CELAPURE

THE EFFECT OF HUMAN AMNIOTIC FLUID ON MANDIBULAR DISTRACTION OSTEOGENESIS
The effect of human amniotic fluid on mandibular distraction osteogenesis


Abstract. The aim of this study was to evaluate the effects of local administration of human amniotic fluid (HAF) on newly formed bone obtained by mandibular distraction osteogenesis (DO) with histomorphometry. A unilateral mandibular osteotomy at the left corpus was performed in 32 adult male rabbits. After a 5-day latency period, the left mandibles were lengthened by mandibular DO over 5 days, at a rate of 1 mm/day, via a custom-made distractor. After the distraction, the rabbits were divided randomly into four groups: 0.3 ml HAF was injected into the distraction gap followed by 21 (group 1) or 45 (group 2) days of consolidation; or 0.3 ml normal saline (NS) was administered followed by 21 (group 3) or 45 (group 4) days of consolidation. Mandibles were removed at the end of the consolidation period and investigated histomorphometrically. The newly formed bone area (NFBA) and number of fibroblasts increased significantly in the HAF groups compared to the NS groups (NFBA: group 1 vs. group 3, P < 0.05; group 2 vs. group 4, P < 0.01; fibroblasts: group 1 vs. group 3, and group 2 vs. group 4, P < 0.05), and also in both 45-day consolidation groups compared to the 21-day consolidation groups (NFBA: group 1 vs. group 2, and group 3 vs. group 4, P < 0.001; fibroblasts: group 1 vs. group 2, and group 3 vs. group 4, P < 0.01). Additionally, the numbers of osteoblasts and capillaries were increased significantly at 45 days of consolidation compared to 21 days in both the HAF and NS groups (osteoblasts: group 1 vs. group 2, P < 0.01; group 3 vs. group 4, P < 0.05; capillaries: group 1 vs. group 2, and group 3 vs. group 4, P < 0.01). Histomorphometric analysis demonstrated that local HAF administration effectively accelerated bone formation. Thus, a HAF injection procedure could improve new bone formation around the bone in maxillofacial operations such as DO.

Key words: human amniotic fluid; distraction osteogenesis; histomorphometry; bone.

Accepted for publication 3 October 2014
Available online 15 November 2014
Distraction osteogenesis (DO), used widely in the treatment of oral and maxillofacial deformities or deficiencies, is a biological procedure that produces new bone formation via gradually separated bone segments, using an external lengthener.1-3 Traditional surgical techniques for skeletal expansion include osteotomies, acute movements of variable magnitude, and the necessity for bone grafts. Problems include donor site morbidity, unpredictable graft resorption, and the risk of relapse because of soft tissue resistance to large skeletal movements.4 Many of these limitations can be avoided with the use of DO to lengthen or expand the skeleton.3,4 The benefits of DO include the minimally invasive nature of the procedure, the ability to achieve movements of great magnitude without the need for a bone graft, and the elimination of donor site morbidity.4 The main advantage of the technique is that the new bone forms together with elongation of the surrounding soft tissue envelope.1 In addition, concurrent soft tissue histogenesis may decrease the relapse.5,6

Although this technique is used to treat several abnormalities of hard and soft tissues, there are some drawbacks, particularly related to long-term consolidation periods.1 To accelerate the maturation of the regenerated bone, numerous approaches including growth factors,7 calcitonin,8 calcium sulphate,9 bisphosphonates,10 and electronic11 and ultrasonic12 stimulation have been researched.

Several growth-promoting factors have been identified after the manifestation of bone defects including those caused by injuries, fractures, and DO; these include platelet-derived growth factor, transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), interleukin (IL)-1, and IL-6. FGF has angiogenic properties and mitogenic activity on the osteoblast lineage.12 A rich content of growth and trophic factors such as epidermal growth factor (EGF), FGF, and insulin-like growth factors I and II (IGF-I and IGF-II), which are critical for development, have been identified in human amniotic fluid (HAF).12-15 Additionally, hyaluronic acid (HA), hyaluronic acid stimulating activator (HASA), chondroitin-4- and 6-sulphate, dermatan sulphate, and heparan sulphate have been identified in HAF.12,16

