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# The Effects of Dexamethasone on Tibia Development of Local Chick-Embryo. I: Computer-Assisted Morphometric Study

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## Abstract

**Background** Dexamethasone is a glucocorticoid as a member of the steroidal anti-inflammatory and immunosuppressant. It has well documented effects on skeletal structures osseous and cartilaginous, commonly used to treat or control diseases.

**Objective** To evaluate by histomorphometric study the effects of dexamethasone on the embryogenesis of long bones in chick embryos.

**Methods** Forty-eight fertile chick eggs of *Gallus gallus domesticus*, were used. The eggs were divided into 2 groups; control and treated groups of 24 eggs each, these groups were subdivided into 4 subgroups (n=6 eggs). On day 10 of incubation, the control group was injected with 25 µl of distilled water while the treated group was injected with 25 µl of distilled water contained 8 µg dexamethasone. In the next days (11, 12, 13, and 14 of incubation), 12 chick embryos were sacrificed in each day. A computer-assisted morphometric/ image analysis (Motic Image Plus version 2.0ML), was used to measure length, area, perimeter of tibiae, and the area and perimeter of the perichondral osseous collar of cross section in mid-diaphyseal zone of these bones.

**Results** These bones of chick embryos treated with dexamethasone, suffered shortening and retardation in length, weight, area and perimeter throughout the period of this study, decline area and perimeter to the perichondral osseous collar in the mid-diaphyseal zone.

**Conclusion** Dexamethasone given at day 10 of incubation caused tibial bones growth retardation at development stages 11, 12, 13, and 14-days; this was observed in the measured parameters: bone length, area, perimeter and weight.

**Keywords** Bones, chick-embryo, dexamethasone, histomorphometry

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**List of abbreviations:** POC = Perichondral osseous collar

## Introduction

The chick embryos (*Gallus gallus domesticus*) are very good models for the study of early vertebrate embryogenesis and later organogenesis [(1)]. Furthermore, the ease of in vivo experimental manipulation is one of the main factors that have made the chick embryo an important animal in developmental research [(2)]. In addition, the poultry models have been proven as a valuable model for human skeletal defects [(3)]. The developmental phase between 4 and 9 days of incubation is characterized by rapid

changes in the wings, legs, and visceral arches [3]. Detailed morphological sequence of events occurring in long bone development from Hamburger-Hamilton stage 32 through stage 44 "7.5-18 days" and 2 days post hatching; the detailed patterning of osteoblasts, osteoid, mineral and vasculature were observed at the mid-diaphysis of the tibia [4]. Dexamethasone is potential glucocorticoid steroid exhibiting both anti-inflammatory and immunosuppressant properties, it is on the WHO model list of essential medicines, the most important medications needed in a basic health system [5]. It is widely used to treat many chronic inflammatory diseases and autoimmune conditions, such as rheumatoid arthritis and bronchospasm [6]. It also can be used postoperatively to reduce pain, wound infection, nausea and vomiting [7]. However, glucocorticosteroids has diverse effects on various systems of the body. Glucocorticoid excess also inhibited osteoblast activities leading to the development of osteopenia and osteoporosis [8]. Glucocorticoids impair the replication, differentiation and function of osteoblasts and induce the apoptosis of mature osteoblasts and osteocytes leading to suppression of bone formation [9].

The present study was conducted to evaluate by histomorphometric study, the effects of dexamethasone drug on embryogenesis of long bone in chick embryo.

## Methods

### Egg collection

This study was conducted using fertilized chick eggs of *Gallus gallus domesticus* chick mothers taken from the Public Authority for Agricultural Research, Ministry of Agriculture, Iraq.

### Incubation of eggs

The eggs were swabbed by gauze with 70% ethanol, incubated in egg incubator with automated turning motor of the egg rack, and a stable temperature of 38 °C, and humidity about 55-60% with regulation circulated fresh air. Turning of eggs was done 8 times/day at 90° about its longitudinal axis.

### Experimental design

Forty-eight fertile chick eggs were randomly divided into two groups; control and experimental (24 eggs per group) each group was subdivided into 4 subgroups of 6 eggs. At day 10 of incubation, control group were injected with 25 µl of distilled water while experimental group were injected with 25 µl of distilled water containing 8 µg of dexamethasone through a hole in the flatter zone of the egg (air cell). Subgroup "A" eggs were opened at day 11, subgroup "B" eggs were opened at day 12, subgroup "C" eggs were opened at day 13, while subgroup "D" eggs were opened at day 14 of total incubation, of control and experimental groups.

