Article de Recherche

TUNISIE ORTHOPÉDIQUE
Année 2011, Vol 4, N° 2
pp 112-117

Accés Libre sur / Free Access on
www.tunisieorthopedique.com

Histological analysis of new bone obtained by two rates of distraction osteogenesis in rabbits

Analyse histologique du régénérat obtenu par deux rythmes différents de distraction chez le lapin.

Trigui M.[1], Bahri A.[2], Zribi W.[1], Aoui M.[1], Rebai T.[3], Fakhfakh Z.[2], Keskes H.[1]

1. Service de Chirurgie Orthopédique et Traumatologique – Hôpital Habib Bourguiba. Sfax – Tunisie.

2. Faculté de Sciences de Sfax, Tunisie.

3. Laboratoire d'Histologie – Faculté de Médecine de Sfax, Tunisie.

CORRESPONDANCE : **Dr. Moez TRIGUI**

Service d'Orthopédie et Traumatologie – Hôpital Habib Bourguiba. Route El-Aïn Km 0,5, 3029, Sfax – Tunisie.

E-mail : dr_moez_trigui@yahoo.fr

ABSTRACT

The aim of this study was to compare histologically the new bone tissue obtained using two rates of distraction in a rabbit model of leg lengthening and evaluate these two methods in terms of their histological characteristics.

Materials and Methods

Twenty-four new Zeeland white (NZW) skeletally immature male rabbits were divided into two groups. Each group of rabbits was further divided into four sub-groups killed at different times of the consolidation period and histological analysis was performed. We investigated the effects of two distraction rates (1.4mm/day and 2.1mm/day) on bone formation during diaphyseal tibial lengthening (at the end of distraction and 1 week, 2 weeks, 5 weeks after the end of distraction) by comparing histological findings.

Results

There were 3 modes of ossification for both rates during distraction osteogenesis. Remodelling of the bone was delayed during rapid distraction. The histological phenomena of ossification of fibrous callus was hindered by acceleration of bone lengthening.

Discussion

Our findings support the contention that, at least in skeletally immature rabbits, an increase in the distraction rate does not improve osteogenesis during bone lengthening. Rapid distraction is not a good method to achieve a shorter period of device fixation during limb lengthening.

RÉSUMÉ

Le but de notre étude était de comparer histologiquement le régénérat osseux obtenu utilisant deux rythmes de distraction différents sur un modèle d'allongement de jambe chez le lapin.

Matériels et Méthodes

Vingt-quatre lapins males, immatures, de race New Zeland White (NZW), ont été répartis en 2 groupes qui ont eu un allongement diaphysaire de jambe avec un rythme d'allongement différent (1,4mm/j et 2,1 mm/j). Chaque groupe de lapin a été subdivisé en 4 sous groupes sacrifiés à des temps différents de consolidation après la fin de l'allongement (à la fin d'allongement, 1 semaine, 2 semaines et 5 semaines après la fin de distraction). Une étude histologique a été réalisée pour chaque sous-groupe.

Résultats

Il y avait 3 modes différents d'ossification pour les deux groupes. Le remodelage osseux a été retardé pour le groupe de distraction rapide. L'ossification du cal fibreux a été retardée par l'accélération du rythme d'allongement.

Discussion

Nos résultats confirment l'hypothèse que au moins pour les jeunes lapins, une accélération du rythme d'allongement n'augmente pas l'ostéogenèse lors de l'allongement et ne réduit pas la durée de fixation externe.

Keywords: osteogenesis, distraction, histology, lengthening, tibia, rabbit

Mots clés : ostéogenèse, distraction, histologie, allongement, tibia, lapin

I. INTRODUCTION

Callus distraction also called callotasis, is a method of lengthening a long bone without grafting. After a diaphyseal osteotomy, the early callus is elongated by slow progressive axial distraction using a dynamic external fixator. Distraction osteogenesis (DO) is the principle of bone lengthening in which new bone tissue is created by progressive distraction of osteotomized bone edges.

