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Mesenchymal Cell Death in Mouse Limb Bud After the Onset of Primary Myogenesis

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Abstract

Background The vertebrate limb bud develops as an outgrowth of mesoderm, which forms all their elements (muscles, nerves, vessels, bone, cartilage, and tendon). Myogenic precursor cells are seen at E11.5 mouse embryo, when the first nerve fascicles begin to enter the limb. The first signs of musculature masses are seen at E12.5 in both fore and hind limb buds. Apoptosis or programmed cell death is essential in the development of the limbs. In vertebrate, the developing limb morphogenesis depends on the appropriate spatial and temporal balance between cell death and cell proliferation.

Objective To perform comprehensive analysis of the proximo-distal pattern of cell death, evaluated by (TUNEL test) in cross sections of mouse limbs during prenatal development after onset of primary myogenesis.

Methods Fifteen pregnant female mice (Musmusculus) were divided into three groups according to the days of pregnancy into day (14, 16 and 19), only two embryos were taken from each mouse. All the limb buds were involved in this study. Paraffin embedded histological cross-sections of the limb buds were prepared, histological staining (using H&E stain) and TUNEL test labeling were done. Assessment of the number of apoptotic cells in the limb bud mesenchyme was done by counting these cells.

Results The H&E stained sections of the limb buds showed less amounts of mesenchymal tissues in older embryos (day 19). The TUNEL stain showed active apoptotic changes at proximal parts of the limb buds at gestational day 19, while the distal parts of the limbs buds showed active apoptotic changes at the early days (day 14). The evaluation of TUNEL test reaction in the proximal regions showed statistical significant increase of apoptotic cells in day 19 compared to day 14 ($p = 0.001$ for both). The mean number of apoptotic cells in the proximal regions were statistically significant ($p = 0.001$) between day 16 and day 19. While the mean number of apoptotic cells of distal regions of the limb buds was higher at day 14 compared to that of day 16 and day 19. These differences between day 14 and day 16 were statistically significant and between day 16 and day 19 while statistically non-significant between day 14 and day 19. Comparison of mean number of apoptotic cells between proximal and distal regions in all the three groups showed a statistically significant higher mean number of apoptotic cells in the distal regions compared to proximal region ($p = 0.001$). The mean number of the apoptotic cells in both regions (proximal and distal) of the limb buds revealed statistically significant differences between day 16 and day 19 ($p = 0.001$).

Conclusion Apoptosis was higher in all parts of the developing limbs during day 19, and that could be associated with degenerative changes occurring at the apical ectodermal ridge. Moreover, apoptosis was higher in the distal part of the limb bud and this may be due to more differentiation of the distal parts than in the proximal part of the limb bud.

Keywords Development, limb bud, mouse, embryo, TUNEL, apoptosis, mesenchyme

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List of abbreviations: AER = Apical ectodermal ridge, H&E = Hematoxylin and eosin

Introduction

Vertebrate limbs develop from undifferentiated homogenous mesenchymal cells that are surrounded by the ectoderm [(1)]. The limb patterning includes three spatial axes; (proximal to distal, anterior to posterior, and dorsal to ventral axes [(2)].

Morphogenesis of the upper and lower limbs is similar; their outer shape is formed when

mesenchymal cells in the buds start to condense. The layer of ectoderm at the distal border of the limb is thickened to form the apical ectodermal ridge which induces limb bud formation and elongation [3].

In mouse, the first sign of the limb development recorded during the E9.5 and E10. At E11.5, the long bones of the limbs develop in proximodistal direction and by the E14.5 the most distal phalanges are formed [4].

The limb buds develop as an outgrowth of mesoderm which form all their elements (muscles, nerves, vessels, bone, cartilage, and tendon) with a supervision of the apical ectodermal ridge (AER), which is formed at the junction between the ventral and dorsal ectoderm [5].

During development, the digits of limbs become separated due to interdigital apoptosis as well as differential growth of the digital tips [6].

The ossification of the limb elements shows different stages which start by the stage of pre-cartilaginous condensations in the presumptive bones, then displaying the proliferating chondrocytes. The chondrogenic precursor cells condense in the center of the limb buds to form chondrogenesis center, in addition for tendons and muscles, which condense in the rims of bud. Myogenic precursor cells are seen at E11.5 mouse embryo, when the first nerve fascicles begin to enter the limb. The first signs of musculature masses were seen at E12.5 in both fore and hind limb buds [4].