The legs (left and right) were removed from each embryo; tibia bone was skinned carefully under dissecting microscope, avoiding any damage or break by using skinning techniques under dissecting microscope. The weight of each tibia bone was taken with digital electric balance. The whole length of was measured by putting the bone at the beginning of ruler picture, the morphometric system was calibrated by comparison with this ruler measurement (Motic Image Plus 2.0ML), then taken the measurement of the length, area and perimeter to all bones, that will be studied in this study. Tibias were fixed in 10% formal saline, dehydrated through graded alcohols, cleared twice with xylene and embedded in paraffin wax. The mid-diaphyseal zone of tibia was sectioned by microtome (7 µm), to obtain the serial sections of the bone in this area then sprouted these sections on slides with the aid of water bath at 37 °C; all of these slides were stained with haematoxiline and eosin. Final examination of these sections at 4X, and serial images of these serial sections were taken by microscope with camera with TV-Based computer (micros), the best 3 images of each bone (N= 96) cross sections (N= 288 images) were entered in the morphometric system

(Motic) in the laptop software to take the measurement of the outside or total Area (A1) and total perimeter (P1), so the area (A2) and Perimeter (P2) of the internal bone cavity, the remain space between two area and two perimeters is the perichondral osseous collar (POC).The experimental animal protocol was approved by Institute Review Board of the College of Medicine, Al-Nahrain University.

**Statistical analysis**

Data were analyzed using, two-way classification with interaction (ANOVA) within SAS statistical Program (version 9.1/ 2010. USA). Means were compared by t-test at P<0.05 level of significant. when the result appears equal to or more than the LSD value, that mean a significant difference, but if the result appears less than the LSD value, that mean the difference in the result is statistically not significant, with keeping the P value on <0.05. Microsoft office excel 2007 programs, was used to illustrate the figure. Data were expressed as Means ± Standard Error of means.

## Results

**General observations**

In the present study, dexamethasone injection on the 10$^{th}$ day of incubation in the air cell of the fertile chick eggs produced retardation on the ossification processes in the mid-diaphyseal zone of the tibia bone (Perichondral Osseous Collar), and in the four bone parameters that have been studied like: length, area, perimeter, and the weight in compare with the control group.

**Perimeter measurement of tibia bone**

No significant difference between the control (26.58±0.91) and treated (25.18±0.76) subgroups. On day 11, the t-test between them was 1.4 mm, this value was less than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (32.03±0.3) and treated (24.63±0.51) subgroups; the t-test between them was 7.4 mm, this value was more than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (36.33±1.65) and treated (27.43±0.48) subgroups; the t-test between them was 8.9 mm, this value was more than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). Also on day 14; a significant difference was observed between the control (46.87±0.99) and treated (28.59±0.84) subgroups; the t-test between them was 18.28 mm, this value was more than LSD value (LSD=2.52) as in (Fig. 1).

**Surface area measurement of tibia bone**

Figure 2, showed no significant difference between the control (12.02±0.86) and treated (11.54±0.68) subgroups on day 11, the t-test between them was 0.48 mm$^2$, this value was less than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (17.64±0.39) and treated (10.95±0.50) subgroups, the t-test between them was 6.69 mm$^2$, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (22.39±1.83) and treated (12.41±0.34) subgroups, the t-test between them was 9.98 mm$^2$, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). Also on day 14, there was a significant difference between the control (34.29±1.27) and treated (13.15±0.67) subgroups, the t-test between them was 21.14 mm$^2$, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase difference in the bone area, while the differences between the means of treated subgroups at different days, the results showed no difference in the area as shown in (Fig. 2).

