating zone indicating delayed ossification process that occurred in the high phytoestrogenic treated foeti (Fig. 8).

Table (2): Morphometric parameters of the foeti of the three examined groups.

Fetal parameters	Casein-based diet group	Low phytoestrogenic diet group	High phytoestrogenic diet group
Total number of foeti	40	30	20
Number of the live foeti	40	30	20
Number of the dead foeti	0	0	0
Percentage of malformed foeti	1.7%	3.4%	80%
Resorped foeti, No (%)	4(4.95%)	7 (10.30%)	15 (26.50%) **
Fetal body weight (gm.)	4.55±0.12	3.9±0.06*	2.3±0.06**
Fetal CVRL (cm)	3.7± 0.058	3.52±0.072*	2.8±0.06**
Fetal head length (cm)	1.33±0.038	1.22±0.025*	0.905±0.032**
Fetal head circumference (cm)	1.8±0.048	1.7±0.047	1.1±0.039**

Values are expressed as means ± S.E.M.; ∗ and ∗∗ denote significant differences from the control value, $P < 0.05$ and $P < 0.01$, respectively (Dunnett).

Table (3): Length of the ossified and unossified portions in the thoracic limb long bones in the foeti of the three examined groups.

Thoracic limb	Humerus			Radius			Ulna		
	Control	Low	High	Control	Low	High	Control	Low	High
Whole bone length (cm)	1.802 ±0.08	1.779 ±0.16	**1.448 ±0.17	1.499 ±0.02	*1.134 ±0.18	**1.089 ±0.28	1.698 ±0.04	1.667 ±0.07	**1.532 ±0.25
Non-ossified proximal epiphysis length (cm)	0.328 ±0.21	0.333 ±0.02	0.427 ±0.13	0.205 ±0.15	0.244 ±0.32	0.236 ±0.29	0.402 ±0.01	0.504 ±0.19	0.486 ±0.36
Non-ossified distal epiphysis length (cm)	0.343 ±0.07	0.36 ±0.12	0.43 ±0.32	0.334 ±0.04	0.302 ±0.34	0.366 ±0.24	0.31 ±0.14	0.43 ±0.03	0.452 ±0.24
Ossified diaphysis length (cm)	1.156 ±0.11	1.09 ±0.25	0.601 ±09	0.952 ±0.16	0.594 ±0.35	0.496 ±0.01	0.998 ±0.06	0.734 ±0.32	0.601 ±0.28
Ossification percentage (%)	63.27	61.13	41.22**	63.85	52.1**	45.17**	58.36	44.00*	39.05**
Ossified diaphysis width (cm)	0.309 ±0.08	0.297 ±0.19	0.24 ±0.14	0.21 ±0.31	0.15 ±0.09	0.109 ±0.18	0.234 ±0.21	0.224 ±0.13	0.213 ±0.29

Values are expressed as means ± S.E.M.; ∗ and ∗∗ denote significant differences from the control value, $P < 0.05$ and $P < 0.01$, respectively (Dunnett).

Table (4): Length of the ossified and unossified portions in the pelvic limb long bones in the foeti of the three examined groups.

Pelvic limb	Femur			Tibia			Fibula		
	Control	Low	High	Control	Low	High	Control	Low	High
Whole bone length (cm)	1.548 ±0.19	**1.469 ±0.25	**1.198 ±0.31	1.268 ±0.16	1.262 ±0.08	**1.128±0.1 1	1.223 ±0.32	1.162 ±0.41	**0.72 ±0.03
Non-ossified proximal epiphysis length (cm)	0.278±0.23	0.54 ±0.15	0.345 ±0.04	0.33 ±0.21	0.43 ±0.23	0.277 ±0.18	0.265 ±0.09	0.321 ±0.04	0.214 ±0.07
Non-ossified distal epiphysis length (cm)	0.295 ±0.019	0.388 ±0.16	0.46 ±0.23	0.145 ±0.16	0.285±0.32	0.288 ±0.12	0.15 ±0.06	0.28 ±0.33	0.194 ±0.02
Ossified diaphysis length (cm)	0.978 ±0.14	0.543 ±0.03	0.413 ±0.43	0.798 ±0.02	0.55 ±0.21	0.565 ±0.09	0.81 ±0.18	0.57 ±0.14	0.315 ±0.12
Ossification length percentage (%)	63.06	36.91 **	33.91**	62.69	43.48*	50.00 *	66.12	48.6**	43.57**
Ossified diaphysis width (cm)	0.229 ±0.16	0.222 ±0.09	0.198 ±0.27	0.185 ±0.04	0.165 ±0.39	0.164 ±0.24	0.116 ±0.11	0.091 ±0.15	0.093 ±0.07

Values are expressed as means ± S.E.M.; ∗ and ∗∗ denote significant differences from the control value, $P < 0.05$ and $P < 0.01$, respectively (Dunnett).