The cell death (apoptosis) is important to balance the cell proliferation during development [7]. The apoptosis is the major cell death pathway for removing unwanted in a clean or silent manner during embryonic development [8]. Apoptosis as a programmed cell death is essential in the development of the limbs [9]. The programmed cell death take place in the developing limb in areas of the undifferentiated mesoderm in connection with the establishment of the pre-chondrogenic condensations of the skeleton, in addition cell death is also observed during the formation of the joints [10]. The areas of apoptosis are recoded with the formation of the shape and skeleton of the limb, it shows a significance difference between species members [11].

The (TUNEL) is a detecting technology for DNA fragmentation by labeling the terminal end of nucleic acids. It uses to recognize DNA fragmentation of apoptotic signaling cascades [12]. The DNA fragmentation might occur after the release of enzymes from cytoplasmic membrane [13].

This study had been done to perform comprehensive analysis of the proximodistal pattern of cell death evaluated by (TUNEL test) in cross sections of mouse limbs during prenatal development after onset of primary myogensis.

Methods

Fifteen pregnant female mice (Musmusculus), aged about 10-12 weeks, weighing between 25-30 g, were divided into three group according to the days of pregnancy into day (14,16 and 19), only two embryos were taken randomly from each mouse. All the limb buds were involved in this study (Table1).

Limb buds were separated according to their age in containers in order to be fixed with formalin 10%, followed by fixation, dehydration, clearing, paraffin embedding and sectioning dewaxing and hydration were done 14. Serial sections of 5-6 µm thickness were cut using the electrical microtome.

The TUNEL-based detection kit was provided by abcam® with kit code ab66108. The fluorescin-labeled DNA was observed with a fluorescent microscope.

Statistical evaluation of the number of apoptotic cells in the limb bud mesenchyme that were showed in a bright yellow fluorescent color was done depending on the counting these cells.

Table 1. Arrangement of Samples groups according to the days of Pregnancy

Days of pregnancy	No. of female mice in each day	No. of embryos that were taken randomly	No. of limb bud that were taken from embryo
14	5	10	40
16	5	10	40
19	5	10	40
Total	15	30	120

Results

The TUNEL test of the limb buds at the variable stages of development showed three different fluorescent colors:

- Yellow color representing the apoptotic cells (positive color).
- Orange color for the non-apoptotic cells (negative color).
- Green color, also a negative color.

The Abcam's in situ Direct DNA Fragmentation Assay Kit used provided two components including positive and negative control cells for conveniently detecting DNA fragmentation by fluorescence microscopy.

The TUNEL test applied in this study showed evaluation of apoptosis ratio in the limb buds.

The variable regions of mesenchymal tissue of the proximal and distal limb bud were evaluated by counting the number of the cells undergo apoptosis. The counting of the positive fluorescent cells in the mesenchymal tissue of the limb buds illustrated the bright yellow colored apoptotic cells (Figures 1, 2, 3, 4).

Evaluations of apoptotic cells in proximal regions of limb bud

Day 14 and Day 16

The counting of the fluorescent cells in the proximal regions of limb bud showed a mean value at day 14 was (90.1±2.7). While that at day 16 was (95.0±4.7) (Table 2); t-test revealed a non-significant difference between the mean value in the proximal regions at day 14 and day 16.

Day 16 and Day 19

The mean value in proximal regions of limb buds at day 16 was (95.0±4.7). While that at day 19 was (180.0±8.3) (Table 3); t-test revealed significant higher values of apoptotic cell in day 19 compared to day 16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value of apoptotic cell in proximal regions of limb bud at day 14 was (90.1±2.7). While that at day 19 was (180.0±8.3) (Table 4); t-test revealed a significant increase of apoptotic cell in day 19 compared to day 14 ($p \leq 0.05$).

Evaluations of apoptotic cells in distal regions of limb buds

Day 14 and Day 16

The mean value of apoptotic cell in distal regions at day 14 was (279.0±10.07). While that at day16 was (203.0±10.56) (Table 5); t-test revealed a significant increase of apoptotic cell in day 14 compared to day 16 ($p \leq 0.05$).

Day 16 and Day 19:

The mean value of apoptotic cell in distal regions at day 16 was (203.0± 10.56). While that at day 19 was (257.0±14.06) (Table 6); t-test showed a significant increase of apoptotic cells in day 19 compared to day16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value in distal regions at day 14 was (279.0± 10.07). While that day 19 was (257.0±14.06) (Table 7); t-test in distal regions

revealed a non-significant variability between the mean value at day 14 and day 19.