HAF, generally obtained by amniocentesis during the second trimester of gestation, contains high molecular weight HA and HASA in high concentrations.14-17 HA has been shown to increase osteoblastic bone formation in vitro, through increased mesenchymal cell differentiation and migration.15,17 Also, HASA has been shown to stimulate and increase the production of endogenous HA. Thus, HAF may increase both endogenous and exogenous HA around the region of application.13

Several studies have investigated the effect of HAF on cell differentiation. HAF has been reported to enhance new cartilage, bone formation, and nerve and tendon healing.12,15,17-19 However, new bone regeneration in the distraction gap after DO of the mandible has not been documented, and there are no data in the literature regarding the acceleration of bone regeneration in the distraction gap with the use of HAF. We hypothesized that HAF would have a positive stimulating effect on bone formation after DO. Hence, the aim of this study was to investigate the effects of HAF, collected between weeks 16 and 24 of gestation, on the acceleration of new bone formation in animal subjects undergoing mandibular DO.

Materials and methods

All procedures were performed in the experimental animal breeding and research centre of the military medical academy. Approval was obtained from the institutional ethics committee and local clinical research ethics committee for the animal research and use of HAF.

Subjects and surgery

The study subjects were 32 adult male New Zealand White rabbits of the same age with an average weight of 2.9 kg (range 2.2–3.5 kg). They had free access to a standard pellet diet and tap water and were adapted to a 12:12-h light–dark cycle in separate cages.

All animals were operated on under general anaesthesia. The rabbits were anaesthetized with a combination of xylazine hydrochloride (5 mg/kg) (Alfazyne 2%; Ege Vet, Izmir, Turkey) and ketamine hydrochloride (50 mg/kg) (Alfamine 10%; Ege Vet, Izmir, Turkey) before the application of distractors. Isoflurane inhalation anaesthetic (10 mg/kg) was used before the various procedures.

After sterilization of all surgical equipment, the left mandible was shaved and disinfected with iodine. After surgical preparation of the experimental side of the mandible, a 2–2.5-cm long submandibular skin incision was made. The subcutaneous tissues were exposed by careful dissection down to the periosteum and the bone was exposed with a periosteal incision. Using a reciprocating saw, a vertical corticotomy line was outlined between the premolars and extended to the inferior mandibular border under saline irrigation. The extraoral bone-borne distraction device used consisted of a 7-mm hyrax expansion screw (Dentalurum GmbH & Co., Ispringen, Germany) with three holes; the retention legs were bent for bone attachment. The distractor device was fixed with two posterior (7 mm) and one anterior (9 mm) titanium screws. The bone cut was completed using a thin osteotome through the vertical corticotomy line, and mobilization of the bone fragments was achieved. Activation of the distraction device was tested. The gap between the bone fragments was narrowed by reverse-directed activation of the distractor. The periosteal flaps were repositioned and closed with 4–0 Vicryl sutures (Fig. 1).

After closure of the mandible, the rabbit was awoken from general anaesthesia and allowed to recover. An Elizabethan collar was placed around the rabbit’s neck to protect the device from dislodgement. A subcutaneous bolus of 30 ml normal saline (NS) was given every 8 h during the first 24–48 h depending on the rabbit’s water intake. After the rabbit had recovered fully as per the facility criteria, it was

Fig. 1. (A) Placement of the distraction device with three parallel fixation pins, which are perpendicular to the lateral surface of the mandible. (B) At the end of the surgery; unilateral placement of the distraction device.
returned to its cage and placed on a soft diet. Prophylactic antibiotics (Coli-
icillin 0.1 ml/kg (100 mg/ml ampicillin-
+ 250,000 IU/ml colistin sulphate); Ege Vet, Izmir, Turkey) were adminis-
tered prior to surgery to prevent any in-
fection that could have resulted from the trauma caused during surgery, and were continued every 12 h for the next 48 h.

**Distraction protocol, administration, and groups**

HAF samples were obtained by diagnostic amniocentesis from normal pregnant women in the second trimester of preg-
nancy, between weeks 16 and 24 of gesta-
tion, under sterile conditions. Samples were then centrifuged at high speeds; the serum was collected and used without being stored. Oral consent was obtained from each woman before HAF collection.