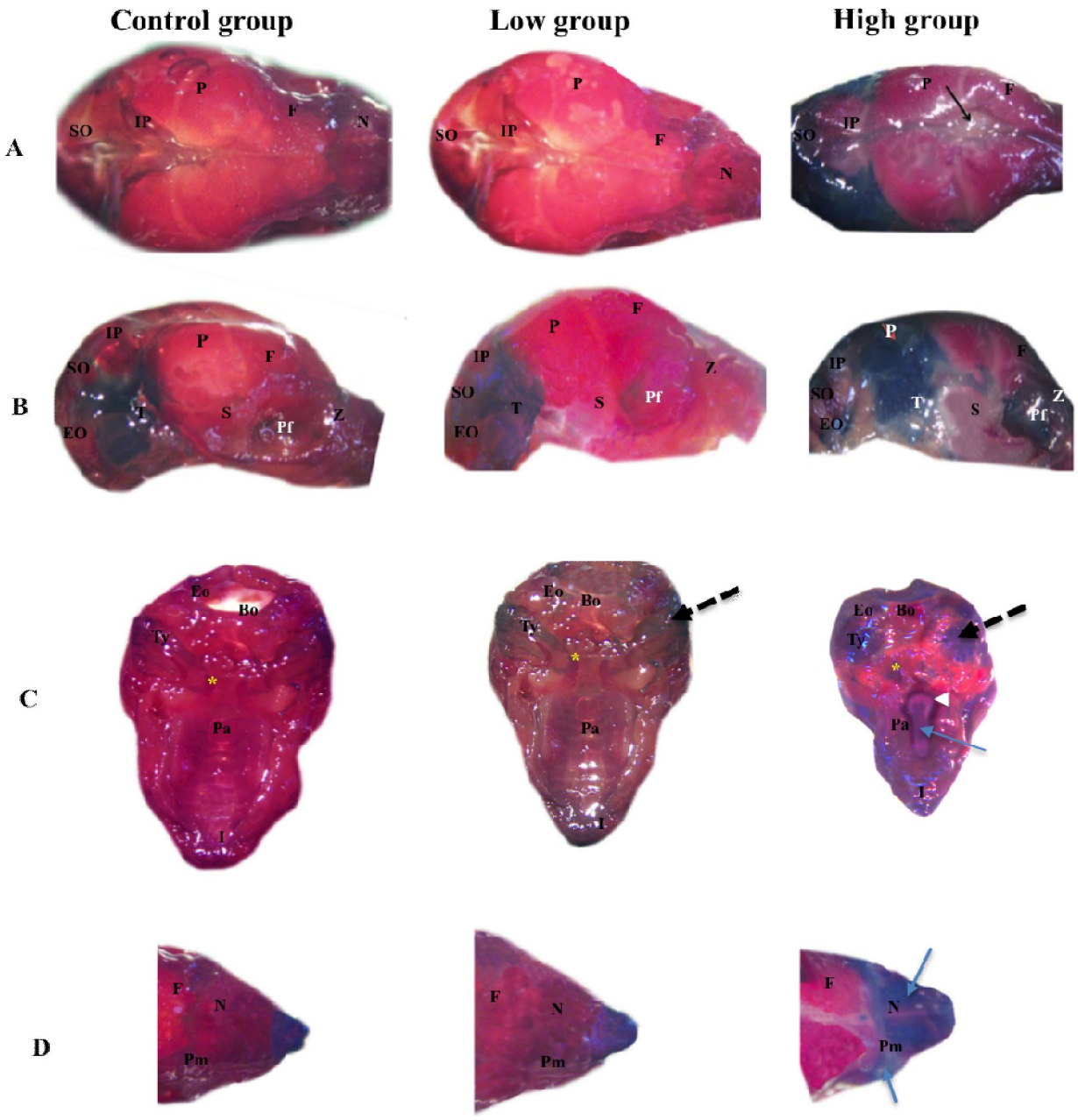

Fig. 1: A photograph of Alcian blue and alizarin red double staining of the skull. (1-A) Dorsal view of foetal rat′s skull showing normal ossification in both control and low treated groups. Delayed ossification (blue stain) in the high treated group with improper closure of the sagittal suture between the frontal bones (F) and between the parietal bones (P) (black arrow). (1-B) Lateral view of the foetal rat′s skull of the high treated group showing incomplete ossification of the parietal (P) bone and interparietal bone (IP), exoccipital bone (Eo) and bones forming the pterygoid fossa (Pf) and zygomatic bones (Z). (1-C) Basal view of the foetal rat′s skull of the high treated group showing incomplete ossification of the pterygoid bone (yellow star) and tympanic bulla (Ty) (dashed black arrow) in both low and high treated groups. Also, a cleft (white arrow head) appeared in the palatine bone (Pa) with incomplete ossification of the same bone (blue arrow) in the high treated group. (1-D) A magnification of the dorsal surface of the nasal bone (N) and premaxilla (Pm) showing improper ossification (blue arrows) in both bones in the high treated group. (SO) supraoccipital bone;(T) temporal bone; (S) squamosal bone; (I) incisive bone; (Eo) exoccipital bone Scale bars: 5 mm.