Although limb lengthening by distraction osteogenesis was first described by CODIVILLA in 1904, the technique did not gain wide acceptance until ILIZAROV in the 1950s identified the physiologic and mechanical factors governing successful osseous regeneration during DO [1, 2, 3]. Further additional works had described the biomechanical, histological, ultrastructural, and molecular changes associated with distraction [2, 4, 5].

Distraction osteogenesis is the mechanism which induces osseous formation between two osseous extremities progressively spread. It develops according to a particular process in the space of distraction and depends on various factors, such as the degree of preservation of periosteum at the osteotomy site, the stability of the external fixation and the timing and the frequency of distraction [2, 6, 7].

Although the rate of distraction recommended by most authors is 1mm/day, as described by ILIZAROV, some uncertainties persist about the effect of fast rate bone lengthening on the quality of the callus [1]. The majority of studies concerned mandibular distraction [1, 4]. The present study was therefore undertaken to elucidate the effects of two frequencies of distraction on the histological aspect of the distraction osteogenesis, using a model of tibial lengthening in rabbits.

II. MATERIALS AND METHODS

The experiments were performed on 24 skeletally immature New Zealand White (NZW) rabbits that weighed between 3.5 and 4.0 kilograms. The animals were kept in the animal house in separate cages at 20±2ºC under a 12h light dark cycle. Tap water and food pellets were available ad libitium. Rabbits were male and randomly separated into two groups of 12 animals each in order to compare two different speeds of tibial lengthening.

A- Operative protocol

The rabbits were anesthetized by intramuscular administration of Ketamine (35 mg/kg) and Xylazine (0, 3 mg/kg). Uniplanar external fixation devices with distraction capability were custom designed, built from brass and weighed 17 g each. Four half-pins were inserted, 2 above and 2 below the osteotomy site. The tibia was exposed subperiosteally, and the osteotomy was performed with an oscillating saw just below the fusion site between the tibia and the fibula. The periosteum was repositioned and the wound closed. Unrestricted activity and weight bearing were allowed postoperatively.

After a 7 day-latency phase post-osteotomy, distraction was performed twice daily at two different rates of distraction. In group 1 (n=12), distraction was performed at a rate of 1.4 mm twice daily for 15 days and in group 2 (n=12), distraction was performed at a rate of 2.1 mm twice daily for 10 days. In both groups, lengthening was of 21mm (approximately 20% of the total length of the tibia). Distraction was followed by a period of 5 weeks, during which the external fixator was held in place with no distraction.

Each group was divided into 4 subgroups of 3 rabbits killed at different times after the end of distraction by intravenous administration of sodium pentobarbitone 1 ml/kg. In subgroup 1, animals were killed at the end of distraction; in subgroup 2, animals were killed 1 week after the end of distraction; in subgroup 3, animals were killed 2 weeks after the end of distraction; and in subgroup 4, animals were sacrificed 5 weeks after the end of distraction.

B- Sample Preparation

Histological and histomorphometric assessments were done according to the suggestions of GERSTENFELD et al [8]. After the rabbits were killed, the distracted right tibia in the two groups was resected and carefully dissected out with the fixator. One nonoperated left tibia was used as a control. The length of the distraction gap size was measured, and then cut with a saw. The samples were longitudinally cut and sized to be used for histological study.

The samples were fixed in a solution containing 750ml of ethanol, 100ml of Formaldehyde and 150ml of $H2O$ for 24 hours. Then, they were dehydratated in increasing concentrations of acetone, deflated in xylene, and embedded in methyl methacrylate resin as recommended by ERBEN et al [9].

Seven micrometer-thick coronal sections were cut in parallel to the long axis of the tibia, of the bone samples, using a heavy-duty microtome (Polycut S Reichert Jung, Germany) equipped with tungsten carbide knives.

Histologic staining was performed using 3 types of colorations: haematoxylin phloxin stain, trichrome modified Goldner's stain to demonstrate the organic phase and the mineral phase of the bone regenerate during the distraction osteogenesis, and toluidine bleue, borax eosin stain to show the cellular nucleus blue, the collagen and fibrosis pink.

