Chapter 3

Formation of New Bone during Vertical Distraction Osteogenesis of the Human Mandible is related to the Presence of Blood Vessels

L. R. Amir¹, A. G. Becking², A. Jovanovic³, F.B.T. Perdijk⁴, V. Everts¹, A.L.J.J Bronckers¹


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¹Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Amsterdam, The Netherlands
²Department of Oral & Maxillofacial Surgery Oral Pathology, Vrije Universiteit Medical Center, ACTA, Amsterdam, The Netherlands
³Department of Oral & Maxillofacial Surgery, Alkmaar Medical Center, The Netherlands
⁴Department of Oral & Maxillofacial Surgery, Gelderse Vallei Hospital, Ede, The Netherlands
Chapter 3

Abstract
We examined the effect of distraction rate on blood vessel growth in intramembraneous ossification after vertical distraction osteogenesis in the human mandible. Six patients (aged 60 ± 9 years) with a severely atrophic mandible underwent bone augmentation with distraction osteogenesis. Two distraction rates (0.5 and 1 mm/day) were compared and for each group three patients were analyzed. Vascular histomorphometry was carried out in two different areas in the distraction gap: (1) in the 1st and (2) in the 2nd one-mm area from the osteotomy line, representing the oldest and younger new-bone area, respectively. Correlation analysis was performed between blood vessel parameters and the amount of new bone formed during distraction.

Histological analysis demonstrated the presence of blood vessels throughout the soft connective tissue in the distraction gap. The volume density of blood vessels between the two investigated areas was significantly lower in the 1 mm/day groups, suggesting a delay in angiogenesis in this group of patients. A positive correlation between blood vessel volume and bone volume density was found in the younger new-bone area but not in the oldest new-bone area. This correlation was due to a higher number of blood vessels rather than to a larger size of the blood vessels.

Our data suggest that the lower blood vessel density found in the patients with 1 mm/day distraction rate may be related to disruption of angiogenesis in the soft connective tissue of the gap or to a less optimal mechanical stimulation of cells involved in angiogenesis. This probably results in the slower rate of osteogenesis seen at the 1 mm/day distraction rate compared to 0.5 mm/day distraction rate. The data support the concept that a positive relationship exists between the density of blood vessels and the formation of bone. For distraction of the human mandible in elderly patients, a distraction rate of 0.5 mm/day seems beneficial.

Introduction
Angiogenesis is crucial for development, remodeling and regeneration of bone. During embryogenesis, endochondral ossification is accompanied by
the invasion of new capillaries sprouting from the neighboring blood vessels into the calcified cartilage (Marks & Hermey 1996). This invasion is followed by recruitment of osteogenic cells and deposition of bone that replaces the cartilage. A similar process occurs during fracture healing. A callus tissue is formed at the fracture site where capillaries will sprout. If there is excess of (micro)motion, blood vessels in the callus will be disrupted and cartilage will form. This tissue survives the hypoxia environment and behaves as a temporary fixator of the bone ends. Later, in a more stable condition, blood vessel formation can occur followed by the formation of new bone replacing the cartilage (Ferguson et al. 1999). During intramembranous bone formation, the role of blood vessels is less thoroughly studied. In distraction osteogenesis, where bone is formed predominantly via intramembranous ossification, osteogenesis takes place only in the area where blood vessels are present (Li et al. 1999). Cells that constitute the blood vessel walls or that are in the vicinity of the blood vessel wall are believed to secrete osteogenic factors and some of these cells appeared to have osteogenic potential (Reilly et al. 1998; Kuznetsov et al. 2001; Lewinson et al. 2001; Bronckers et al. 2005).