After a latency period of 5 days, dis-
traction was started at a rate of 0.5 mm twice a day for 5 days. The animals were divided randomly into four equal groups consisting of eight rabbits per group. The experimental groups (groups 1 and 2) were treated with a single dose of 0.3 ml HAF, and the control groups (groups 3 and 4) were treated with single dose of 0.3 ml NS solution. The HAF or NS was injected into the distraction gap with a micro-syringe (Hamilton Injection Syringe; Hamilton Company, Reno, NV, USA) at the end of the distraction process. The group pro-
tocols were as follows: group 1, 0.3 ml local HAF administered and sacrifice at day 21 of consolidation; group 2, 0.3 ml local HAF administered and sacrifice at day 45 of consolidation; group 3, 0.3 ml local NS administered and sacrifice at day 21 of consolidation; and group 4, 0.3 ml local NS administered and sacrifice at day 45 of consolidation. After the consolidation,
period, all animals were sacrificed by in-
jection of 200 mg/kg sodium thiopental (Pentothal; Abbott Laboratories, Abbott
Park, IL, USA). The mandibles were dis-
sected subperiosteally and fixed in a 10% formalin solution for histomorphometric evaluation.

In order to investigate the distraction process and the newly formed bone area (NFBA) at the distraction gap, serial cone beam computed tomography (CBCT) scans were obtained for all rabbits at the time of placement of the distractor, at the end of the distraction period, and at the end of the consolidation period (after 21 or 45 days) postoperatively. A 3D Accui-
tomo 170 (J. Morita Manufacturing Corp.,
Kyoto, Japan) was used, with a voxel size of 0.08 mm (field of view (FOV) 170 × 120 mm). The tube settings were set to 65 kV, 2.0 mA, and an exposure time of 30 s. After scanning, the CBCT datasets were transferred to an independent computer workstation with Sim-
Plant-OMS software (Materialise Dental, Leuven, Belgium), and three-di-
 dimensional (3D) virtual models were gen-
erated for macroscopic comparisons be-
 tween groups. The same investigator eval-
uated all CBCT images and generated
3D models at the medical design and
manufacturing centre.

**Histomorphometric evaluation**

The histomorphometric evaluation was carried out in the pathology department. All materials were fixed in 10% buffered paraformaldehyde for 48 h then decalcif-
ied in ethylenediaminetetraacetic acid
(EDTA) solutions. Tissue specimens were prepared, embedded in paraffin, and sec-
tioned with a microtome. The sections
were stained with haematoxylin and eosin.
Stained specimens were investigated un-
der a Nikon Eclipse E400 light microscope
(Nikon, Tokyo, Japan). For each speci-
men, the same area was photographed
after staining using a Nikon Coolpix
5000 camera attachment (Nikon, Tokyo,
Japan). A photograph of the Nikon mi-
crometer microscope slide (MBM11100
Stage Micrometer Type A) was also
obtained during the procedure. All photo-
graphs were then transferred to a PC and
analyzed by Clemex Vision Lite 3.5 image
analysis programme (Clemex Technolo-
gies Inc., Longueuil, Quebec, Canada).

The length was calibrated by comparing
the photograph of the specimen with
the photograph of the Nikon micrometer
microscope slide, which was obtained at the
same magnification. A 0.5-mm² area was
designated using the Clemex Vision Lite
3.5 image analysis programme, and oste-
oblasts, osteoclasts, fibroblasts, and vessels
were marked with the same image analysis
programme in an area of 445,928.3 mm².

Damaged cells were not evaluated. The
marked cells were counted automatically
with the same image analysis programme.
The NFBA regions per unit area were
measured with the same image analysis
programme in an area of 445,928.3 mm².
The reader was blinded to the origin of the
specimen (Fig. 2).