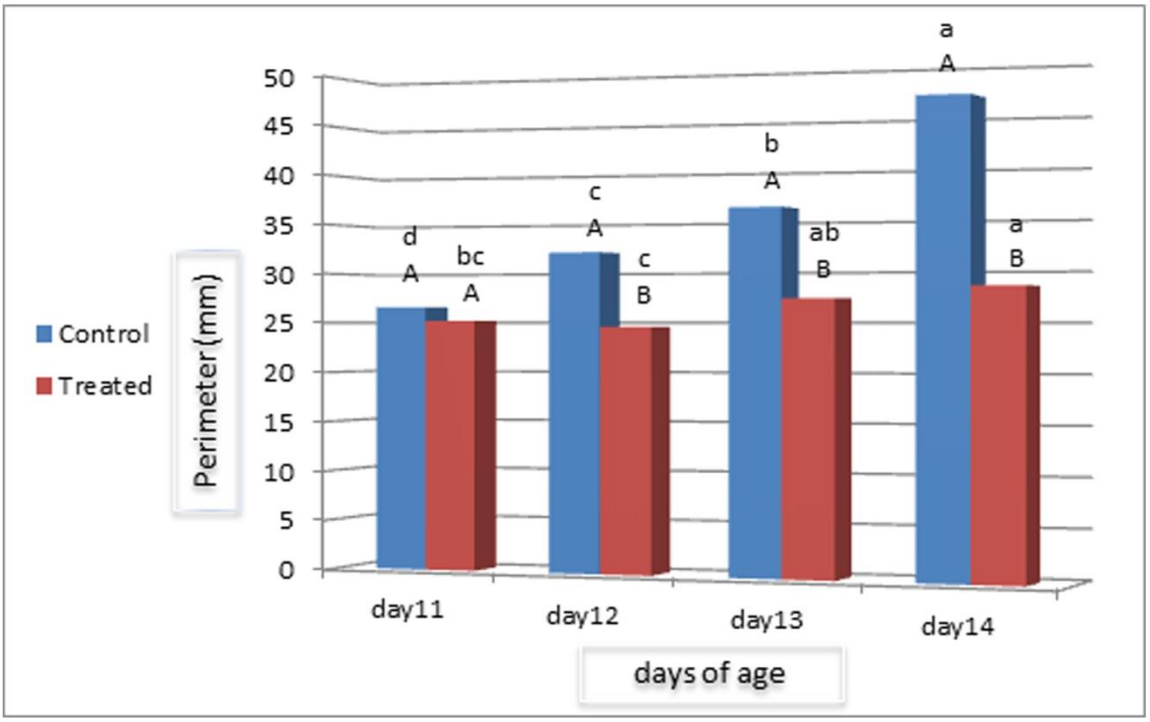


**Figure 1. Comparison between the mean of growth of the Perimeter of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between any two days differ significantly. Means with same small common letter in between any two days differ insignificantly.**

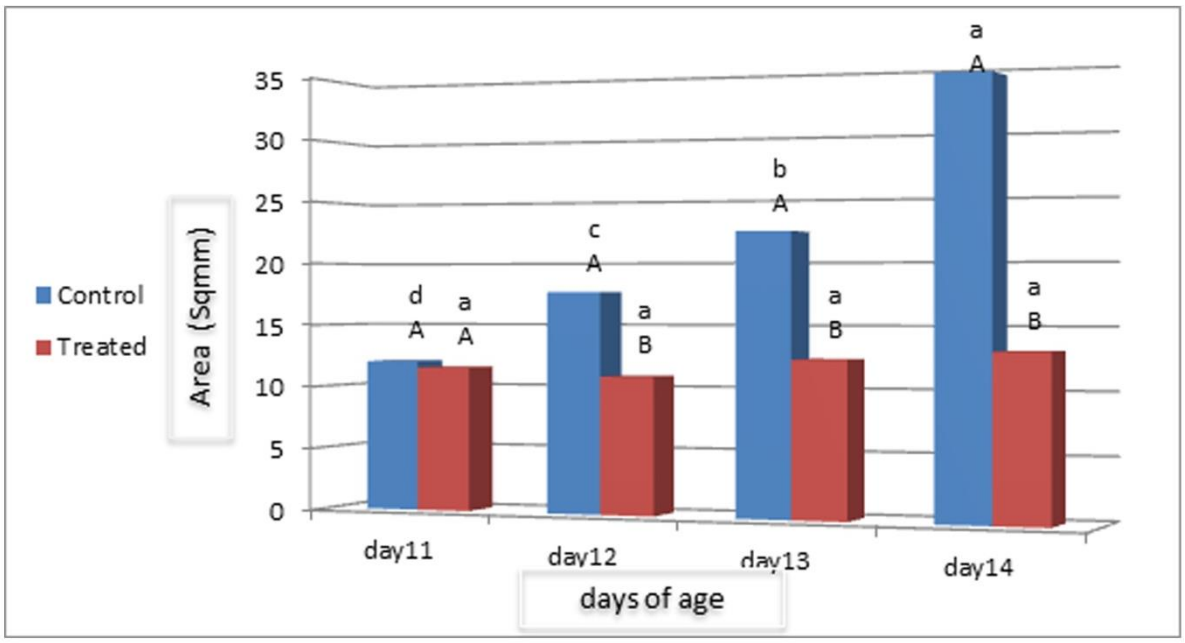


**Figure 2. Comparison between the means of Area growth of 12 tibia bones of the control and treated subgroups from day 11 - 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between any two days differ significantly**

**Weight measurement of tibia bone**

No significant difference between the control (0.0098±0.0009) and treated (0.0072±0.68) subgroups on day 11 (Fig. 3), the t-test between them was 0.0026 g, this value was less than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). On day 12, there was a significant reduction in the weight between the control (0.016±0.0003 g) and treated (0.0077±0.0005 g) subgroups the t-test between them was 0.0083 g, this value was more than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). On day 13, there was a significant increase in the weight between the control (0.024±0.002) and treated (0.010±0.0002) subgroups, the t-test between them was 0.014 g, this value was more than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). Also on day 14, there was a significant reduction in the weight between the control (0.045±0.002) and treated (0.013±0.0006) subgroups, the t-test between them was 0.032g, this value was more than LSD value (LSD=0.0037). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone weigh, while there were no significant differences between the means of treated subgroups on day 11 with the day 12 and 13, so between 13 and 14, but it's a significant between day 14 and day 11, 12 (Fig. 3).