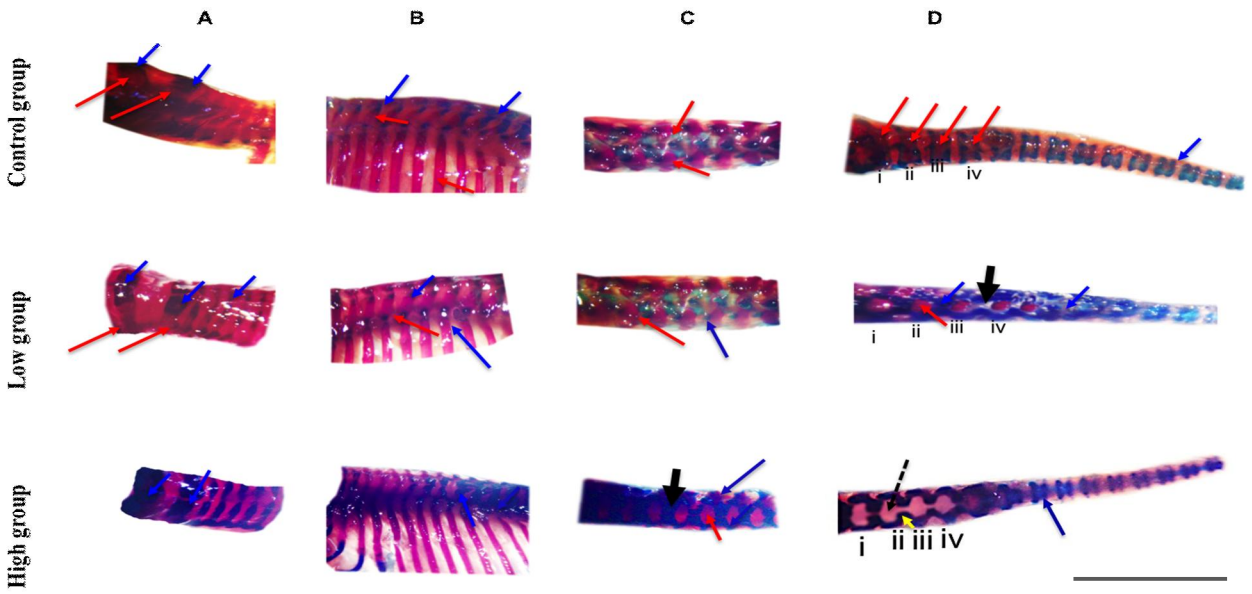

Fig. 2: A photograph of Alcian blue and alizarin red double staining of the vertebral column regions: lateral view in the cervical region (A); thoracic region (B) and lumber region (C); and ventral view in sacro-coccygeal region (D). Red arrows referred to the ossified area; blue arrows indicated the cartilaginous areas; black arrows referred to the area of fusion between the vertebral bodies appeared in some regions in the low and high treated groups and yellow arrow referred to the defect in formation of some vertebral bodies and absence of their centrum (dashed black arrow). (i,ii,iii,iv) denoted the four sacral vertebrae. Scale bars: 5 mm.

