Histological Analysis Of The Newly Formed Bone Secondary To Distraction Osteogenesis Of The Mandible In Goat

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Abstract: A unilateral mandibular distraction osteogenesis (DO) was performed in 16 healthy adult male goats. The mandibular body was lengthened by gradual distraction. After 10 days of distraction periods, using a protocol of 0-day latency and a 1-mm/day rate for 10 days, the mandible was held in external fixation for 8 weeks consolidation. The histological features and pattern of the healing process was described after 4 and 8 weeks consolidation. Contrary to the prevalent concept, the results indicated that bone formation in the mandible of this model was principally via the intramembranous-type of ossification. No evidence of cartilage tissue was seen in the gap healing. Five phases could be observed during the healing process: 1) Initial phase 2) Collagenic phase 3) Osteogenic Phase 4) Lacuna Formation Phase, and 4) Early modeling - remodeling phase of the bony regenerate.

Key Words: distraction osteogenesis, osteodistraction, mandibular body lengthening, consolidation, osteogenic cells, intramembranous ossification, Bone remodeling.

1 Introduction
DO is the biologic process of new bone formation between the surfaces of bone segments that are gradually separated by incremental traction [1]. The traction generates tension on the skeletal and surrounding soft tissue structures, which stimulates new bone formation parallel to the vector of distraction [2,3]. DO was first described by Codivilla in 1905 to elongate the femur [1]. In 1951, Ilizarov developed a technique for repairing complex fractures of long bones. This technique was divided into five sequential periods: osteotomy, latency, distraction, consolidation and remodeling [4, 5, 6]. When the desired or possible length is reached, a consolidation phase follows to allow maturation of the regenerate after traction forces are discontinued [7]. Cope and Samchukov [8] found zones of endochondral ossification and transchondroid bone formation at 4 weeks of consolidation in dog mandible. In rabbit mandible, new bone was formed in the distraction gap (DG) by both intramembranous and endochondral ossification [5] and by intramembranous ossification only [6, 9]. Sawaki et al. [7] in children reported new bone formed by intramembranous ossification with some fibro-cartilage islands. The aim of this study was to analyze the sequence of histological features and cell behaviors during DO of the mandibular body.

2 Materials and Methods
Sixteen adult male goats, divided into two groups, were used in this study. They underwent unilateral mandibular body DO using a protocol of 0-day latency and a 1-mm/day rate for 10 days. The mandible (in one group) was held for 4 and (in the other group) for 8 weeks consolidation periods to allow distraction gap regeneration. Plain radiographs were performed before the mandibles of both groups were harvested. Also an arteriogram had been made to show the pattern and distribution of the inferior alveolar artery in the distraction area. Sagittal sections of The 10 mm distraction regenerate was osteomized and fixed in 10% buffered formalin for 24 hours. Then decalcified in 10% EDTA for about 3 weeks before paraffin embedding. Sections, 5 – 7 Um thick, were cut and stained with hematoxylin and eosin and Crossmon’s trichrome stain as outline by Bancroft and Stevens [10].

3 Results
When a fracture induced, the soft and bony vasculatures were disrupted. A hematoma floods the wound as a result, with many blood borne elements providing the first population of cells at the fracture site. As the clot was formed, platelet deposition occurred and a fibrin scaffold developed. Too few mesenchymal cells (Fig. 1) migrated from the adjacent marrow and bony tissue. They contributed to the earliest formation of the DR. In addition, osteoclasts in the form of syncytial consolidation of monocytes of hematological origin were observed (Fig. 2). This stage lasted from 1 to 5
days, at which time the clot was replaced by a granulation tissue. One week after osteotomy, the reparative tissue was filling the DG. It was soon reinvaded by an influx of undifferentiated cells. This stage lasted for approximately 2 weeks.

As the granulation tissue continued to organize, endothelial cells and smooth muscle cells migrated along the fibrin scaffold as vascular buds. They provided the pool needed for the ongoing replacement of cells during regeneration. As healing progressed, ossification centers became apparent to start an intramembranous-type of bone. They were randomly scattered in the DR. Each center appeared as an area of mesenchymal condensation within a homogeneous ground substance (Fig.3).