A number of studies have suggested a relationship between mechanical loading and angiogenesis (Li et al. 1999; Brown & Hudlicka 2003; Moore et al. 2003; Yoshino et al. 2003; Yao et al. 2004). Angiogenesis in bone marrow tissue is enhanced in response to exercise and leads to more bone formation and less bone resorption (Moore et al. 2003). It has been postulated that exercise training increases blood flow that enhances shear stress, cyclic strain and tension in the blood vessel wall (Resnick & Gimbrone, Jr. 1995; Brown & Hudlicka 2003). These haemodynamic forces act as a stimulus for endothelial cells to migrate and proliferate and eventually form new vessels (Ando et al. 1987; Resnick & Gimbrone, Jr. 1995; Lehoux & Tedgui 1998; Rowe et al. 1999). The biological response to mechanical stimulation is mediated by a variety of angiogenic factors, most importantly vascular endothelial growth factor (VEGF) (Brown & Hudlicka 2003; Yao et al. 2004).
Distraction osteogenesis provides an *in vivo* system of bone formation by means of controlled mechanical force. During active distraction, blood flow in the distracted tissue is four to five times higher than in non-distracted controls as shown by a quantitative technetium scintigraphic study in the dog model (Aronson 1994). This increase persists during post distraction period (neutrofixation) up to seventeen weeks after osteotomy and is approximately two-fold higher than in non-distracted controls (Aronson 1994). Recently, we examined the influence of distraction rate on the amount of bone formed during vertical distraction of the mandible of elderly patients (Amir et al. 2006). We found more bone formation in the distraction gap at a distraction rate of 0.5 mm/day than at 1 mm/day. In biopsies of two patients, a very low amount of bone was formed. Those areas were characterized by a very scarce vascularisation. Given the relevance of blood vessels in the bone formation process, we speculated that the beneficial effect of a slow distraction rate on osteogenesis might have been caused by a better blood supply in the distraction gap.

In the present study we examined more closely and also quantified the blood vessels present in the distraction gap of both groups of patients (0.5 and 1 mm/day). We hypothesize that (1) a slow distraction rate has a stimulatory effect on blood vessel formation in the distraction gap and (2) vascularisation is positively correlated with a faster bone formation as seen at a low distraction rate.

**Material and methods**

*Clinical procedure*

Patient selection and surgical procedures have been described in detail in a previous paper (Amir et al. 2006). Briefly, completely edentulous patients with severely atrophic mandibles (approximately 8 mm residual height) underwent anterior alveolar bone augmentation by vertical distraction osteogenesis using extraosseous distractors (KLS Martin and Mondeal Medical System, Tuttlingen, Germany). After performing osteotomy and placing the distractor, a latency period of 5-7 days was introduced to enable the soft tissue to heal. Next, the
distractor was activated, moving the bone segment approximately 10 mm upward. The rate of distraction was either 0.5 or 1 mm/day (2x0.5 mm) and was applied for 20 or 10 days of distraction period, respectively. The bone segments were then maintained in neutral position without activation for 8 - 11 weeks (neutrofixation period) to allow the formation of new bone in the distraction gap. Patients should have good general physical and mental health, no drug and/or alcohol abuse, no systemic diseases that could interfere with normal wound healing and good oral health. The selection of patients in two groups (0.5 and 1 mm/day groups) was non-randomized, assigned by two different surgeons who performed the surgery. Treatment protocols for each surgeon were exactly the same. One surgeon applied 0.5mm/day rate and the other 1 mm/day (see discussion Amir et al. 2006).

At the time of removal of the distractor at the end of neutrofixation period, biopsies were taken. A hollow trephine burr with 3.5 mm (outer) diameter (Straumann, Switzerland) was used for implant preparation vertically through the bone segments and distracted area. The inner core of the trephine was collected as a biopsy sample and the dental implants were placed at the site prepared with the trephine burr. The bone biopsies were used for histology and histomorphometric analysis. The Medical Ethical Committee of Northern Holland gave approval for research and all patients gave informed consent.

**Histological procedure**

The biopsies were fixed in 4% formaldehyde solution in 0.1 M phosphate buffer pH 7.3 at 4°C for 24-48 hours and rinsed in 0.1 M phosphate buffer pH 7.3, 2 times for 1 hour at ambient temperature. Specimens were dehydrated through ethanol series at 4°C and embedded in low temperature polymerising methylmetacrylate (MMA) without decalcification. Longitudinal sections of 5 μm thick were cut on a Jung heavy-duty microtome. In total, 5 groups of 5 consecutive sections from each biopsy, with an interval of 125 μm between the groups were mounted on glass slides and stained with Goldner’s trichrome. This procedure results in the following staining: green for mineralised tissue,
red for osteoid, blue or black for cell nuclei and light orange or red for cytoplasm. The sections were analysed by light microscopy.

**Biopsy selection**

From available materials obtained in an earlier study (see Amir et al. 2006) a selection of biopsies from six patients was made to form two groups (0.5 mm/day and 1 mm/day distraction). The selection of biopsies was based on the quality of the biopsies (as intact and complete as possible with intact soft connective tissue in the distraction gap) and obtained after approximately the same healing time (mean ± SD: 0.5 mm/day group: 87 ± 9.8 days; 1 mm/day group: 87±2.0 days, Table 1). Selected patients were all female with age ranging between 47 – 73 years (mean ± SD: 60 ± 9); mean age of both groups was comparable (mean ± SD: 0.5 mm/day group: 66 ± 8.2; 1 mm/day group: 55.7 ± 8.1, Table 1).