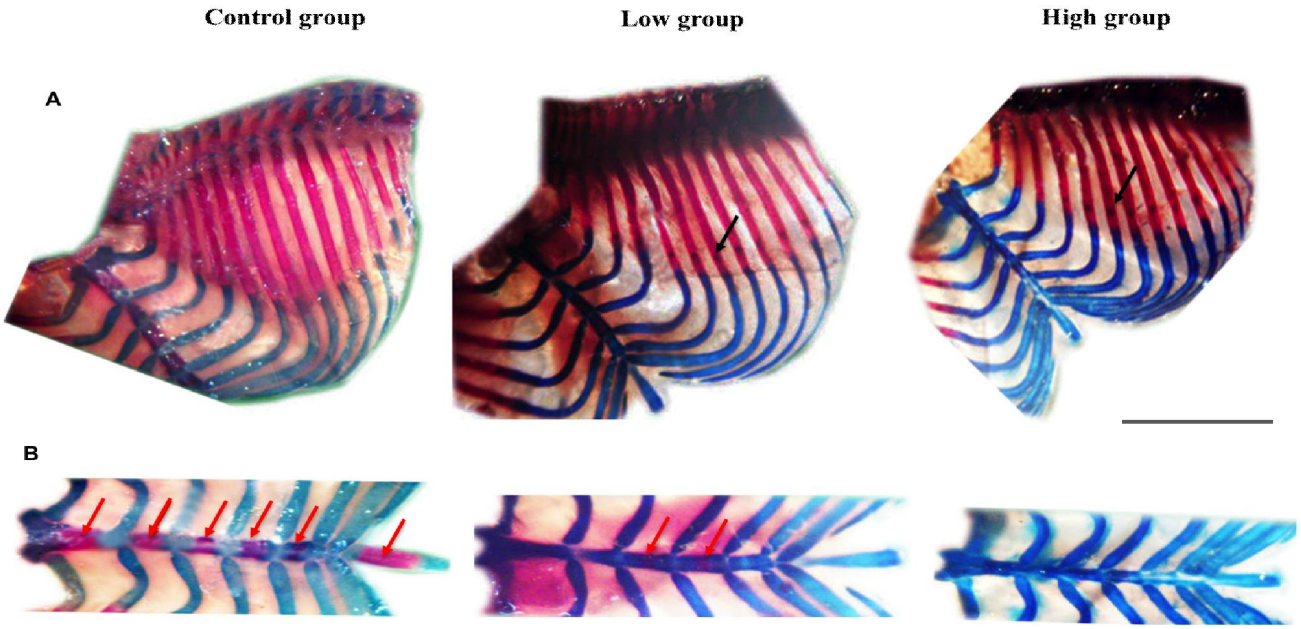

Fig. 3: A photograph of Alcian blue and alizarin red double staining to the bony thorax. (3-A) showing same number of the ribs among the three groups, black arrows referred to unossified area in the distal extremity if the ribs of the low and high treated groups. (3-B) showing six ossified sternebrae in control group and two in low group (red arrows). Scale bars: 5 mm.