Pronounced development occurred when fibroblasts differentiated from the mesenchymal cells. They produced collagen fibers and extracellular ground substance. Later, randomly scattered collagen fibers were seen among the cells. Progressive development of the collagen produced identifiable bony specules. In the meantime, some of the mesenchymal cells became osteoprogenitor cells and preosteoblasts (Fig.4). Soon after, these preosteoblasts underwent maturation and became active osteoblasts. Each appeared as a large oval cell with a basal spherical nucleus, basophilic cytoplasm and a prominent large pale apical Golgi zone (Fig.4). It seems possible that maturation of these osteoblasts was enhanced by the presence of pre-existing osteoclasts in the ossification centers (Fig.5).
An osteoclast appeared as a giant multinucleated cell (Fig.6). Their nuclei varied in number, 6-15.

Their cytoplasm was "foamy" with many vacuoles. As osteoblasts deposit initial matrix (osteoid), some of them became entrapped in their secretion as the future osteocyte (Fig.7). This osteoid became mineralized after a time lag the so called osteoid maturation period. As healing advanced, the reparative area showed many irregular primitive trabeculae or specules. Later on, progressive expansion of these developing trabeculae occurred.

Gradually, the new regenerate became modeled and remodeled, by both the osteoblasts and the osteoclasts, to acquire the architecture of neighboring bone. Although, newly formed bone was achieved by the 4th weeks and became more remodeled by the 8th weeks of consolidation, the reparative gap did not yet attained the neighboring bony architecture.

Analysis and Cell behaviors in the Distraction regenerate (DR):

After 4th and 8th weeks consolidation, the new regenerate revealed only an intra-membranous-type of ossification which occurred simultaneously in the following phases:

2.1 Initial phase:

Examination of the newly formed DR revealed multiple ossification centers scattered randomly in the reparative framework. These foci could be considered as the osteogenic forerunner in the DG. Adjacent to the DR, the outer surface of the edges of the DG was invested by the periosteum. Its inner osteogenic layer was sharing actively in invading the DR by an influx of osteoprogenitor cells. Each ossification center appeared as an aggregation of mesenchymal and osteoprogenitor cells intermingled with minute blood spaces and randomly arranged collagen fibrils. The mesenchymal cells appeared crowded and hyperplastic, forming mesenchymal condensations. A mesenchymal cell had a small cell body with a few long and thin cell processes. The nucleus was large spherical with clear appearance. Many mesenchymal cells begun to differentiate very rapidly into preosteoblasts. The cells enlarged and gradually became polyhedral or rectangular. Their processes were retained or disappeared. Their cytoplasm stained lightly basophilic on account of its high content of rER. Gradually, these centers became more vascular as they
were actively invaded with numerous capillaries derived from periosteal and endosteal. These new capillaries were essential for the ongoing reparative process. As these ossification foci became highly vascular, their activity became more pronounced.

2.2 Collagenic phase:
The intercellular spaces in the ossification center were occupied by a ground substance containing wavy collagen fibers. As more fibroblasts differentiated from the mesenchymal cells, more collagen fibers became randomly scattered within the ground substance. They formed interlacing fibers within which many cells were located. Moreover, many collagen fibers coalesced together to form bundles and became embedded in the initial secretion (osteoid) of the newly differentiated osteoblasts to form primitive specule or trabecula. The osteoid was in the form of a hyaline jelly-like material. This secretion was partially surrounding the osteoblast and was mostly extending towards the collagen bundles.