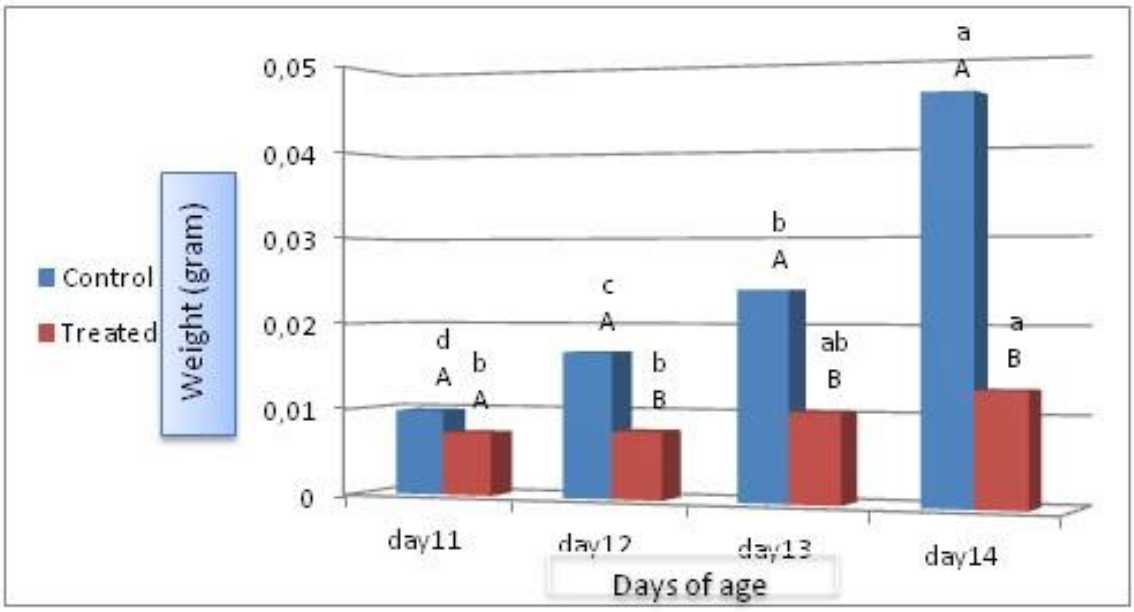


**Figure 3. Comparison between the means of growth of the weight of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly**

**Tibia bone Length measurement**

No significant difference between the control (10.11±0.35) and treated (9.51±0.28) subgroups was seen on day 11, the t-test between them was 0.6 mm, this value was less than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (12.22±0.14) and treated (9.38±0.21) subgroups, the t-test between them was 2.84 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (13.77±0.53)

and treated (10.50±0.18) subgroups, the t-test between them was 3.27 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (18.62±0.43) and treated (10.87±0.31) subgroups, the t-test between them was 7.75 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05), (Fig. 4). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone length, while there were no significant differences between the means of treated subgroups on day 11 with the day 12, so between 13 and 14, but it's a significant between day11 and day13, 14, so between day 12 and day 13, 14, it's a significant (Fig. 4).

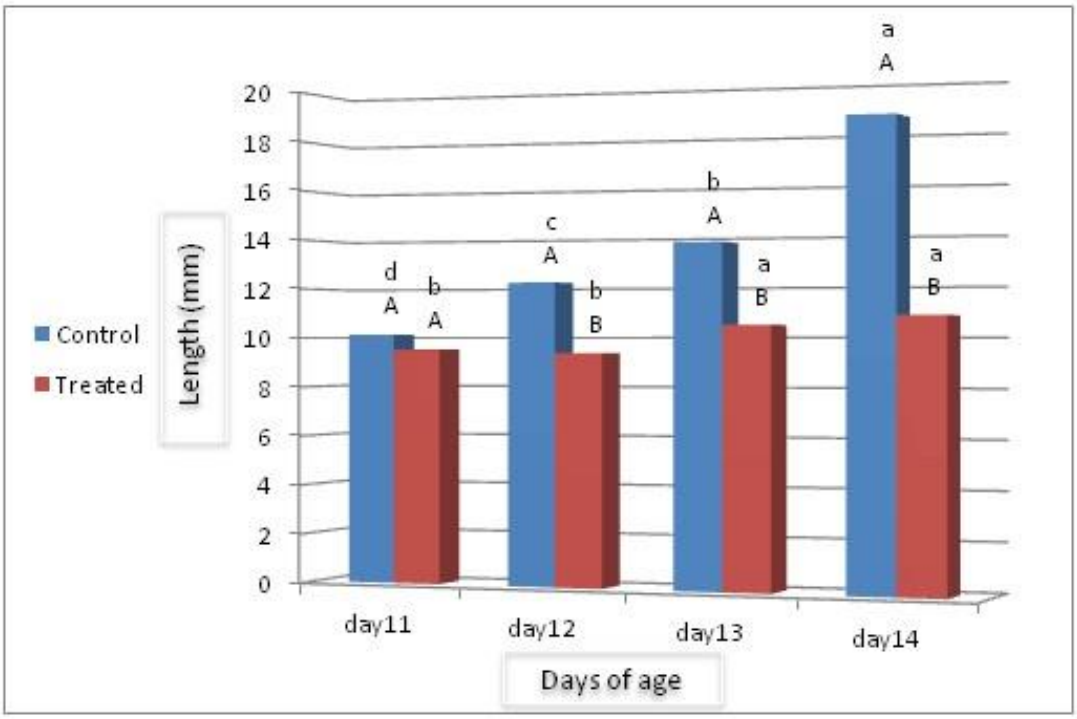


**Fig. 4. Comparison between the means of growth of the length of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly**