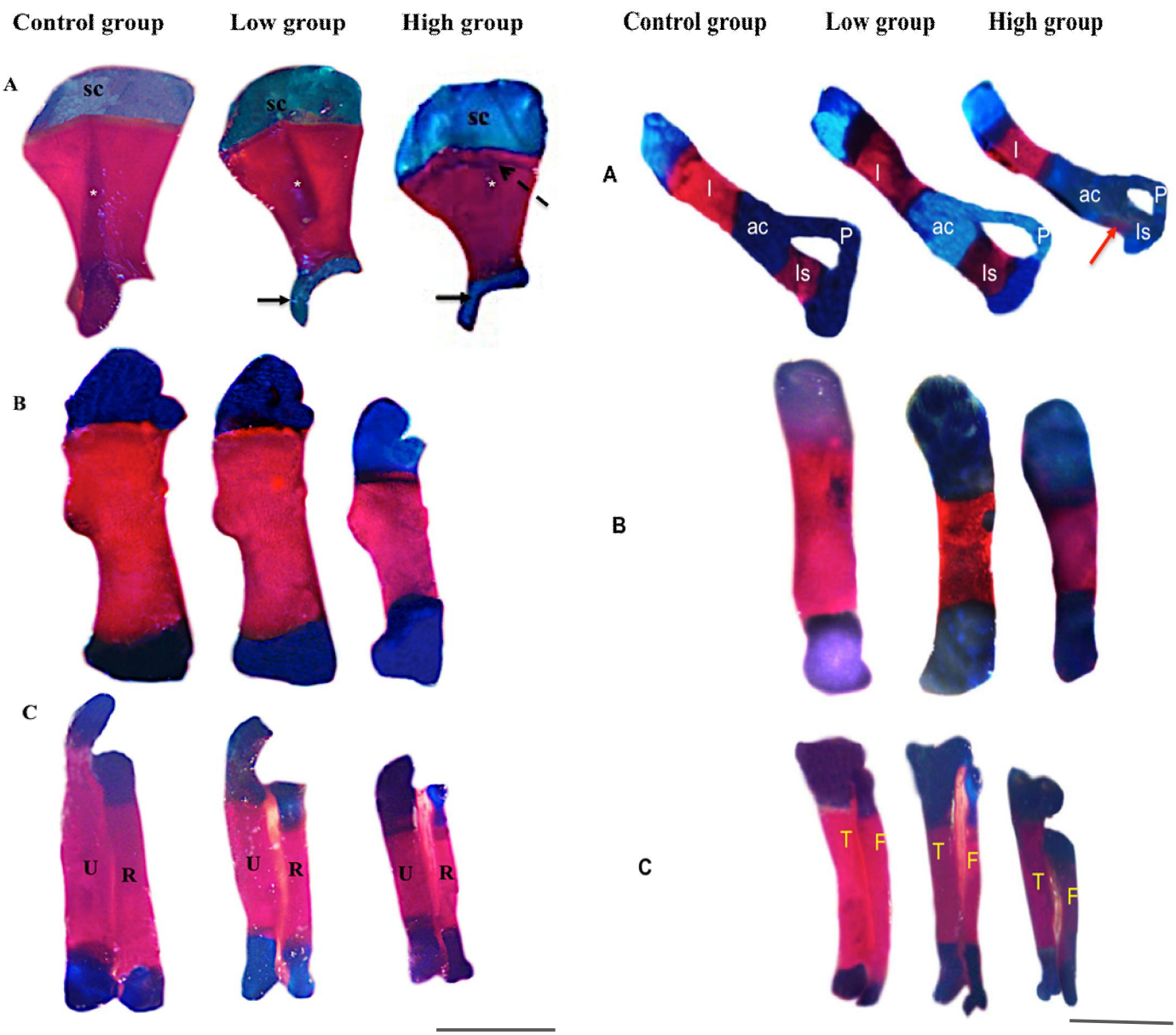

Fig. 4: A photograph of Alcian blue and alizarin red double staining to the bones of the thoracic limb. (A) Scapula; (Sc) Scapular cartilage ;(B) Humerus; (C) Radius (R) and Ulna (U). Black arrow indicated unossified ventral angle and glenoid cavity of the scapula in low and high treated group. Dashed black arrow referred to unossified line- like part on the dorsal border of the scapula in the high treated group. A short scapular spine (white star) in the low group while extremely short in the high group. Scale bars: 5 mm.

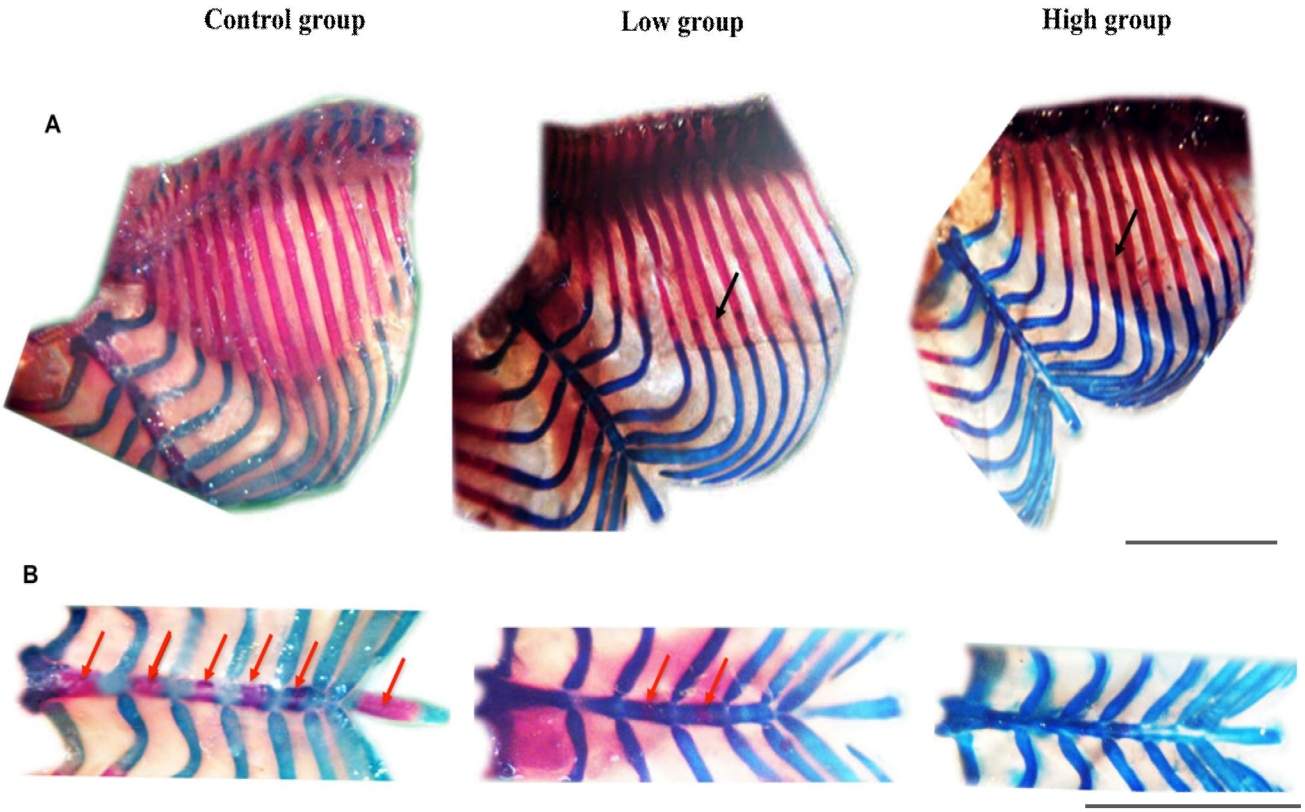

Fig. 5: A photograph of Alcian blue and alizarin red double staining to the bones of the pelvic limb (A) Coxal bone; (B) Femur and (C) Tibia (T) and Fibula (F). Red arrow referred to the small-ossified area in the ischium of the high group. (I) Ilium; (ac) Acetabulum; (Is) Ischium; (P) Pubis.