Scanning electron microscopy (SEM) was performed on the regenerate bone obtained in the gap created after distraction of the tibia in the two groups. The callus was cut longitudinally with a scalpel, immersed in 50% sodium hypochloride for 3h to remove organic material. It was rinced for 30mn in distilled water and fixed overnight in 1% osmium tetroxyde dissolved in 01 M cacodylate buffer (pH 7.2). Subsequently, the samples were then rinsed for 30mn in distilled water, dehydrated in an ascending series of 70, 95, 100% ethanol. Further treatment was conducted in hexamethyldisilazane (HMDS) for 1h, followed by airdrying using a filter paper. They were SEM analysed at 20kV (SEM Philips series XL30) as described by LIBOUBAN et al. [10].

III. RESULTS

A- Clinical evaluation

All animals were fed a soft diet, and all gained weight. All the experiment animals loaded their weight on the operated limb already during the day following the operation. All animals fed well and exhibited a normal pattern of activity. There was no postoperative infection. The length of the distraction gap size in the 2 groups was approximately 21mm. All subjects completed the surgical procedure without incident. There were no wound infections, but localized and encapsulated abscesses were later found in some animals. None of the animals experienced device dislodgement, and the distractors remained stable until the animals were killed.

B- Histological examination

The chronological evolution of the histological aspects of bony regenerate was comparable for the groups of fast and slow distraction.

At the end of distraction, we observed a soft callus made of fibrous tissue developing in the space created by distraction. The collagen fibers of this soft callus were well organized and disposed in the same way recalling the structure of the tendons (Figure 1). An early phase of mineralisation existed at this stage (new bony tissue), developing on a fibrous collagenic tissue next to the bony surfaces (place of the osteotomy). This regenerate was disposed in a circumferential way and was born from mesenchymatous tissue organized in fibro-cellular membrane. Ossification was thus membranous.

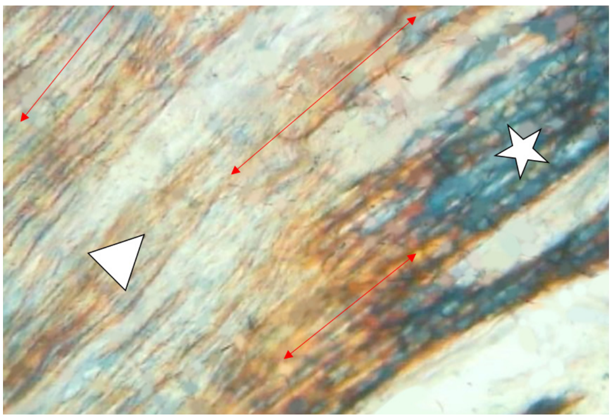

Figure 1*: initial callus formed of soft tissue and collagen fibers. These fibers (◁) are disposed parallel along the distraction site, following distraction forces. (☆) They appear in the pre-existing fibrous tissue (Trichrome modified Goldner's stain (X250))*

One week after the end of distraction, we observed in the callus the arrangement of three zones divided between the peripheral and central part of the space created by the distraction: a bony anastomized trabeculae, a cartilaginous forehead, and a fibrous zone (Figure 2). The precursor cells, taking a round shape, turned into chondrocytes and began to produce a cartilaginous matrix. This particular cellular disposition translates a cartilage in growth and is seen therefore more frequently in a young cartilage. This passage by a cartilaginous stage corresponds to the classic enchondral ossification. The cellular multiplication was intense with appearance of the osteoid substance that was deposited progressively on the trabecules of the calcified cartilaginous matrix.