2.3 Osteogenic Phase:
Osteoclast was the first bone cell observed early in the regenerate. As the growing ossification centers initially invaded by the osteoprogenitor cells from the neighboring tissue, many of these cells differentiated into preosteoblasts which soon became mature osteoblasts. This maturation was enhanced by the effect of the pre-existing osteoclasts. An active osteoblast deposited the initial matrix, the osteoid amorphous substance. This secretion was in the form of a hyaline jelly-like material. It was given off unidirectional from the cell end away from the nucleus. The osteoid became mineralized after a time lag the so-called osteoid maturation period. The osteoid was mostly extending towards the collagen bundles in the form of thin bars (Fig. 5) which coalesced together. This secretion masked the collagen fibers and imparted them a hyaline or lightly basophilic appearance. Some osteoblasts became entrapped in their secretion as the future osteocytes. Adjacent collagenic bundles embedded in the osteoid with some entrapped osteoblasts formed a specule or a trabecula of early forming bone. Numerous osteoblasts congregated on the surface of this newly formed bony trabecula (Fig. 9). As more osteoblasts became incarcerated within their secretion as osteocytes, they were replaced by newly differentiated osteoblasts at the surface of the trabeculae. The presence of some osteoclasts attached to the surface of the newly formed trabeculae, in association with osteoblasts (Fig.7). This supported the theory that these two cells are working together in a synchronizing manner and were responsible for normalizing the newly formed regenerate.

2.4 Lacuna Formation Phase:
The osteoblasts eventually formed a row of cells on the surface of the developing bony specules. They deposited their osteoid secretion. Some cells, that failed to join the surface, became entrapped within the newly deposited matrix at the periphery of the trabecula (Fig.10). These trapped osteoblasts became osteocyte when it was completely encased by matrix in a lacuna. These first osteocytes assisted in osteonal development and its mineralization. An osteocyte lay in its own lacuna could contacting its neighboring osteocytes through fine primitive canaliculi. This contact would maintain its vitality by passing nutrients and metabolites from the blood, regulating ion homeostasis, and transmitting signals. Maturation of
them appeared flattened with their long axes parallel to the surface of the developing trabecula. The matrix was generally acidophilic near the surface of the trabecula. However, some trabeculae showed a mottled appearance due to the difference in staining properties of both the core and the edges of the trabecula.

2.5 Early Modeling-remodeling phase:
During the consolidation period, there was continual modeling and remodeling of the newly formed bony trabeculae and specules. They were subjected to the synchronizing activities of both osteoblast and osteoclast. It is well known that the osteoblast and osteoclast are integral parts of the bone formation. Moreover, it was believed that the osteoclast is only lodging in a concavity called Howship's lacuna. This lacuna, in the present study, was not found as the osteoclast was present in a very active form at the early reparative phase.

2.5.1 Role of osteoblast:
The osteoblasts produced layer after layer of bone inward on the surface of the developing trabecula. As the trabeculae enlarged in size, its structure was altered by internal reconstruction and by remodeling. Remodeling resulted from alteration in certain areas and deposition of new bone elsewhere.

2.5.2 Role of osteoclast:
a) Bone remodeling:
Osteoclasts were usually found in contact with the surface of a collagenic bundle (Fig. 11). Osteoclasts refine the affected bony edges. This enhanced the newly formed regenerate largely to retain the architectural shape of the preexisting bone.

b) Haversian canal remodeling:
The lytic or erosion-refining effects of the osteoclasts resulted in the formation of cylindrical cavities or tunnels, the forerunner of the Haversian canal (Fig. 12). The latter were lined by osteoblasts which deposited successive bony lamellae to increase the diameter of the canal.

c) Phagocytic activity:
Some osteoclasts acted as a giant cell. This was illustrated by phagocytosing and digesting osteocyte together with its surrounding bony lacuna.

Radiographic findings:
The regenerate filling the distraction gap after 4 weeks consolidation, showed a variable density when compared with the adjacent bone segments (Fig. 13).

After 8 weeks this regenerate showed homogenous bone densities (Fig. 14).
Angiographic results:
After 4 weeks consolidation, the inferior alveolar artery appeared patent and thin with remarkable decrease in its diameter (Fig.15). After 8 weeks consolidation, it showed a relatively thicker diameter. However, it still had a less diameter than the normal one (Fig.16).