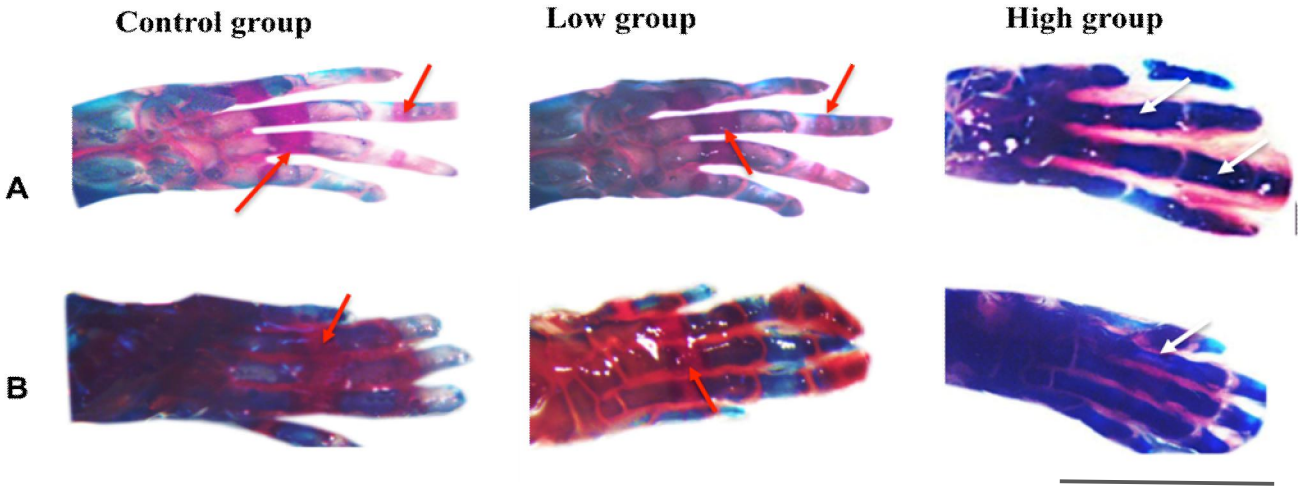

Fig. 6: Alcian blue and alizarin red double staining of (A) manus (anterior paw) and (B) pes (posterior paw) regions showing the pattern of ossification. Red arrows referred to the ossified areas of the metacarpal and metatarsal bones as well as the phalanges in the control and low treated groups. White arrows referred to delayed ossification of the same areas in high treated group.