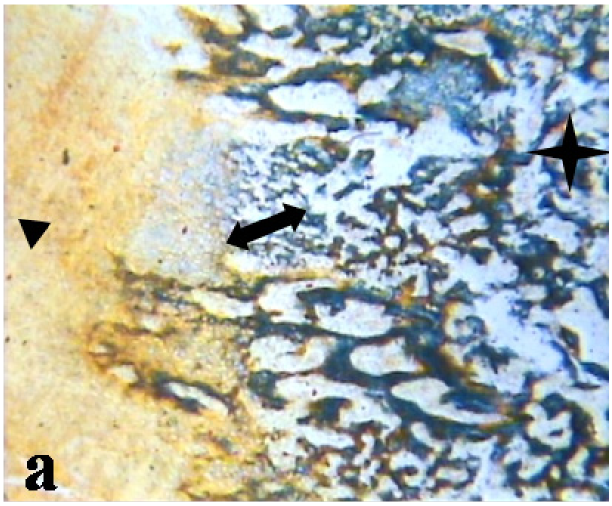

Figure 2a*: Callus aspect on the fast distraction group, one week after the end of distraction. We see the presence of three zones: fibrous zone (▶), cartilaginous front (✦) and an area colonized by uncontrolled primary bone (bone spans anastomosis) (⟷). (Trichrome modified Goldner's stain (X250))*

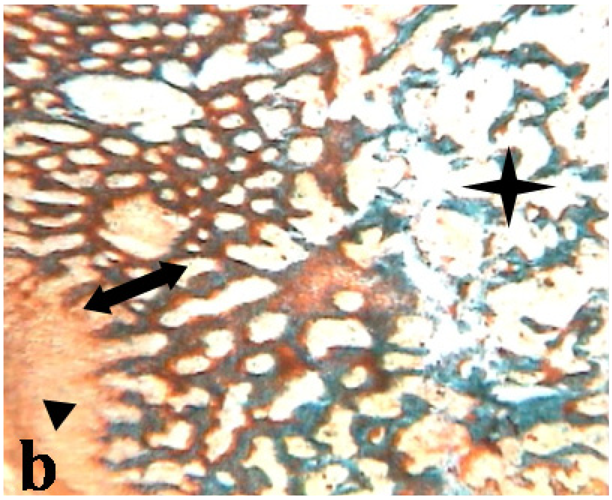

Figure 2b*: Callus aspect on the slow distraction group, similar to the fast distraction group. (Trichrome modified Goldner's stain (X250))*

Two weeks after the end of distraction, some differences were observed between the two groups: in the slow distraction rate group, we observed an immature bone muff (woven bone), composed of homogeneous bays (Figure 3a), that increases in the distraction site. There was a mixture of cartilaginous and bony tissue due to the calcification of an osteoïd tissue directly formed from the cartilaginous matrix. This bony tissue was formed by a transchondroid ossification and was amplified in the extremities of the distraction site. Haematoxylin phloxine stain showed the presence, in the trabecular tissue, of a hematopoietic tissue. There was also in the immature bone, the presence of many small cells that would seem to be osteoblasts.

In the fast distraction rate group, we noted the persistence of a fibrous zone in the central part of the distraction site. The bays, having big ridges, presented a parallel oriented aspect to the distraction axis (Figure 3b).

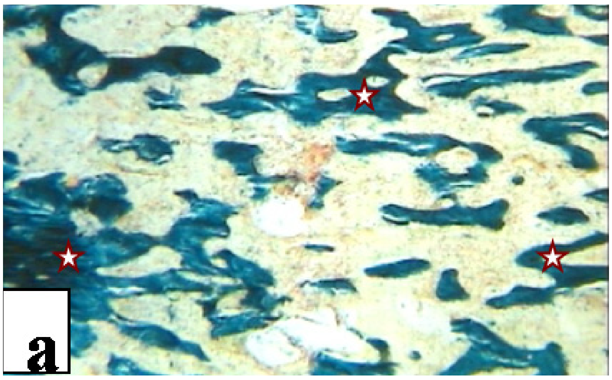

Figure 3a: *Initial callus formed of immature bone (woven bone), 2 weeks after the end of distraction. Trabecular immature bone oriented according to the distraction forces. (Trichrome modified Goldner's stain (X250))*