**Table 1. Vascular histomorphometric results**

<table>
<thead>
<tr>
<th>Distraction rate</th>
<th>Patient number</th>
<th>Age (year)</th>
<th>Healing time (days)</th>
<th>Bone volume density (%)</th>
<th>Blood vessel parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Volume density (%)</td>
</tr>
<tr>
<td>0.5 mm/day</td>
<td>3</td>
<td>68</td>
<td>76</td>
<td>32.0 ± 1.4</td>
<td>4.7 ± 1.9</td>
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<tr>
<td></td>
<td>5</td>
<td>57</td>
<td>90</td>
<td>24.7 ± 36.0</td>
<td>11.1 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>73</td>
<td>95</td>
<td>32.0 ± 31.3</td>
<td>2.5 ± 6.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>66±8.2</td>
<td>87±9.8</td>
<td>29.6 ± 4.2</td>
<td>29.6 ± 7.5</td>
<td>6.1 ± 4.4</td>
</tr>
<tr>
<td>1 mm/day</td>
<td>12</td>
<td>47</td>
<td>85</td>
<td>25.2 ± 17.1</td>
<td>6.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>63</td>
<td>87</td>
<td>44.0 ± 13.1</td>
<td>9.1 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>57</td>
<td>89</td>
<td>23.2 ± 24.2</td>
<td>7.4 ± 3.2</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>55.7±8.1</td>
<td>87±2.0</td>
<td>30.8 ± 11.5</td>
<td>18.1 ± 5.6</td>
<td>7.6 ± 1.4</td>
</tr>
</tbody>
</table>

*The same patient numbers have been used as reported (Amir et al. 2006). † Healing time encompasses distraction period and neutrofixation period. ‡ Bone volume (%) = (bone area / tissue area) x 100. Data were collected in a previous study (Amir et al., 2006). § Blood vessel volume density (%) = (blood vessel area/soft tissue area) x 100. ¶ Number of blood vessels/soft connective tissue area (#/mm²). ‡ Blood vessel area/number of blood vessels (μm²). * p=0.02, volume density of blood vessels in 1 mm/day group was significantly lower in the younger new-bone area than in oldest new-bone area. ** p=0.02, number of blood vessels per mm square area in the 1 mm/day group was significantly lower in the younger new-bone area than in oldest new-bone area.
Since patients with a 0.5 mm/day of distraction rate underwent 10 more days of distraction than patients with a distraction rate of 1 mm/day, for the purpose of comparison, the neutrofixation time of the 1 mm/day group was extended with 10 days, making the healing time (i.e., distraction time plus neutrofixation time) of both groups equal.

**Vascular histomorphometry**

Quantitative measurements of blood vessels were carried out using the Leica Qwin image analysis system (Leica Imaging System, Ltd, Cambridge, England) (Zerbo et al. 2003). In a previous study we investigated the amount of bone in the distraction gap in two areas: the 1st and the 2nd one-mm area distant from the osteotomy line over the full width of the biopsy. These areas represent the oldest and younger newly formed bone within the distraction gap, respectively (Fig. 1). In the present study, we analyzed for the incisal region blood vessel parameters for the same two areas. We were thus able to correlate the results of this study with the previous results on bone formation in the distraction gap. Pictures of the areas of interest were taken in which soft connective tissue and blood vessels were identified. A drawing of the area of how blood vessels and soft connective tissue were measured is presented in Fig 2A, B.

![Fig. 1. Schematic drawing of the areas of analysis. The regions of interest are indicated by dotted boxes.](image)
The vascularisation was quantified in three ways:

1. Determination of the number of blood vessels in the soft connective tissue around the bone trabeculae per unit of soft connective tissue (number of blood vessels/mm²). A blood vessel was defined as a lumen of various sizes, lined by elongated endothelial cells, sometimes surrounded by flattened or cuboidal smooth muscle cells. Occasionally, identification was facilitated by the presence of red blood cells in the lumen.

2. Determination of the volume density of blood vessels, defined as the percentage of total soft connective tissue area taken in by blood vessels. Blood vessel volume was calculated as (total blood vessel area/total soft tissue area) x 100%.

3. Determination of the blood vessel size. This was calculated by the sum of the areas of the separate blood vessels/number of blood vessels, expressed as μm².

