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Bone regeneration in mandibular fractures after the application of autologous mesenchymal stem cells, a randomized clinical trial

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Abstract – *Background/Aim*: Facial injury in adults can commonly result in fractures of the mandible. Autologous mesenchymal stem cells (AMSCs) transplantation is proposed as an alternative to conventional graft treatment to improve bone regeneration. The aim was to evaluate the effectiveness of AMSCs application in mandibular fractures to reduce regeneration time and increase bone quality. *Materials and methods*: This study was a single-blind controlled clinical trial conducted in patients with mandibular angle fractures. Patients were divided into two groups: study group fracture reduction plus application of AMSCs and control group only fracture reduction. AMSCs were obtained from adipose tissue 24 h before the procedure. Intensity and density were evaluated in normal bone and fractured bone at 4 and 12 weeks after surgery using panoramic radiography and computed tomography. *Results*: A total of 20 patients, 10 in each group, were included. The study group had a mean age of 31.2 ± 6.3 years, and the control group mean age was 29.7 ± 7.2 years. All patients were male. Bone quality measured in grey levels at week 4 was 108.82 ± 3.4 vs 93.92 ± 2.6 ($P = 0.000$) using panoramic radiography and 123 ± 4.53 vs 99.72 ± 5.72 ($P = 0.000$) using computed tomography. At week 12, the measurements were 153.53 ± 1.83 vs 101.81 ± 4.83 ($P = 0.000$) using panoramic radiography and 165.4 ± 4.2 vs 112.9 ± 2.0 ($P = 0.000$) using tomography in the study and control groups, respectively. *Conclusion*: Similar ossification values were obtained after 4 weeks when the use of AMSCs was compared to simple fracture reduction. However, after 12 weeks, the AMSCs group had a 36.48% higher ossification rate.

Facial injury in adults can commonly result in fractures of the mandible, and they often occur in the third decade of life (1). The most frequent localization is in weak regions where the bone structure has lower resistance, such as the mandibular condyle. In adults, one of the most frequent fractures is the condyle fracture, which is often associated with contralateral symphyseal fractures, and underlying lesions are generally found. The compromise of soft tissue, nerve and vascular structures is commonly presented (2).

Imaging studies are used to confirm the diagnosis. Panoramic radiography offers a first general vision. The mandibular series include an anteroposterior projection, a Towne's projection and a right/left oblique projection (3, 4). Computed tomography (CT) is essential in the horizontal visualization of the mandible and has become the most used method for diagnosis (5). Also, diagnosis should be supported by clinical analysis, characterized mostly by pain, joint functional impotence, mandible deformity (malocclusion), crepitation,

abnormal movement, swelling, facial asymmetry, tear of the mucosa, sublingual haematoma and paresthesia, dysesthesia or anaesthesia of the lips caused by injury of the inferior alveolar nerve (1).

When these fractures are not treated appropriately, the soft tissue rapidly adopts the shape of the underlying bones which are displaced. This hinders the restoration of the original form of the face once the acute phase has ended. Thus, it is fundamental to accomplish early alignment of the facial skeleton (6, 7).

Only those fractures, which are not displaced, stable or incomplete, and with no objective changes in occlusion, can be treated conservatively with soft diet, absolute joint rest and expectant attitude. Normally functional and conservative treatments are preferred. The objective of conservative treatment is to allow functionality without complete reduction caused by early mobilization, while the aim of surgical treatment is to correct the position in the best way on a case-to-case basis (2).

Previously, for the management of non-condyle mandibular fractures, intermaxillary fixation was performed although internal fixation is usually needed in displaced fractures. Nowadays, internal fixation is performed using miniplates and mono- or bi-cortical screws. If surgical treatment is the procedure of choice, the mandible is only fixated during the trans-operative period.