**Cross section of tibia bone measurements**

***1. External Perimeter measurement of cross section (P1)***

No significant difference between the control (3.56±0.10) and treated (3.40±0.10) subgroups was observed on day 11, the t-test between them was 0.16 mm, this value was less than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (4.31±0.05) and treated (3.29±0.07) subgroups, the t-test between them was 1.02 mm, this value was more than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (4.69±0.15) and treated (3.73±0.04) subgroups, the t-test between them was 0.96 mm, this value was more than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (5.42±0.06) and treated (3.71±0.04) subgroups, the t-test between them was 1.71 mm, this value was more than LSD value (LSD= 0.252)

when the P-value was constant at (P<0.05), (Fig. 5). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase difference in the external perimeter of cross section (P1), while there were no significant differences in the means of treated subgroups on day 11 with the day 12, so between 13 and 14, but between day11 and day13, 14, as well between day 12 and day 13, 14, it's a significant (Fig. 5).

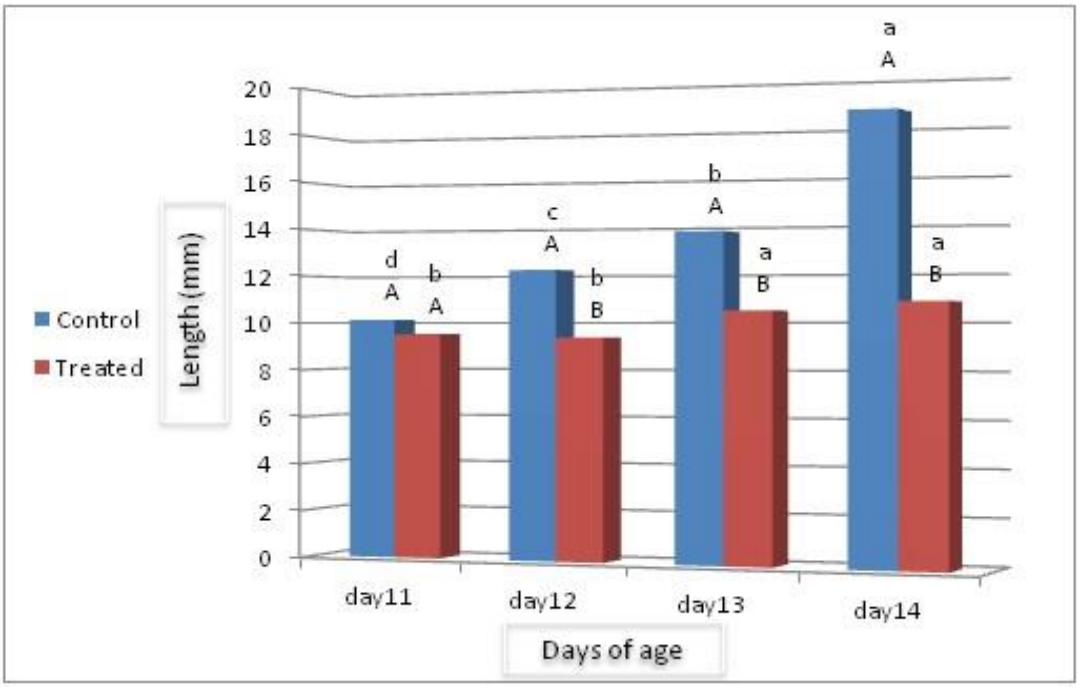


**Figure 5. Comparison between the means of grow of the external perimeter (P1) of the cross section in mid- diaphyseal zone of the12 tibia bones of chick embryos in the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly**

***2.Internal Perimeter measurement of cross section (P2)***

No significant difference between the control (2.14±0.06) and treated (2.19±0.06) subgroups on day 11 (Fig. 6), the t-test between them was 0.05 mm, this value was less than LSD value (LSD=0.155) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (2.34±0.03) and treated (2.00±0.08) subgroups, the t-test between them was 0.34 mm, this value was more than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). On day 13, there was no significant difference between the control (2.22±0.05) and treated (2.29±0.02) subgroups, the t-test between them was 0.07 mm, this value was less than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (2.40±0.05) and treated (2.14±0.02) subgroups, the t-test between them was 0.26 mm, this value was more than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a variable difference in the bone Internal perimeter of cross section (P2), it increased significantly on day 12, reduced insignificantly on day 13 than that of day 12 and increased on day 14 than other days. While there was a significant difference between the means of

treated subgroups at day 11and 12, as well as between day 12 and 13, but between day11,13 so day13, 14, and day 12,14, was no significant, also between day14 and day 11, 12 and 13 (Fig. 6).