No attempt was made to quantify the blood vessels in the soft connective tissue in bone alveoli that were completely surrounded by bone. The measurements were performed at x100 magnification by two investigators (LA

Fig. 2A & 2B. Drawing of the area of blood vessels for vascular histomorphometry. Hatched = blood vessel (BV) area. Goldner’s trichrome staining. Green area = mineralized bone, red area = osteoid; white area = soft connective tissue (SCT) area. x100.
and VE) who were unaware of the origin of the sections. Of each biopsy, four sections at different depth were measured and the values were averaged.

Statistical analysis
Values were analysed using GraphPad prism 4 for Windows and presented as means and standard deviations. The statistical significance of the blood vessel

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Fig. 3. Histological appearance of blood vessels in the soft tissue area around bone trabeculae in the distraction gap.
(A) Dense fibrous tissue (DFT) and loose fibrous tissue (LFT) in the distraction gap. B= bone, BV= blood vessels x200.
(B) Blood vessels (BV) and bone (B) trabeculae are oriented along the inciso-apical direction of distraction force. x100. Erythrocytes (Ec) were occasionally found within the lumen of a blood vessel (Inset).
(C) Deposition of osteoid (O) by osteoblasts (Ob) in close vicinity to blood vessels (BV). x200.
values between 0.5 mm/day and 1 mm/day groups was tested by the Students T-test. The mean values of blood vessel volume in the oldest and younger new-bone area of the same biopsies were tested for differences by the paired T-test. Pearson correlation test was used to examine the correlation between the amount of new bone and the blood vessel volume. The significance was accepted when p<0.05 (two-tailed).

**Results**

**Vascular histology**

Blood vessels were found throughout the soft connective tissue area in the distraction gap. They were readily seen in the loose connective tissue between the newly formed bone trabeculae but were difficult to find in the center of the gap filled with very dense and cell-rich soft connective tissue (Fig. 3A). Soft connective tissue, the forming bone trabeculae and blood vessels were all aligned along the inciso-apical direction of the distraction (Fig. 3B). Nearly in all areas where osteoid was found being deposited by active osteoblasts, blood vessels were present in the close vicinity (Fig. 3B,C).

**Vascular histomorphometry**

The total volume of the vascular bed (blood vessel volume density) in the oldest new-bone area was the same for both groups of patients (Table 1). The blood vessel volume density in the younger new-bone area was almost three fold higher in the 0.5 mm/day than in the 1 mm/day, but due to a high variation this value was not significantly different (Fig. 4, p=0.19).

However, there was a difference in the effect of distraction rate on the volume density of blood vessels in the two areas within the same group of patients. In the 1 mm/day group, the blood vessel volume density was significantly lower in the younger new-bone area compared to the oldest new-bone area (Fig. 4, p=0.02). No such difference between these areas was measured in the 0.5 mm/day group. Both values were high and not different from the oldest new-bone area in the 1 mm/day group.
We investigated whether in the 1 mm/day group the difference in blood vessel volume density between oldest and younger new-bone area was due to a difference in the number of blood vessels or to a difference in their size. The number of blood vessels was significantly lower in the younger bone area than in the oldest area (Fig. 5, p=0.02). The mean size of the blood vessels tended to be lower in the younger new-bone area but this difference was not significant (Fig. 6, p=0.08).

Furthermore, we analyzed the correlation between the blood vessel parameters and bone volume data from the same biopsies. The data of the bone volume densities were obtained in a previous study and are presented in Table 1. A positive correlation was found between the volume density of bone and blood vessels (Fig. 7, r=0.90, p=0.01). This correlation was seen only in the younger new-bone area (from the combined groups), not in the oldest new-bone area (r=0.03, p=0.96, data not shown). When the number of blood vessels

![Graph showing the effect of distraction rate on blood vessel volume density in two areas of the distraction gap.](image)
(combined groups) was correlated with the amount of bone, the correlation was almost significantly positive (Fig. 8, r=0.79, p=0.06) while no correlation was found between the size of the blood vessels and the amount of bone (Fig. 9, r=0.69, p=0.13).

**Fig. 5.** Number of blood vessels per mm² of soft connective tissue in the 1 mm/day group. Significant difference was found between the two investigated areas (p=0.02).

**Fig. 6.** Blood vessel size in soft connective tissue in 1 mm/day group (μm²). No significant difference was found between the two investigated areas.
Fig. 7. Relationship between blood vessel volume density (%) and bone volume density (%) in the youngest new bone from the 0.5 mm/day and 1 mm/day groups combined. Bone volume data were collected in a previous study (Amir et al. 2006). These parameters were positively correlated with each other ($r=0.90$, $p=0.01$).