**Statistical analysis**

The sample size for each group was cal-
culated and based on the estimated power of the study (=0.90) according to the effect size (=0.65), with a significance level of α = 0.05 and β = 0.20. The sample size calculations showed eight rabbits in each
group to be sufficient. So, each group
consisted of eight rabbits. All statistical
evaluations were performed with SPSS
version 15.0 for Windows (SPSS Inc.,
Chicago, IL, USA). Results of the evalua-
tion were given as the mean ± standard
deVIation (SD), median, minimum, and
maximum. The Kolmogorov–Smirnov
test was used at baseline to determine whether all the values were normally distributed. The NFBA data had a normal distribution; thus the independent samples $t$-test was used for the comparison of NFBA values between the groups. Differences amongst the four groups with regard to the numbers of osteoblasts, osteoclasts, fibroblasts, and capillaries were evaluated using the Mann–Whitney $U$-test. A value of $P < 0.05$ was considered as statistically significant.

Results

Clinical evaluation

During the study period, no serious weight loss was seen in any of the rabbits. No deep mucosal infection, dehiscence, or other adverse effects were observed in most of the animals. However, due to a faulty distractor device and infection, two rabbits in the NS control group and one rabbit in the HAF group were excluded from the study and substituted with new ones.

Bone lengthening in the range of 4–7 mm (mean 4.2 mm) was achieved in the mandible of all animals. Laterognathia and malocclusion were observed in all subjects due to the unilateral lengthening of the mandible. However, this malformation did not seem to affect the rabbits’ nutrition.

Macroscopic evaluation

A total of 32 distracted hemimandibles were examined. Differences in the gross appearance between the groups were not evident. While all distracted mandibles revealed a fibrous tissue-filled gap and had a similar appearance to the surrounding bone tissue, the same bone tissue formation was observed in the experimental and control group animals (Fig. 3).

The 3D digital models formed by the initial CBCT scans, demonstrated that the distractors were placed successfully (Fig. 4A). At the end of the distraction period, on day 5, gaps were observed in the distraction region of all groups (Fig. 4B). At the end of the each consolidation period (21 and 45 days), callus formation had occurred in the distraction gap in all samples. The digital models revealed evidence of ossification, which was increased substantially in the experimental groups when compared with the control groups after the consolidation periods (Fig. 4C–F).

Histomorphometric findings

Histological findings indicated that bone formation was sparse in all groups and the distraction gaps were filled with intra-membranous new bone and fibrovascular tissue. The results showed that HAF (groups 1 and 2) effectively accelerated the new bone formation when compared with the NS control groups (Fig. 5). All parameters were compared by group and consolidation period, separately (Tables 1 and 2).

Newly formed bone area (NFBA)

In the comparison of NFBA between the two 21 days of consolidation groups (groups 1 and 3), the increase was significant in group 1 (HAF) ($P = 0.044$). In the comparison between the two 45 days of consolidation groups (groups 2 and 4), the increase was significant in group 2 (HAF) ($P = 0.001$). The results indicated that the increase in new bone formation in the HAF groups was significant when compared to the NS groups (Table 1).

NFBA was significantly greater at 45 days of consolidation in both the HAF and NS groups (groups 2 and 4) when compared to NFBA at 21 days of consolidation (groups 1 and 3) ($P < 0.001$) (Table 2).

Number of osteoblasts

The HAF and NS groups were compared for the number of osteoblasts; there were increases in the HAF groups both at 21 and 45 days of consolidation (groups 1 and 2) when compared to the NS groups (groups 3 and 4) respectively; however the increases were not statistically significant ($P > 0.05$) (Table 1). Histological findings revealed that the number of osteoblasts was significantly higher at day 45 of consolidation in comparison with day 21 in both the HAF and NS groups ($P = 0.002$ and $P = 0.03$, respectively) (Table 2).

Number of osteoclasts

Comparison of the HAF and NS control groups at both 21 and 45 days of consolidation showed no statistically significant differences for the number of osteoclasts ($P > 0.05$) (Table 1). Furthermore, the number of osteoclasts did not change with the duration of consolidation in either group ($P > 0.05$) (Table 2).
Number of fibroblasts

When the HAF and NS groups were compared for the number of fibroblasts, a statistically significant increase was found in the HAF groups at both 21 and 45 days of consolidation versus the NS groups (<0.05) (Table 1). Also, the number of fibroblasts increased significantly with time in both the HAF and NS groups; the number of fibroblasts was significantly higher in groups 2 and 4 than in groups 1 and 3, respectively (P = 0.007 and P = 0.009, respectively) (Table 2).