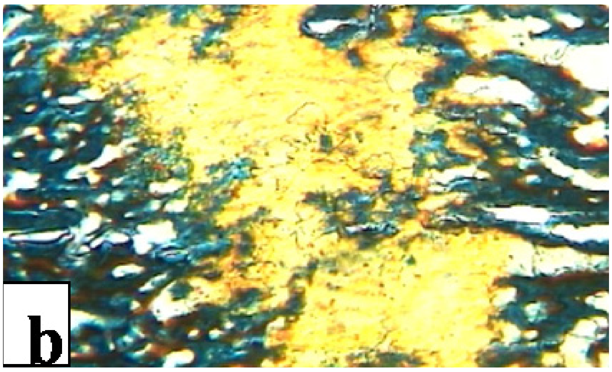

Figure 3b: *Persistence of fibrosis in the central part of the distraction site. (Trichrome modified Goldner's stain (X250))*

Five weeks after the end of distraction, in the slow distraction rate group (Figure 4a), we noted a new developing cortical bony tissue that begins to take haversian structure. Otherwise, in the medullary cavity, under individualization in the central part of the callus there was a fibroadipous tissue and a haematopoietic tissue.

So far, the fast distraction rate group presented a less homogeneous and less organized aspect, giving a less compact tissue and including more important remaining bony bays (Figure 4b). The haversian structure was incomplete and the overhaul was therefore incomplete. The bony overhaul during fast distraction rate was delayed.

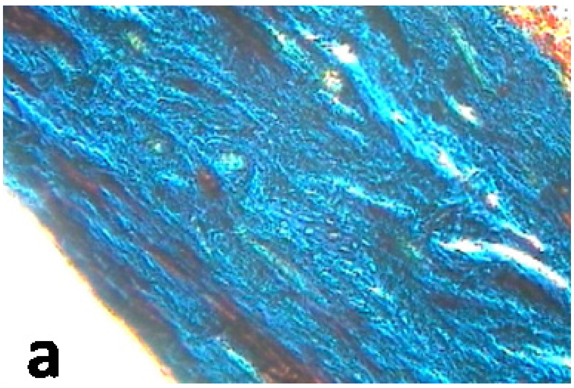

Figure 4a: *Aspect of the compact lamellar bone in the group of slow lengthening five weeks after the end of distraction. Presence of haematopoietic tissue. (Trichrome modified Goldner's stain (X250))*

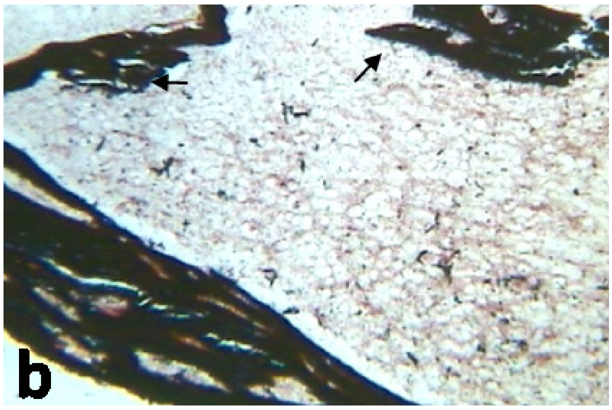

Figure 4b: *Less compact lamellar bone in the group of rapid distraction (Trichrome modified Goldner's stain (X250))*

C- Ultra structural study

The SEM examination was a descriptive study that provided callus morphology and its external surface structure. The study concerned only the hard samples of each group; some soft samples of the end of the distraction did not resist treatment.

At the end of distraction, there was a large number of spans with a riddled, and undifferentiated aspect recalling a spongious bone in the two groups, however, it was more structured and homogenous in the slow distraction group. Two weeks after the end of distraction, spans of reticular bone had a longitudinal enlargement in the direction of the distraction forces however, in the fast distraction group, reorganization of the spans was less obvious (Figure 5). Five weeks after the end of distraction, the callus evolved to a haversian bone in both group, but more organized in the group of slow lengthening.