Fig. 8. Relation between density of blood vessel number and bone volume density (%) in the youngest new-bone area from the 0.5 mm/day and 1 mm/day groups combined. The correlation was almost significant ($r=0.78$, $p=0.06$).
Discussion

In the present study we analyzed the effect of distraction rate on blood vessel volume in the distraction gap of the human mandible. We assessed the vessels in the distraction area formed during the early phase of distraction (oldest new-bone area) and in the distraction area that was formed later (younger bone area). In the 0.5 mm/day group, a comparable (high) blood vessel density was found in both areas. However, in the group of 1 mm/day of distraction, the blood vessel volume was lower in the younger new-bone area than in the oldest new-bone area, due to a significantly lower number of vessels and a tendency of the vessels to be smaller, though not statistically significant. Considering that the distracted tissues were left for ten weeks in neutral position to heal before biopsies were taken, it is likely that the younger bone tissue had not yet formed during the actual distraction phase, indicating that the tissue at time of distraction was still a soft connective tissue. Therefore, the data suggests that it may be the soft connective tissue of the gap in which angiogenesis is negatively affected by the faster distraction rate. This eventually will reduce the number

Fig. 9. Relation between mean blood vessel size and bone volume density (%) in the younger new-bone area from the 0.5 mm/day and 1 mm/day groups combined. These parameters were not significantly correlated (r=0.69, p=0.13).
of vessels in the bone forming area. However, as soon as bone has formed the (low) blood vessel volume will catch up as suggested by the finding that the values in the oldest area in the 1 mm/day group are higher and in the same range as in the 0.5 mm/day group.

This lower blood vessel volume density in the area with younger bone of the 1 mm/day group may be explained in several ways. First, it may be that distraction of 1 mm/day disrupts the delicate initial capillaries that have just sprouted in the fibrous soft connective tissue in the distraction gap (Li et al. 1999). This dense connective tissue bears the total distraction force as long as no mineralized tissue has been formed to carry the load. After formation of the first bone trabeculae, the bone will gradually take over the mechanical load from the soft connective tissue. This will change the surrounding connective tissue from a dense collagenous tissue into a more open soft tissue, now allowing the capillaries to expand in size and to grow. This expansion may represent the catch-up effect of blood vessel volume density from younger to oldest area.

A second explanation for a better blood vessel growth in our studies is that a low distraction rate stimulates angiogenesis in the soft connective tissues of the gap more optimally than a faster rate. Distraction increases angiogenesis (Resnick and Gimbrone 1995; Moore et al. 2003; Carvalho et al. 2004; Yao et al. 2004) and enhances blood flow in the distracted tissue as long as seventeen weeks after osteotomy (Aronson 1994). Studies in long bones of young rabbits have demonstrated that the distraction rate significantly influences blood vessel formation in the fibrous tissue area (Li et al. 1999). For the rabbit long bone model a moderate distraction rate (0.7 and 1.3 mm/day) stimulates angiogenesis maximally while lower and faster rates give a lower response (Li et al. 1999). No effect of distraction rate was found in the area adjacent or distant to fibrous tissue where bone had formed or just started to form. Our study concurs with these data and suggests that also in the human mandible of aged patients there may be a stimulating effect of a slow distraction on angiogenesis in comparison to a faster rate.
An important finding in this study is the positive correlation between the amount of bone and the volume density of blood vessels in the younger new-bone area but not in the oldest area. Immunostaining with specific markers for vessel formation have shown that more precursor cells of new capillaries were present in fibrous tissue area near locations where more bone was formed (Li et al. 1999; Lewinson et al. 2001), implying that the formation of blood vessels precedes and codetermines the growth of bone in the gap. Early bone formation necessitates a high energy consumption, accordingly an adequate blood supply is required. Taken together, our findings are in line with other studies on the role of blood vessels in the bone forming activity (Li et al. 1999; Lewinson et al. 2001; Choi et al. 2002; Carvalho et al. 2004) and provide a likely explanation why in a few of our patients with minimal vascularisation of the transport bone segment, the new bone was very poorly developed (Amir et al. 2006).

In conclusion, we found that the faster bone formation at a slow distraction rate (0.5 mm/day) was accompanied by a higher blood vessel volume than at a distraction rate of 1 mm/day. Our findings support the view that a relationship exists between angiogenesis in soft connective tissue and bone formation. The clinical relevance of this finding is that in vertical distraction osteogenesis in elderly patients, decreasing the standard distraction rate of 1 mm/day down to 0.5 mm/day may be beneficial for bone growth.

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Reference list