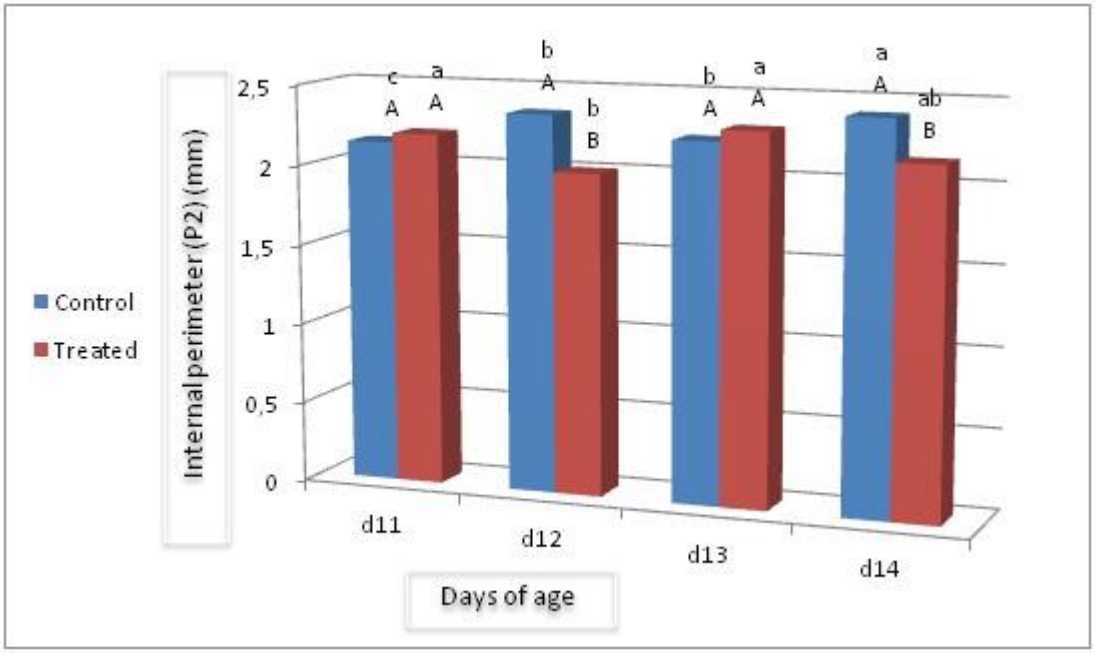


**Figure 6. Comparison between the means of growth of the internal perimeter (P2) of the cross section in mid-diaphyseal zone of the 12 tibia bones of chick embryos in the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly**

**Surface area of POC measurement**

No significant difference between the control (2.20±0.14 mm) and treated (1.85±0.13 mm) subgroups on day 11 (Fig. 7), the t-test between them was 0.35 $mm^2$, this value was less than LSD value (LSD= 0.552) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (3.65±0.11 mm) and treated (1.75±0.10 mm) subgroups, the t-test between them was 1.9 $mm^2$, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (4.71±0.42 mm) and treated (2.41±0.06 mm) subgroups, the t-test between them was 2.3 $mm^2$, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05). On day14, there was a significant difference between the control (6.82±0.21 mm) and treated (2.52±0.09 mm) subgroups, the t-test between them was 4.3 $mm^2$, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05).Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone Area of POC while, there was no significant differences in the bone area of POC of means of treated subgroups on day 11 with the day 12, so between 13 and 14, but between day11 and day13, 14, as well between day 12 and day 13, 14, it's a significant (Fig. 7).