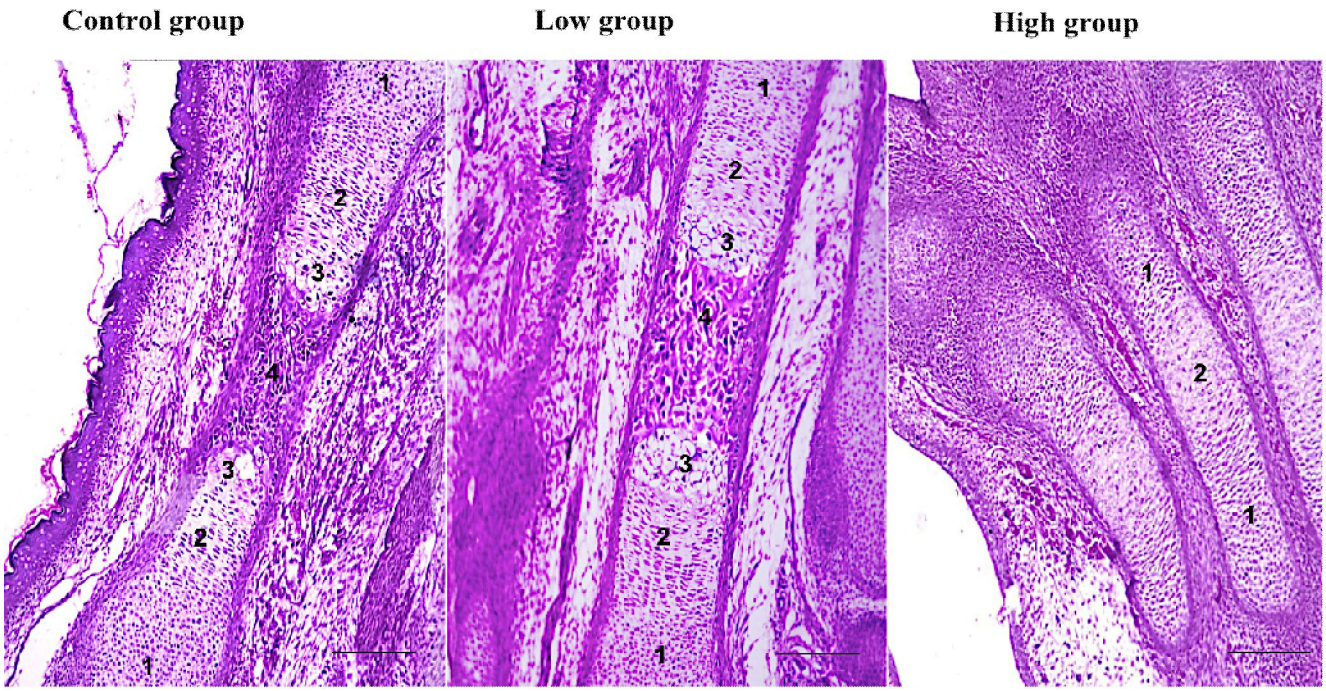

Fig. 7: A photomicrograph of longitudinal section in the metatarsal bones showing normal zones of ossification process within their shafts in the control and low groups including: zone of resting (1); zone of proliferation (2); zone of maturation and hypertrophy (3) and zone of cartilage calcification and ossification (4). Black arrow indicated the developing periosteum. Yellow arrow indicated the perichondrium. (H&E), Scale bars: 100 μm.