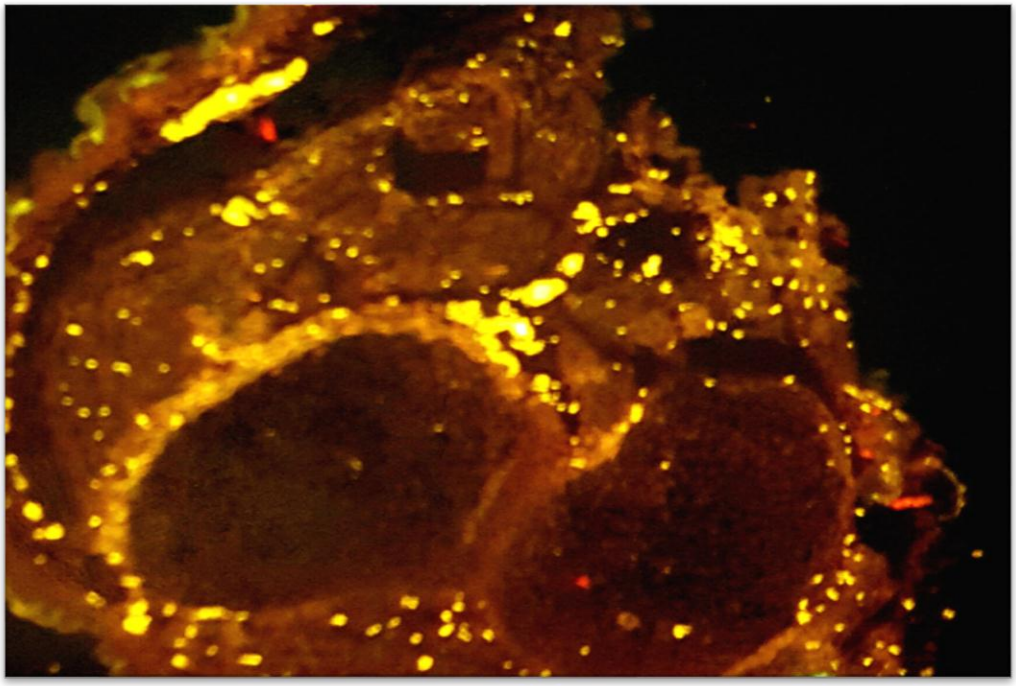

Figure 1. Cross section of the proximal regions at day 16 showing bright yellow color of apoptotic foci TUNEL test (100X)

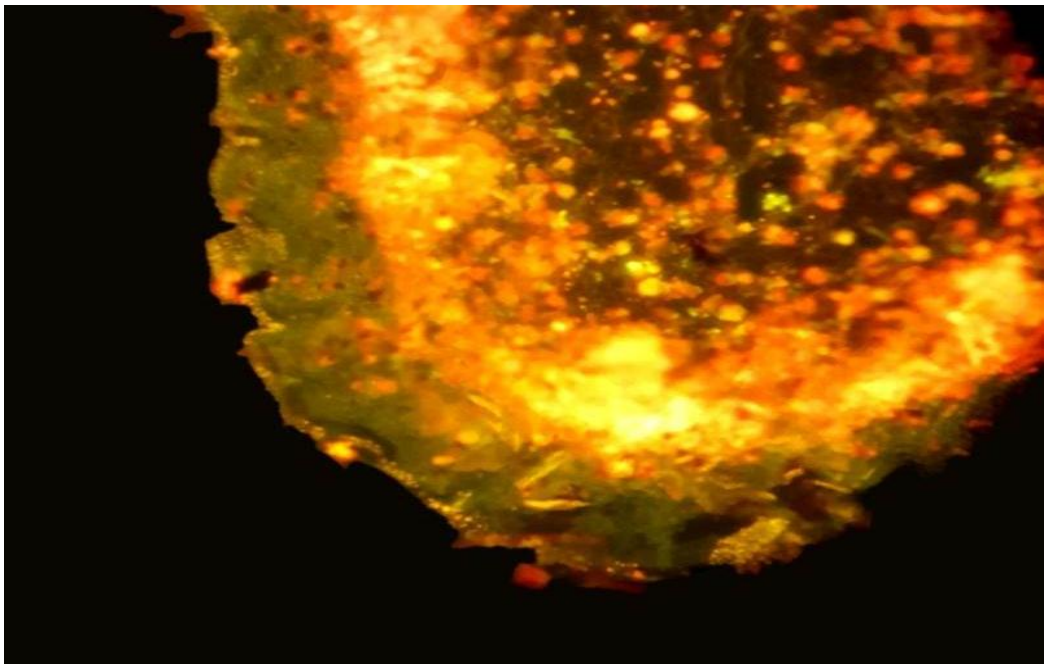

Figure 2. Cross section of the proximal regions at day 19 showing bright yellow color of apoptotic foci TUNEL test (400X)