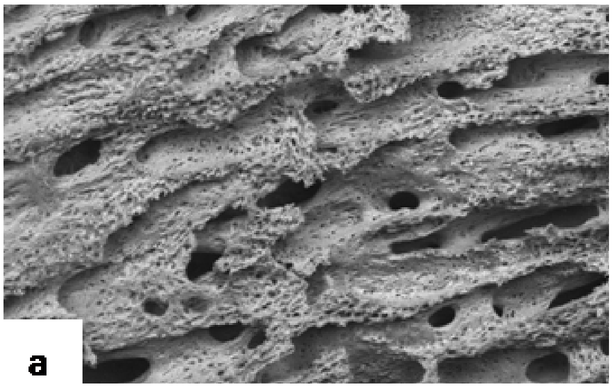

Figure 5a: *Ultra structural aspect of the callus in the group of slow distraction two weeks after the end of lengthening. Longitudinal enlargement of the spans in the direction of the distraction forces (x62)*

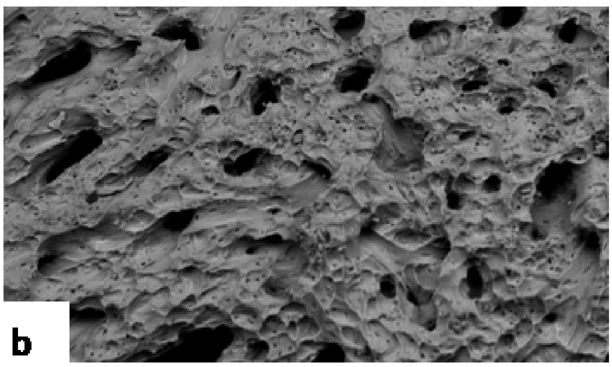

Figure 5b: *The callus is less organized recalling the appearance of a spongious bone (x61)*

IV. DISCUSSION

Distraction osteogenesis requires a considerable length of time to achieve the desired bone lengthening. Therefore, any approach that increases the rate of new-bone formation reduces the duration of consolidation, and permits the earlier removal of the external fixator would be desirable. STEWART et al. [11] did not detect any difference in the level of osteogenesis between rapidly and slowly distracted rabbit mandibules using bone densitometry and biomechanical testing, but they found the presence of histological non union in half the rapidly distracted group.

Studies about diaphyseal tibial lengthening were rare. LI et al. [12] investigated the angiogenic response to four varying rates (0.3, 0.7, 1.3, and 2.7mm/day) of distraction in a rabbit model of leg-lengthening. They found that a slow rate of distraction (0.3mm/day) did not maximally stimulate angiogenesis in the central fibrous zone, whereas high rates (2.7mm/day) appeared to impair this response. The vascularization process in the central fibrous zone was maximally stimulated at distraction rates of 0.7 and 1.3mm/day. LI et al. [13] demonstrated that bone formation is intramembranous independently of the rate of distraction, but the cellular differentiation is affected by the rate of distraction. Chondrocytes are found only at rates of 0,7 and 1,3mm/day. A comparison of different distraction rates in the mandibular rabbits was investigated by Al RUHAIMI et al. [1]. Their results indicated that a distraction rate of 1.0mm per day produced the best osteogenesis. However, 0.5mm distraction may result in immature bone healing and distraction of 2.0mm once a day resulted in fibrous union.

In our series, we did not find any histological differences between the two groups at the end of distraction and one week after the end of distraction. Differences were observed only after two weeks after the end of distraction. These differences were more obvious five weeks after the end of distraction. The bony overhaul during fast distraction rate was delayed, whereas for the slow distraction rate group, regenerate was strongly ossified and contained a well-organized haversian bone, with the presence of hematopoietic tissue. In the ultrastructural study, we demonstrated that the two types of lengthening gave different spongious aspects, leading to a corticalized bone, more organized in the group of slow lengthening and with a delayed remodelling process in the group of fast lengthening.

The different steps of healing during bone lengthening are comparable to those observed during fracture healing [14]. According to RICHARDS et al. [15], the ossification of the distraction callus is endochondral and endoconjunctive. According to SCHINDELER et al. [14], if the stability of the distraction callus is sufficient, a lamellar bone develops directly. On the contrary, if the stability and the vascularisation are insufficient, a fibrous or a cartilaginous tissue is formed.