4 Discussion
Intramembranous ossification was the main and only mechanism of bone formation in the distraction gap. There was no evidence of cartilaginous tissue in the newly formed bone. This finding was also described by Sencimen et al. [9] in rabbits. The finding of Carter et al. [11] that distraction was associated with cartilage only in very small discrete regions at the periphery of the distraction gap adjacent to the osteotomy ends is denied by the present study. Also in agreement with Rahn et al. [12] no inflammatory phase, no cartilaginous intermediate, nor callus formation were found in primary bone healing. Wornom and Buchman [13] were of the opinion that in some instances, bone may undergo primary healing after a fracture without a cartilage intermediate. In rabbit mandible, Komuro et al. [5] reported new bone formed by both intramembranous and endochondral ossification whereas Sawaki et al. [7] found new bone formed by intramembranous ossification, with some fibrocartilage islands. At the initial time of osteotomy, Ashton and Allen [14] and Brighton [15] found precursor mesenchymal cells and osteoprogenitor cells migrating into the gap from the adjacent muscle, marrow and from the periosteum. Tonna and Cronkite [16] mentioned that these cells contribute to the earliest formation of bone at the fracture site. The present findings showed an influx of mononuclear cells invading the reparative zone. This was derived from the marrow, blood and deepest layer of periosteum. These local precursor cells differentiate into fibroblasts and osteoblasts to produce collagen and osteoid in the gap. This finding is parallel with that of Nemeth et al. [17]. Moreover, these pluripotential cells differentiate into osteoblasts which underwent cementing to the cutting edges of bone at the fracture site. Others congregate on the surface of the newly formed bony trabeculae. They are responsible for depositing a new matrix. Their activity results in the formation of small lacunae in which the osteoblast resides to become osteocyte. In agreement with Lane et al. [18] type-1 collagen was initially deposited to form the framework on which mineralization occurs. According to Robling, Castillo, and Turner [19] bone matrix consists mainly of type-I collagen fibers (approximately 90%) and noncollagenous proteins or osteocalcin, (makes up 1% of the matrix). The latter may play a role in calcium binding and stabilization of hydroxyapatite in the matrix and/or regulation of bone formation. Within lamellar bone, the collagenic fibers are forming arches for optimal bone strength. These lamellae can be parallel to each other or concentrically arranged. Our findings supported the view of the interplay of the three bone cells as a team, which is called basic multicellular unit (BMU). This BMU is a mediator mechanism bridging individual cellular activity to whole bone morphology [20,21,22]. Units of the osteoclasts and the osteoblasts were activated in sequences of both bone resorption and formation to convert the contours of the reparative zone to the original bony architecture [13]. Recent evidence suggests that the normal mechanism of internal bone resorption may occur under the influence of the mature osteocytes. Osteocytes live in fluid-filled hollows within the bone matrix and are interconnected by numerous long cell extensions through microscopic tunnels. They are believed to be the cells that sense mechanical strain. Osteocytes respond to this strain by sending signals that either cause new bone formation or bone remodeling [23]. Glass, Bialek, Starbuck and Patel [24] described that osteoblast secretes collagenous and non-collagenous bone matrix proteins, including type-I collagen. Osteoblasts also produce a range of growth factors under a variety of stimuli. The most prominent features of the osteoclast is the ruffled

Fig.15: Arteriogram – 4 weeks consolidation.

Fig.16: Arteriogram – 8 weeks consolidation.
border facing the bone matrix. Osteoclasts are able to produce both phosphatase and protease which are necessary to dissolve bone minerals and to activate enzymes that break down collagen fibers. Furthermore, the cell secretes collagenase and gelatinase-β which are involved in bone matrix digestion [25]. Attachment of the osteoclast to the bone surface is essential for bone resorption. This attachment is performed via integrin receptors, which bind to specific Arginine-Glycine-Aspartate (RGD) sequences found in matrix proteins [26]. This process involves transmembrane adhesion receptors of the integrin. According to Parfitt [27] and Weinbaum, Cowin and Zeng [28] integrins attach to specific amino acid sequences at the surface of the bone matrix.

**Conclusion:**
The intramembranous type of ossification was the main mechanism of bone reformation in the distraction gap in mandible. No evidence of pre-existing cartilage tissue during gap healing. The term soft or hard callus is a surgical term and is not longer valid in the field of histology. Apoptotic and not necrotic changes occur during induction and healing of fracture. The role of osteoclast is to refine the bony surface and remodeling the newly formed bone. Both osteoblast and osteoclast are doing their work in a synchronizing manner under a genetic encoding which regulates their functions.

**References:**
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