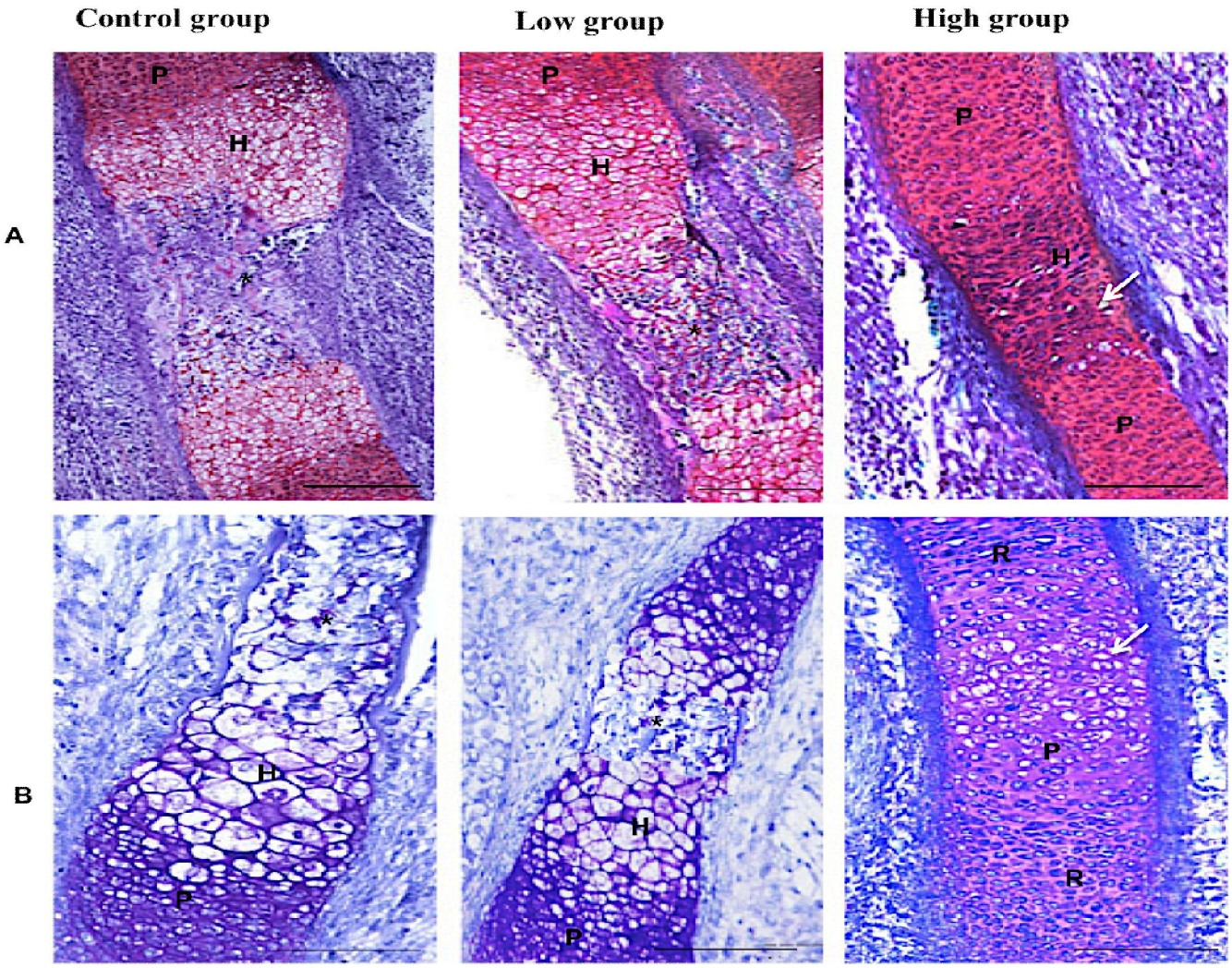

Fig. 8: A photomicrograph of the ossification centers in longitudinal section of the metatarsal bones using Safranin-O (A) and toluidine blue (B) staining technique showing the presence of zone of proliferating (P) and hypertrophic (H) chondroblasts in both control and low treated groups with a middle non-stained ossified center (black star), White arrow indicated resting and proliferated zones with scattered few number of small hypertrophic chondroblasts in the sections of the high group foeti of both stains. Scale bars: 100 µm.

Discussions

The aim of this teratogenicity study is to investigate the potentiality of dietary isoflavones as a factor inducing skeletal abnormalities in Albino rat foeti. Our previous published data **(El-Mahdy et al., 2017)** showed that there was no lethal effect on the pregnant mothers with an increase in the resorption rate of foeti resulting in marked decrease on the pregnancy outcome in the high phytoestrogenic group compared with the other experimental groups. In addition, marked decrease in many foetal parameters such as body weight, CVRL and head parameters in a dose dependent manner in both low and high phytoestrogenic groups compared with the control group with severe abnormalities in different body organs that appeared in the high maternally treated foeti. So, the current work provided more detailed approach to elucidate the possible risks especially on the rat fetus skeleton development after exposure to a mixture of phytoestrogens that present in soybean during the prenatal period.

The current data revealed that there was no evidence of bone absence in any of the treated groups. On the other hand, severe defects in the bone ossification were very clear in the high treated group together with marked decrease in length of long bones of the limbs. A cleft in the palatine bone was the most characteristic skeletal anomaly in the high

treated group of foeti with presence of a wide non-stained area that observed at the interfrontal and interparietal junctions which might be due to a defect in closure of the sagittal suture between these bones. Moreover, delay in the ossification process was noticed in many bones of the skull including interparietal, temporal, supraoccipital, exoccipital, pterygoid fossa, zygomatic, pterygoid and nasal bones as well as the tympanic bulla, the borders of the parietal bones and the palatine process of the maxillary bone.

The vertebral column ossification appeared to be moderately affected in the low phytoestrogenic group where delay in ossification of the vertebrae of some regions with abnormal fusion between the bodies of the sacral and the first four caudal vertebrae. On the other hand, the effect was more obvious in case of the high treated group foeti as most of the vertebral column regions showed severe delay in ossification. Furthermore, incomplete formation of the vertebral bodies and absence of their centrum together with ventral fusion of the bodies of the sacral vertebrae and the first two coccygeal vertebrae as well as dorsal fusion of their laminae were observed in this group. Ossification of the sternum also was mildly affected in the low phytoestrogenic treated group while no ossification was noticed in the high treated group.

The histological and histochemical examination of the metatarsal bones suggested the dose-dependent manner of the phytoestrogen treatment on the foeti of the pregnant females.

According to Amal et al. (2014) after using Alizarin red staining, there was incomplete ossification of the cranial bones as well as absence of the sternebrae, phalanges and the coccygeal vertebrae in Albino rat foeti. However, the recent study didn't show any bone absence, the result which might be due to the use of Alcian blue and Alizarin red double staining technique that able to stain both cartilaginous and bony elements of the skeleton.

Microscopic examination of the metatarsal bones, as representatives of long bones, suggested a dose-dependent manner of the phytoestrogen treatment on the range of progress of the ossification process of these bones in the foeti of the pregnant female Albino rats. These findings confirmed what stated by McClain et al. (2007) who recorded minor abnormalities in the sternum and presence of rudimentary extra ribs in rat foeti in case of high dose of genistein compared to the controls as well as unossified certain bones of the paws in the low dose treated group. In this connection, Zou et al. (2012) studying the effect of genistein in rat limb bud cell cultures at different concentrations concluded that the high concentration caused reduced size and number of cartilage modules.

In conclusion, our entire data suggested that phytoestrogen treatment during the prenatal period affected in a dose dependent manner on the skeleton ossification, growth and development, where low phytoestrogen exposure during pregnancy led to delay in ossification in different bones of the skeleton with mild decrease in growth of some bones compared with the control foeti. On the other side, exposure of the rat foeti to high phytoestrogenic diets showed severe signs of delay in ossification together with anomalies such as evidence of cleft palate and defect in the closure of the sagittal suture between the parietal bones as well as between the frontal bones of the skull.

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