Number of capillaries

No statistically significant differences were found in the number of capillaries between the HAF and NS groups (P > 0.05) (Table 1). However, the number of capillaries increased significantly with time in both groups; the number of capillaries was significantly higher in groups 2 and 4 than in groups 1 and 3, respectively (P = 0.001 and P = 0.009, respectively) (Table 2).

Discussion

A great deal of attention has recently been directed towards mandibular DO in the light of expanding demand for minimally invasive orofacial reconstructive techniques. However, an adequate understanding of the biological events during DO is necessary to effectively modulate and manipulate the bony regeneration. The clinical goals of DO research include improving the speed of new bone formation, a shorter fixation time, enhanced bone quality, and a minimization of the risk of non-union of the osteotomized edges. Many investigators have attempted to define the optimal parameters of mandibular DO using animal models. Dogs, sheep, pigs, rabbits, and rats have been used with varying degrees of success and failure as a model for mandibular DO. The rabbit model is a well-established model in DO of the mandible.

According to a review by Swennen et al., 12.9% of the total mandibular DO model studies were performed on rabbits. Furthermore, the rabbit has been found to be an appropriate model for the study of human bone physiology, because

Table 1. Comparison of the newly formed bone area (NFBA) and the numbers of osteoblasts, osteoclasts, fibroblasts, and capillaries between the experimental (HAF) and control (NS) groups.

<table>
<thead>
<tr>
<th>Consolidation period</th>
<th>Group</th>
<th>NFBA</th>
<th>Osteoblasts</th>
<th>Osteoclasts</th>
<th>Fibroblasts</th>
<th>Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 days</td>
<td>HAF (group 1)</td>
<td>Mean 143,924.23 SD 22,625.78</td>
<td>37.43</td>
<td>2.86</td>
<td>32.29</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median 148,251.60 Minimum 100,654.60</td>
<td>39</td>
<td>1</td>
<td>3.68</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximum 165,211.40</td>
<td>43</td>
<td>3</td>
<td>4.05</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>NS (group 3)</td>
<td>Mean 118,885.38 SD 19,535.81</td>
<td>32.60</td>
<td>2.00</td>
<td>27.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median 111,457.60 Minimum 98,755.20</td>
<td>34</td>
<td>1</td>
<td>2.88</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximum 149,748.10</td>
<td>36</td>
<td>3</td>
<td>4.08</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>P 0.044 SD 0.0149</td>
<td>0.149</td>
<td>0.755</td>
<td>0.048</td>
<td>0.106</td>
</tr>
<tr>
<td>45 days</td>
<td>HAF (group 2)</td>
<td>Mean 201,511.72 SD 10,691.14</td>
<td>49.71</td>
<td>1.86</td>
<td>40.29</td>
<td>7.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median 199,543.20 Minimum 184,589.20</td>
<td>49</td>
<td>2</td>
<td>4.42</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximum 214,873.90</td>
<td>56</td>
<td>2</td>
<td>4.03</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>NS (group 4)</td>
<td>Mean 159,124.40 SD 21,922.03</td>
<td>45.83</td>
<td>1.67</td>
<td>34.67</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median 154,457.45 Minimum 134,582.30</td>
<td>46</td>
<td>2</td>
<td>3.46</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximum 198,657.60</td>
<td>54</td>
<td>2</td>
<td>4.03</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>P 0.001 SD 0.366</td>
<td>0.628</td>
<td>0.035</td>
<td>0.138</td>
<td></td>
</tr>
</tbody>
</table>

HAF, human amniotic fluid; NS, normal saline; SD, standard deviation.
rabbits show patterns of bone accretion and peak bone mass profiles similar to those of humans. Similarily, the quest for optimization of HAF clinical parameters has led to the development of a variety of animal models. Many have used the rabbit as the animal model to investigate the effects of HAF. To define the biological sequence of events that occur during the distraction process with HAF and to compare them with those that occur during physiological bone wound healing, the rabbit mandible sample was preferred in the current study.