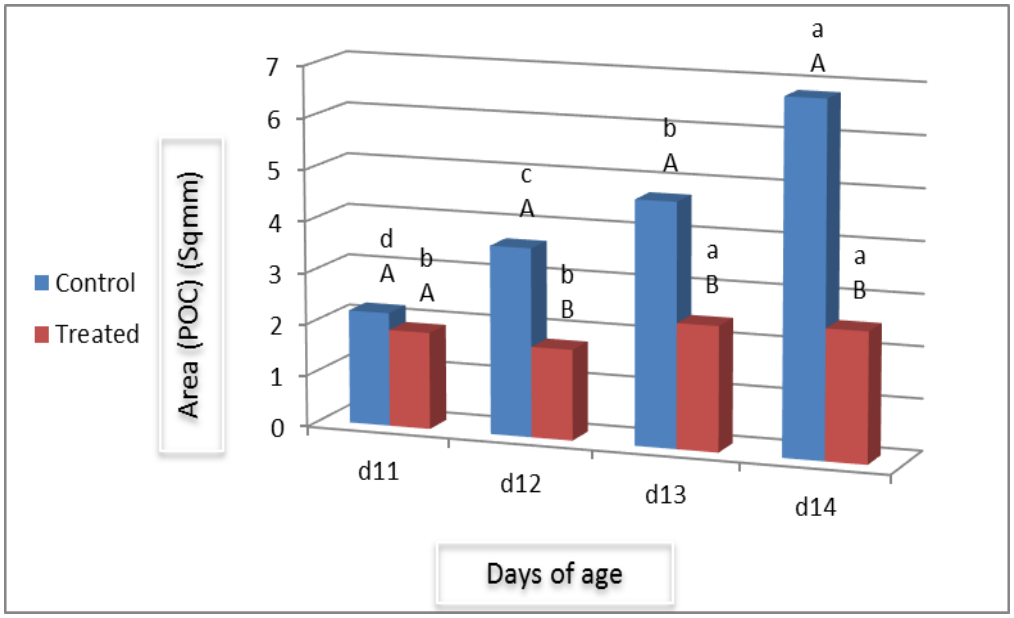


**Fig. 7. Comparison between the means of grow of the Area of the perichondral osseous collar [A (P.O.C)] cross section in mid-diaphyseal zone of the 12 tibia bones of the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly**

**Perimeter of POC measurement**

No significant difference in the POC perimeter between the control (1.42±0.05) and treated (1.20±0.05) subgroups on day 11 (Fig. 8), the t-test between them was 0.22 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (1.97±0.04) and treated (1.23±0.04) subgroups, the t-test between them was 0.74 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (2.46±0.15) and treated (1.43±0.03) subgroups, the t-test between them was 1.03 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (3.03±0.08) and treated (1.56±0.04) subgroups, the t-test between them was 1.47 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone perimeter of POC perimeter, except at day 11, while the difference between the means of treated subgroups at day 11 was not significant with the day 12, so between 12 and 13, also between 13 and 14, but is a significant between day 11 and day 13, 14, and between day 12 and day 14.

## Discussion

Dexamethasone is a synthetic glucocorticoid that has been used clinically as an anti-inflammatory drug. Long-term therapy with dexamethasone or other steroids may cause or exacerbate osteoporosis [10]. Glucocorticoid stimulates osteoclast-mediated bone resorption and reduces osteoblast-mediated bone formation, which results in increased overall net bone resorption [11].

The present work has demonstrated a significant decrease in tibia length of chick embryos treated with dexamethasone; this is in agreement with previous study that observed shorter femora and humeri in newborn piglets

treated with dexamethasone during their prenatal and neonatal life in comparison with controls [12]. This may be due to the inhibition of osteoblasts development and the inhibition of bone specific osteocalcin (OC) gene expression which arrested trabecular bone formation and likely contributes to glucocorticoid-induced osteoporosis [10].

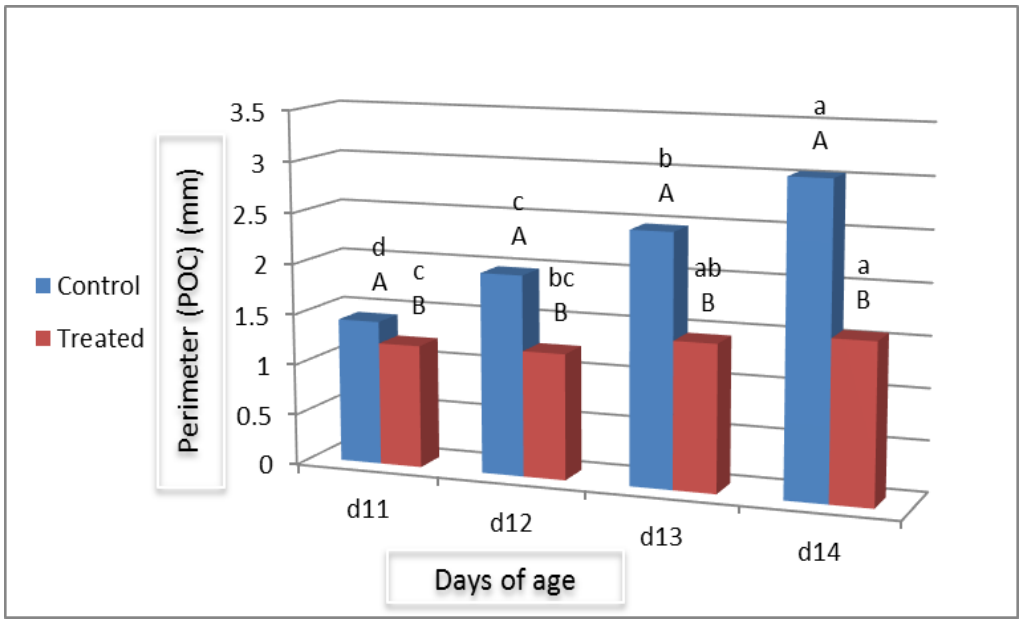


**Figure 8. Comparison between the means of growth of the Perimeter of perichondral osseous collar [P (P.O.C)] cross section in mid-diaphyseal zone of the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly**