Our study has provided information about the basic mechanisms involved in bone lengthening by callus distraction. Reproducible and homogeneous results were obtained because the experimental conditions were controlled by a rigid system of external fixation. We obtained similar information for the two groups concerning the basic mechanisms involved in bone lengthening. Slow or fast distraction produced a zonal structure: a central zone separating 2 zones containing a matrix of calcified bone, indicating that the ossification during lengthening is centripetal and that the distraction happens only at the central zone.

Distraction osteogenesis using long bones in rabbits and rats has demonstrated cartilaginous callus formation in the distraction gap during the latency period [16, 17].

During distraction, YASUI et al. [18] demonstrated, in a rat model, that endochondral ossification was predominant in the early stage of distraction especially in the circumferential region where cartilaginous callus had been formed during the latency period between osteotomy and distraction. This endochondral ossification is replaced by an intramembranous ossification between 10 and 20 days of distraction. In our study, we observed that the ossification at the callus extremities at the end of distraction remained membranous. During this time, a fibrous tissue is built to fill the space of distraction. During the following phases, the ossification becomes endochondral with columnar arrangement of chondrocytes at the borderline of cartilaginous and osseous tissue. This ossification takes over the membranous ossification, becoming predominant since the second week after the end of distraction for the two groups. According to ALI et al. [4], the ossification was intramembranous during the active distraction phase and the consolidation period; endochondral ossification was observed only during the early consolidation phase (first week to third week) and only at the central portion of the distraction gap.

Apart from endochondral and intramembranous ossifications, a third mechanism has also been proposed by YASUI et al. [18]: the “transchondroid bone formation,” in which the gradual transition from cartilage to bone via chondroid bone occurs. The observation of histological sections allowed us to confirm this third mechanism of ossification. During this ossification, the transition between fibrous tissue and bone is made progressively without vascular invasion. The cells of the chondoid bone do not have the same morphological characteristics as chondrocytes, but they are compared to young chondrocytes in which differentiation to osteocytes is more rapid.

Our study suggests that attempts to shorten the duration of external fixation in clinical tibial distraction osteogenesis should be done by methods other than more rapid distraction. Methods to reduce the duration of external fixation, other than fast rate of distraction, were attempted. MIZUMOTO et al. [19] demonstrated the benefits of recombinant human bone morphogenetic protein-7 on the acceleration of regenerate ossification. YAMANE et al. [20] demonstrated that ED-71 increases callus volume during the early period after the completion of lengthening. SHIMAZAKI et al. [21] found that treatment by ultrasound was highly effective in achieving maturation of bone of distracted callus. TAKUSHIMA et al. [22] used cultured periosteal cells to facilitate osteogenesis in bone defect created by distraction in rabbits.

There are probably several other factors that influence the distraction osteogenesis, such as the age, the stability of fixation, the level of corticotomy, the type of operative procedure and the timing of distraction [5-7, 23].

Acknowledgments: The Authors wish to thank professor Moufida BOUYAHIA for her aid in reviewing this paper.

Conflict of interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Ethical Board Review statement: This study was approved by the Institutional ethical board review of Habib Bourguiba Hospital.