Iliizarov documented that the ideal rate of lengthening is 1 mm/day, with faster rates resulting in fibrous or non-union. The consolidation period for DO is at least twice the distraction distance (mm) in days. In the present study, distraction was started at a rate of 0.5 mm twice a day for 5 days, and consolidation periods of 21 and 45 days were selected for comparison.

The rabbits own amniotic fluid was not used in this experimental study, because similar amounts of amniotic fluid (0.3 ml for each rabbit) would be difficult to obtain from pregnant rabbits. It was easy to collect sufficient HAF for the study, because amniocentesis is a common procedure performed for prenatal diagnosis at 16–24 weeks of gestation in many obstetrics and gynaecology departments. Furthermore, the variations in concentration of HA, HASA, and other factors, like growth factor and the extracellular matrix precursors, in the HAF of women during pregnancy are already known based on data from previous studies. The composition of the amniotic fluid includes many growth factors (FGF, EGF, IGF-I, and IGF-II), mucopolysaccharides (HA, HASA, chondroitin-4- and -6-sulphate, dermatan sulphate, and heparan sulphate), and extracellular macromolecules (fibronecrtin and laminin). In experimental studies by Ozgenel et al. it was reported that a preoperative injection of HAF underneath the free perichondrial grafts promoted the proliferation and differentiation of chondrocytes. Similarly, Kavakli et al. showed that the administration of HAF into the perichondrial bed increased chondrogenesis in adult rabbits. All of the authors mentioned are in agreement that HAF, which is a polysaccharide, has a positive effect on cell differentiation and migration, and the invasion of various cell types. The presence of HA provides a mesenchymal signal for healing in bone, cartilage, nerve, and tendon. It has also been reported that HA and HASA are associated with an acceleration of new bone formation.

In the present study, we hypothesized that HAF, which includes growth factors like HA and HASA, would have a positive stimulating effect on bone healing after DO. As affirmed previously, the mean area of newly formed bone (NFBA) and the number of fibroblasts at the distraction gap in groups 1 and 2, which were treated with HAF, were significantly higher when compared with the respective control groups (groups 3 and 4). It is assumed that the rich HA and growth factor content possibly contributed to this result. Our results are in accordance with those of the studies mentioned above, which demonstrated a positive stimulating effect of HAF on bone or cartilage healing.

HAF contains high concentrations of high molecular weight HA during the second trimester of gestation. The presence of HA assists the healing process through regeneration and growth rather than scarring and fibrosis. In a recent study, it was reported that HA at a concentration of 19 mg/ml and a molecular weight of 6 x 10⁵ significantly limited the formation of adhesion. The concentration of HA in HAF decreases during gestation (approximately 20 mg/l between 16 and 20 weeks and 1 mg/l at week 30).

<table>
<thead>
<tr>
<th>Group</th>
<th>Consolidation period</th>
<th>NFBA</th>
<th>Osteoblasts</th>
<th>Osteoclasts</th>
<th>Fibroblasts</th>
<th>Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAF</td>
<td>21 (group 1)</td>
<td>Mean 143,924.23</td>
<td>37.43</td>
<td>2.86</td>
<td>32.29</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>22,625.78</td>
<td>5.74</td>
<td>0.48</td>
<td>3.68</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>148,251.60</td>
<td>39</td>
<td>2</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>100,654.60</td>
<td>28</td>
<td>1</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>165,211.40</td>
<td>43</td>
<td>3</td>
<td>38</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>201,511.72</td>
<td>49.71</td>
<td>1.86</td>
<td>40.29</td>
<td>7.00</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>109,691.14</td>
<td>4.19</td>
<td>0.38</td>
<td>4.42</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>199,543.20</td>
<td>49</td>
<td>2</td>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>184,589.20</td>
<td>44</td>
<td>1</td>
<td>36</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>214,873.90</td>
<td>56</td>
<td>2</td>
<td>48</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Sig.</td>
<td>P</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.799</td>
<td>0.007</td>
<td>0.001</td>
</tr>
<tr>
<td>NS</td>
<td>21 (group 3)</td>
<td>Mean 118,885.38</td>
<td>32.60</td>
<td>2.00</td>
<td>27.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>19,535.81</td>
<td>3.85</td>
<td>0.71</td>
<td>2.88</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>111,457.60</td>
<td>34</td>
<td>2</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>98,755.20</td>
<td>26</td>
<td>1</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>149,748.10</td>
<td>36</td>
<td>3</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>159,124.40</td>
<td>45.83</td>
<td>1.67</td>
<td>34.67</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>21,922.03</td>
<td>6.97</td>
<td>0.52</td>
<td>4.03</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>154,457.45</td>
<td>46</td>
<td>2</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>134,582.30</td>
<td>33</td>
<td>1</td>
<td>29</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>198,657.60</td>
<td>54</td>
<td>2</td>
<td>41</td>
<td>7</td>
</tr>
<tr>
<td>Sig.</td>
<td>P</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.537</td>
<td>0.009</td>
<td>0.009</td>
</tr>
</tbody>
</table>