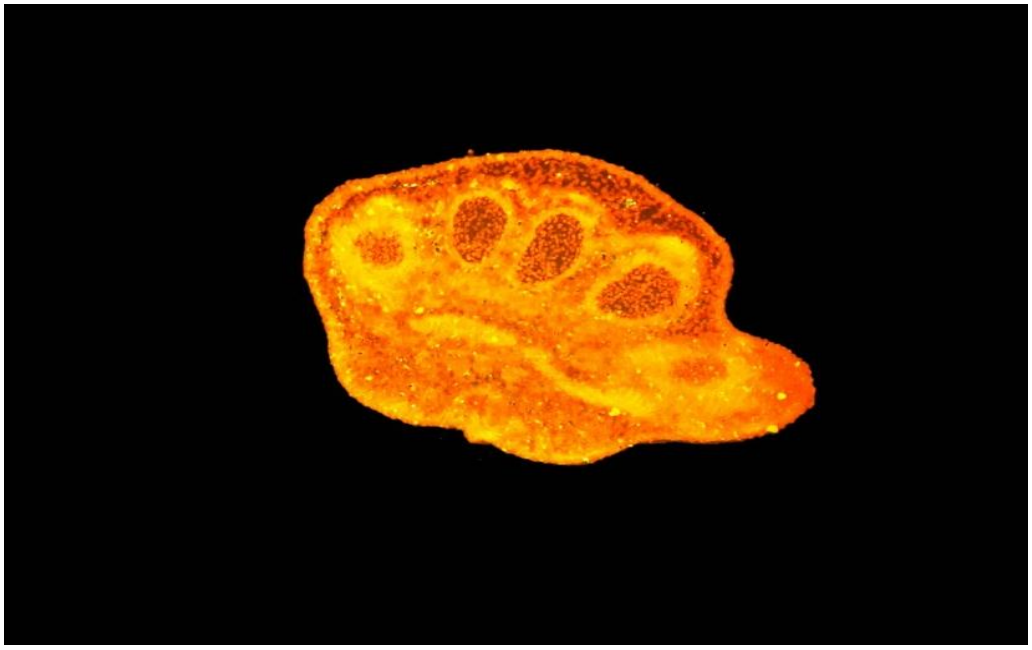

Figure 3. Cross section of the distal regions at day 14 showing bright yellow color of apoptotic foci TUNEL test (100X)