V. REFERENCES

1) Al Ruhaimi K.A. Comparison of different distraction rates in the mandible: an experimental investigation. Int J Oral Maxillofac Surg 2001; 30:220-7.
2) Aronson J., Harrison B.H., Stewart C.L., Harp J.H. The histology of distraction osteogenesis using different external fixators. Clin Orthop Relat Res 1989; 241:106-16.
3) Ilizarov G.A. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. Clin Orthop Relat Res 1989; 239:263-85.
4) Ali M.N., Ejiri S., Kobayashi T., Anwar R.B., Oda K., Oshima H. et al. Histologic study of the cellular events during mandibular distraction osteogenesis. Oral Surg Oral Med Oral Path Oral Radiol Endod 2009; 107:325-35.
5) Krawczyk A., Kuropka P., Kuryszko J., Wall A., Dragan S., Kulej M. Experimental studies on the effect of osteotomy technique on the bone regeneration in distraction osteogenesis. Bone 2007; 40:781-91.
6) Aronson J., Shen X. Experimental healing of distraction osteogenesis comparing metaphyseal with diaphyseal sites. Clin Orthop Relat Res 1994; 301:25-30.
7) Fischgrund J., Paley D., Suter C. Variables affecting time to bone healing during limb lengthening. Clin Orthop Relat Res 1994; 301:31-7.
8) Gerstenfeld L., Ronski T.J., Hollinger J.O., Einhorn T.A. Application of histomorphometric methods to the study of bone repair. J Bone Miner Res 2005; 20:1715-22.
9) Erben R.G. Embedding of bone samples in Methylmathacrylate: an improved method suitable for histomorphometry, histochemistry, and immunohistochemistry. J Histochem Cytochem 1997; 45:307-13.
10) Libouban H., Morceau M.F., Lesourd M. et al. Osteolytic bone lesions in the multiple myeloma model: radiographic scanning and microtomographic studies. J Histotechnol 2001; 24:81-6.
11) Stewart K.J., Lvoff G.O., White S.A., Bonar S.F., Walsh W.R., Smart R.C. et al. Mandibular distraction osteogenesis: a comparison of distraction rates in the rabbit model. J Cranio-Maxillofacial Surg 1998; 26:43-9.
12) Li G., Simpson A.H., Kenwright J., Triffitt J.L. Effect of lengthening rate on angiogenesis during distraction osteogenesis. J Orthop Res 1999; 17:362-7.
13) Li G., Virdi A.S., Ashhurst D.E., Simpson A.H.R.W., Triffitt J.T. Tissues formed during distraction osteogenesis in the rabbit are determined by the distraction rate: Localization of the cells that express the mRNAs and the distribution of types I and II collagens. Cell Biol Int 2000; 24:25-33.
14) Schindeler A., McDonald M.M., Bokko P., Little D.G. Bone remodelling during fracture repair: the cellular picture. Seminars in Cell and Developmantal Biology 2008; 19:459-66.
15) Richards M., Goulet JA, Schaffler MB, Goldstein SA. Temporal and spatial characterization of regenerate bone in the lengthened rabbit tibia. J Bone Miner Res 1999; 14:1978-86.
16) Kojimoto H., Yasui N., Goto T., Matsuda S., Shimomura Y. Bone lengthning in rabbits by callus distraction. The role of periosteum and endosteum. J Bone Joint Surg 1988; 70B:543-9.
17) Yasui N., Kojimoto H., Sasaki K., Kitada A., Shimizu H., Shimomura Y. Factors affecting callus distraction in limb lengthening. Clin Orthop Relat Res 1993; 293:55-60.
18) Yasui N., Sato M., Ochi T., Kimura T., Kawahata H., Kitamura Y. et al. Three modes of ossification during distraction osteogenesis in the rat. J Bone Joint Surg 1997; 79B:824-30.
19) Mizumoto Y., Moseley T., Drews M., Cooper V.N., Reddi H. Acceleration of regenerate ossification during distraction osteogenesis with recombinant human bone morphogenetic protein-7. J Bone Joint Surg 2003; 85A, Suppl 3:124-9.
20) Yamane K., Okano T., Kishimoto H., Hagino H. Effect of ED-71 on modelling of bone in distraction osteogenesis. Bone 1999; 24:187-93.
21) Shimazaki A., Inui K., Azuma Y., Nishimura N., Yamano Y. Low-intensity pulsed ultrasound accelerates bone maturation in distraction osteogenesis in rabbits. J Bone Joint Surg 2000; 82B:1077.
22) Takushima A., Kitano Y., Harii K. Osteogenic potential of cultured periosteal cells in a distracted bone gap in rabbits. J Surg Res 1998; 78:68-77.
23) White S.H., Kenwright J. The timing of distraction of an osteotomy. J Bone Joint Surg 1990; 72B:356-61.