HAF, human amniotic fluid; NS, normal saline; SD, standard deviation.
In addition to HA, HAF also contains HASA, which stimulates the wound to increase production of endogenous HA. Therefore, HAF may increase both endogenous and exogenous HA at the region of application. The presence of both HA and HASA in HAF would explain the increased NFBA and fibroblasts in groups 1 and 2. Additionally, the growth factors in HAF may also explain the observed acceleration in bone formation area and fibroblasts in the HAF groups. Moreover, the current study revealed that the consolidation period plays an important role in the new bone formation after DO. In this study, a consolidation period of 45 days (groups 2 and 4) resulted in significantly higher new bone formation when compared to a consolidation period of 21 days (groups 1 and 3); increases in all parameters related to new bone formation (NFBA and numbers of osteoblasts, fibroblasts, and capillaries), except the number of osteoclasts, were statistically significant in both groups at 45 days of consolidation. However, the differences were more evident in the HAF groups according to the consolidation period. Since single-dose injection of 0.1 ml or 0.2 ml or 0.3 ml HAF has been demonstrated to be sufficient to exhibit effectiveness in many previous studies, the maximum dose (0.3 ml) of HAF was selected in order to be sure that an effective quantity of the causative substances was present and to fill the under-surface of the distraction gap adequately in the present study. However, the fluid may quickly flow away and the effectiveness of the substance may be diluted due to the lack of any obstruction around the distraction area. This is a criticism applicable to all topical fluid applications to the distraction gap. However, applying the solution repeatedly to the surgical area would greatly increase the risk of infection.

In conclusion, this experimental study suggests that the local injection of HAF in the distraction gap after DO increases new bone formation and the number of fibroblasts significantly. Prior to human application, further animal investigations using different amounts of HAF preparations, with varied consolidation periods, are necessary to determine the ideal dose of HAF for bone regeneration acceleration and to examine the possible side effects. Additionally, studies are also needed to assess the roles of the various factors in HAF in modulating the process of new bone formation after DO.

Funding
None.

Competing interests
None declared.

Ethical approval
Approval for the use of animals was obtained from the Ethics Committee of Gulhane Military Medical Academy in Ankara, Turkey (#2010-26). Approval for the use of HAF was obtained from Kecioren-Ankara Nr 1 of the Clinical Research Ethics Committee (2010/01-204).

Patient consent
Not required.

References
22. Norris SA, Pettifor JM, Gray DA, Buffenstein R. Calcium metabolism and bone mass in female rabbits during skeletal maturation.
The content provided in this white paper is intended solely for general information purposes, and is provided with the understanding that the authors and publishers are not herein engaged in rendering medical, clinical or other professional advice or services. The information in this report is intended to help health care decision makers—patients and clinicians, health system leaders, and policymakers, among others—make well-informed decisions and thereby improve the quality of health care services. This report is not intended to be a substitute for the application of clinical judgment. Consequently, any use of this information should be done only in consultation with a qualified and licensed professional who can take into account all relevant factors and desired outcomes. The information in the following white paper was written, prepared and distributed with reasonable care and attention. However, it is possible that some information in the following white paper is incomplete, incorrect, or inapplicable to particular circumstances or conditions. We do not accept liability for direct or indirect losses resulting from using, relying or acting upon information in the following white paper.