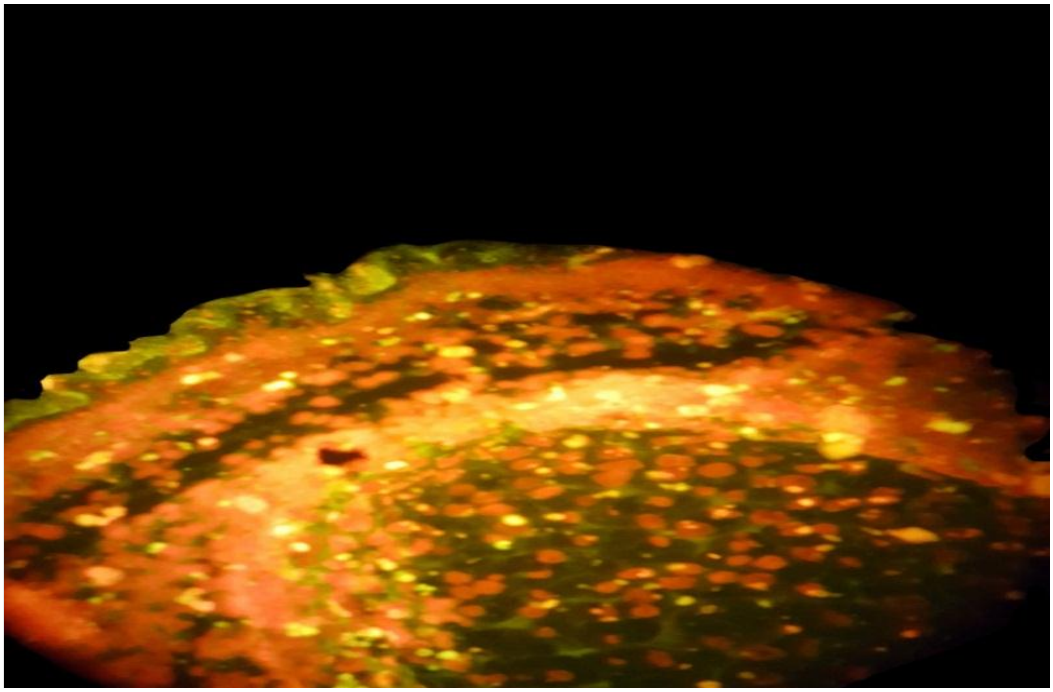

Figure 4. Cross section of the distal regions at day 16 showing bright yellow color of apoptotic foci TUNEL test (400X)

Table 2. Mean number of apoptotic cells revealed in proximal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	90.1±2.7	0.2
Day 16	95.0±4.7	

Table 3. Mean number of apoptotic cells revealed in proximal regions in E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	95.0±4.7	0.001
Day 19	180.0±8.3	

Table 4. Mean number of apoptotic cells revealed in proximal regions in E14 and E19 by TUNEL test

Age	Mean± SE	P value
Day 14	90.1±2.7	0.001
Day 16	180.0±8.3	

Table 5. Mean number of apoptotic cells revealed in distal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	279.0±10.07	0.004
Day 16	203.0±10.56	

Table 6. Mean number of apoptotic cells revealed in distal regions in E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	203.0±10.56	0.004
Day 19	257.0±14.06	

Table 7. Mean number of apoptotic cells revealed in distal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	279.0±10.07	0.2
Day 19	257.0±14.06	

Comparison of mean number of apoptotic cells between proximal and distal limb bud

The mean value of apoptotic cell in proximal regions at days E14, E16 and E19 were (180.0 ±16.5). While that in distal region at days E14, E16 and E19 were (246.3±7.83) (Table 8). T-test showed significantly higher mean values of the apoptotic cells in the distal regions compared to proximal region ($p \leq 0.05$).

Table 8. Mean number of apoptotic cells revealed in proximal regions and distal regions by TUNEL Test

Region	Mean± SE	P value
Proximal	121.7±6.5	0.001
Distal	246.3±7.83	

The mean number of apoptotic cells in both regions of limb buds

Day 14 and Day 16

The mean value of apoptotic cells at day 16 was (149.0 ±15.3). While that at day E14 was (184.5 ±15.9) (Table 9); t-test showed a non-significant variability was in the mean value of the counted cells during day 14 and 16.

Day 19 and Day 16

The mean value of apoptotic cells at day 19 was (305.5±11.8). While that at day 16 was (149.0 ±15.3) (Table 10); t-test showed significantly higher mean values of the apoptotic cells in the day 19 compared to day 16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value of apoptotic cells at day 19 was (305.5±11.8). While that at day 14 was (184.5±15.9) (Table 11); t-test revealed a significant value of apoptotic cell which was higher in day 19 compared to day 14, ($p \leq 0.05$).

Table 9. Mean number of apoptotic cells revealed in both regions at E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	184.5±15.9	0.2
Day 16	149.0 ±15.3	

Table 10. Mean number of apoptotic cells revealed in both regions at E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	149.0 ±15.3	0.001
Day 19	218.5 ±11.8	

Table 11. Mean number of apoptotic cells revealed in both regions at E14 and E19 by TUNEL test

Age	Mean± SE	P value
Day 14	184.5±15.9	0.001
Day 19	218.5 ±11.8	

Discussion

It was reported that the developing vertebrate limb morphogenesis depends on the appropriate spatial and temporal balance between cell death and cell proliferation [15].

The results of this study indicate that the proximal parts of the limb bud are more actively involved with apoptotic changes at gestational day 19. The pattern of apoptosis in the proximal parts of the limb buds at the earlier developmental stages was found to be steady. The distal parts of the limbs showed active apoptotic changes at the earlies day 14.

The myogenic differentiation was reported to be associated with cell death to eliminate excess cells around the developing muscles [16]. However, the evaluation of proximodistal sequential localization of the phenomenon of apoptosis was not described.