The decrement in bone length may also be due to inhibition of protein and glycoprotein synthesis and reduction of the proliferating cells number in dexamethasone treated rats [13], it may also be due to the inhibition effect of the drug on the proliferation of the growth plate chondrocytes [14]. Dexamethasone administration in young male albino rat led to alteration in the structure of the epiphyseal plate growth with an observable reduction thickness, less frequent chondrocytes with wide matrix areas, thus corticosteroids might slow longitudinal bone growth and induced growth retardation [15]. Dexamethasone has been demonstrated to accelerate the deposition of calcium salts, inhibited the proliferation of chondrocytes, and increased apoptosis of chondrocytes and osteocytes that lead to shortening of the developing long bone of chick embryos [16]. The decline in bone formation in mice and humans receiving glucocorticoids is mediated by direct inhibition of osteoblast proliferation and differentiation and by an increase in the apoptosis rates of mature osteoblasts and osteocyte [17,18].

In the present investigation, decrease in the tibia bone weight was observed in the dexamethasone treated chick embryos; this is coinciding with Sultana [19], who observed a reduction in tibial weight in dexamethasone administrated immature female mice. Maternal treatment with dexamethasone decreased the weight of the tibia and led to thinning of articular and growth plate cartilages

and trabeculae thickness and reduced the serum GH concentration in male piglets [20]. Dexamethasone suppresses osteoblast function and bone morphogenetic protein "BMP" pathways by enhancing the expression of mRNA of BMP antagonists, and bisphosphonate and PTH exert pharmacologic effects [21].

The present study showed that dexamethasone caused a decrease in tibia bone area and perimeter of the bone as a whole from 11-day of incubation. Cross-sectional, cortical and trabecular areas were reduced by 30% in the hemimandible of dexamethasone treated female Sprague-Dawley rats during the growth phase, suggesting that the corticosteroid exerts a combined, negative action on bone geometry (mass and architecture) and volumetric bone mineral density of cortical bone [22]. Dexamethasone induced osteoporosis, growth retardation both in long bones and in the vertebral column and induced reduction in bone volume in the (three-week-old) mice [23]. Dexamethasone administration in both prenatal and neonatal life of the piglets led to reduction of volumetric bone density and mechanical and geometric properties of their bones [12].

In both controlled and treated groups, a morphometric system was organized to be able for compatible reading of the best 3 images cross section in the mid-diaphyseal zone of each bone, then comparison between the mean values of external and internal parameters, that gives us the idea of the development of the perichondral osseous collar that grow with the ossification process and affected this process with the retardation effects that occurred by used dexamethasone drug. So that the effect of this drug on the process of invasion to the connective and vascular tissue into the bone body. Greater of the differences between the external and internal perimeters, and area; refer to the greater of the perimeter and area of the collar.

The perichondral osseous collar in the mid-diaphyseal zone cross-section of tibia bone of the treated embryos of this study, showed a significant reduction when compared with normal control, however a significant increase in the area of perichondral osseous collar in the mid-diaphyseal zone cross-section of tibia bone during 13 and 14-day of incubation in embryos treated with dexamethasone of the present study when compared with those of days 11 and 12. This is coinciding with Gaytan et al. [24] who observed a rapid increase in cartilage volume from day 12 to day 13, with rapidly increased of the invading connective and vascular tissue volume from day 11 to day 14 whereas the rate of cartilage resorption increased until day 13 reaching perichondral osseous collar after this age. Treated embryos with dexamethasone showed a delay in the tibia longitudinal growth as well as in the growth of bone collar and have been related to the scarcity of resorptive cells found in the cartilage-marrow interface [25]. Perichondral tissues and blood vessels in particular influence chondrocyte maturation in a positive manner and may cooperate with hypertrophic chondrocytes in dictating the normal pace and location of the transition from cartilage to bone [26], the blood vessels of the dexamethasone treated rabbits have been detected to be irregular with disrupted endothelial cells and congested with blood that might be responsible for the delay of collar formation [27]. Morphological studies are in progress to observe the effect of dexamethasone administered during embryonic period on the histological processes changes in the bone development such as osteoblast proliferation, osteoclast activities, reduction in the bone collar thickness, impairment of matrix synthesis together with the negative effect on the whole-body mass as well.

In conclusion, the present investigation demonstrated that administration of dexamethasone caused tibial bones growth retardation at development, leading to reduction in the bone length, area, perimeter

and weight and retardation of the perichondral osseous collar.

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E. Revision of the manuscript for important intellectual content: Ali, Dr. Al-Ani, Dr. Al-Salih.

## Conflict of interest

The authors have no conflicts of interest.

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