The localization of cell death along the arthogonal axes in relation with the organization of the skeletal pattern of the limb described in this study was also reported in a study on early avian limb, the avian limbs showed regional variability with an anterior and posterior areas of cell death [11].

The results of this study do not agree with Milaire and Roze in 1983 [17] who mention that no regional variability in different zones of early developing limb of mammals. This disagreement could be related to the more accurate enlightenment of apoptosis by TUNEL technique used in this study, or it may be related the different chronological development of the limb bud as this study evaluate more later developmental stages.

The more massive apoptosis in the distal regions found in this study during the gestational day 14 was supported by the Hurle et al in 1996 [18] who reported massive programed cell death at the distal mesoderm that serve in phenotyping of digits.

Many physiological triggering signals for apoptosis were reported in the developing limb buds including the expression of BMP-2, BMP-4 and BMP-7 [19].

Also, previous studies support the coexistence of different cell death effectors in association with apoptosis of the mesodermal tissues of the developing limb buds [20-22].

The evaluation of the programmed cell death in both the upper and lower limbs buds was based on the assumption reported in the literatures that apoptosis in the upper and the lower limb buds provides a valuable model that mold the limb bud tissues to present the morphological and functional specialization in different vertebrated including ducks, turtles, bats, chickens, humans, and lizards [23,24].

The programmed cell death detected by the TUNEL stain in this study after the onset of primary myogenesis after day 14 was higher in all parts of the developing limbs during day [19]. This result is supportive to the finding of, casps-3 and -9 began was found to be detected from 13.5 gestational day and become more strengthened during the later period of embryogenesis suggesting that the Casp family starts to be expressed in accordance with onset of myogenesis [25].

It was concluded that signaling (as fibroblast growth factor) from the most distal part of the limb buds (the apical ectodermal ridge) at different developmental stages regulates cellular growth located at the most proximal parts. This signaling was essential to ensure normal skeletal elements, and perhaps other limb tissues [26]. Also, it was reported that the mesenchymal region underlying the distal ectoderm was found to show massive cell death after removal of the apical ectodermal

ridge [27,28]. This finding is supportive to the results of this study, the later stage involved in this study (day 19) is associated with degenerative changed of the apical ectodermal ridge [29] and this may the underlying factor leading to massive cell death at this developmental stage as cell death might be due to an indirect effect of loss of signaling associated with absence of the apical ectodermal ridge in later developmental stages.

The results of this study evaluated the reactivity of TUNEL test labeling apoptotic cell in mesenchymal tissues seen in the transverse sections of limb around the various tissue components. This approach was established after the reports that ensure uncertainty whether the labeling of dying cells could be a progenitor of chondrocytes or other cell types in the limb [30].

The higher apoptotic activity at the distal regions of the developing limb was determined in all the three developmental stages involved in this study. This result was in agreement with the with previously suggested limb patterning of the progress zone model [31], which had been the most important model of limb proximal–distal patterning since decades. This model postulated that limb progenitors in a 'progress zone' in the limb bud distal tip become progressively 'distalized'. As cells proliferate but progress zone size remains constant, cells must be continually exiting the progress zone. Once they leave, they cease distalizing. Therefore, cells exiting early form proximal skeletal elements whereas those exiting late form distal elements. Signaling from the apical ectodermal ridge was thought to be the accommodating factors that allow progress zone cells to continue distalizing. Therefore, the proximal regions of the limb buds showing active apoptosis in this study as a phenomenon associated with early maturation of the proximal musculoskeletal elements, the late distal increase of labeled apoptotic cells found in this study is related to maturation of the distal musculoskeletal elements of the limbs. This interpretation was supported by the establishment reported previously that distal region is specified only after proximal [32].

The methodology of t-test statistical analysis has been used in this study to compare variables associated with the subsequent chronology of mesenchymal cell apoptosis, this scheme of statistical analysis provides simplicity of interpretation and ease for calculation in the manner that inaugurate with the aims of the study [33].

This study concluded that the active apoptosis during myogenesis showed proximodistal pattern regulating limb morphogenesis.

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Authors Contribution:

Al-Musawi: Performing the laboratory research work. Dr. Mubarak: Performing the production and interpretation of the results.

Conflict of interest

The authors disclose no any financial and personal relationships with other people or organizations that inappropriately influence (bias) our work.

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