The popularity of resorbable materials for mandible fixation (such as polyglycolic acid, polylactic acid and polidoxanona) has increased, but they are not used frequently for mandibular fractures. Their capacity for biological degradation avoids the need for further surgery to remove the plates. However, an inflammatory response to the foreign body has been described when using polylactic and polyglycolic acid implants in patients with diabetes (1). Saman et al. (8) reported that mandibular fixation in all trauma patients after open reduction and internal fixation (ORIF) of the mandible is not required and may not be an advantage in the treatment of patients with non-comminuted symphyseal, parasymphyseal or mandibular angle fractures.

Nowadays, treatment with stem cells is experimental. The multipotentiality of stem cells obtained from adipose tissue has been demonstrated. These cells have the capacity to differentiate into chondrocytes and osteogenic cells, and they can be placed in bone defects (9–13). Autologous mesenchymal stem cells (AMSCs) transplantation is proposed as an alternative to conventional graft treatment (14). The expansion of these cells can be performed *in vitro* using various osseous morphogenetic proteins (15–17). Multiple studies have demonstrated the AMSCs capacity to improve bone regeneration in large bone defects in animal models (18,19).

The aim of this clinical trial was to evaluate the effect of AMSC application on bone quality after surgery to repair mandible fractures.

Materials and methods

The study was conducted according to the principles of the Helsinki Declaration of 1989 with all its amendments, the guidelines of the General Health Act of Mexico and under the regulations of the Mexican Institute of Social Security for Health Research. The local ethics and health research committee from the High Specialty Medical Unit of the Specialties Hospital of the Western National Medical Center – Mexican Institute of Social Security – approved the research protocol with registration 2010-1301-27. Full, written informed consent was obtained for all individual participants included in the study. ClinicalTrials.gov (ID: NCT02755922; last verified: April 2016).

This single-blind randomized clinical trial was carried out in the Maxillofacial Surgery Service, in the Specialties Hospital of the Western National Medical Center, Guadalajara (Jalisco, Mexico), during the period between 3 April 2015 and 30 September 30 2015. The inclusion criteria were as follows: patients with mandibular condyle fractures associated or not with other initial fractures that required ORIF, aged 17–59 years, female or male gender and patients who gave their informed consent. The exclusion criteria were as follows: patients older than 59 years and younger than 17 years; chronic degenerative diseases, active smoking, collagen disorders, patients with signs of infection in the area to be treated and in whom the fracture occurred in more than 10 days before the surgery.

This was a pilot study in which a sample size of 10 patients for each group was calculated. They were divided into two groups: study group (SG) which included patients with the application of AMSCs and control group (CG) only fracture reduction. The allocation method used was a sealed envelope chosen by a person outside the trial and with no previous knowledge about the patients.

The AMSCs were obtained from adipose tissue (50 cc) 24 h before the surgery. The adipose grafts were sent to a cellular biotechnology laboratory where procedures to obtain the stem cells were performed (Fig. 1). The procedure to harvest AMSCs was the following: 50 cc of adipose tissue were washed with saline solution. Collagenase 0.1% was applied after it was dissolved and filtrated. Then, the sample was incubated at 37°C in constant motion. Once adipose tissue was digested, it was centrifuged to a Relative Centrifugal Force of 2.400 RCF during 10 min. Fifteen millilitre of Dulbecco's Modified Eagle Medium (DMEM) low glucose, previously supplemented with foetal bovine serum (FBS) 10%, and a solution of antibiotics were added. The medium was harvested in a carbon dioxide 5% incubator. AMSCs (representing about 37% of the culture) adhere to the walls (usually at the bottom) of the bottle of culture, hence facilitating their separation and extraction from the rest of the culture. Approximately 400 000 cells of the non-adipose tissue were obtained. Quantification and viability were evaluated using flow cytometry. The processing of 50 cc of adipose tissue can result in approximately 1×10^7 to 6×10^8 cells with more than 90% viability (20).

The primary outcome was to measure bone regeneration which was defined as the presence of bone calcification at both ends of the fracture and on its contour. This was measured by analysing bone intensity and density on a panoramic radiograph and a CT scan.

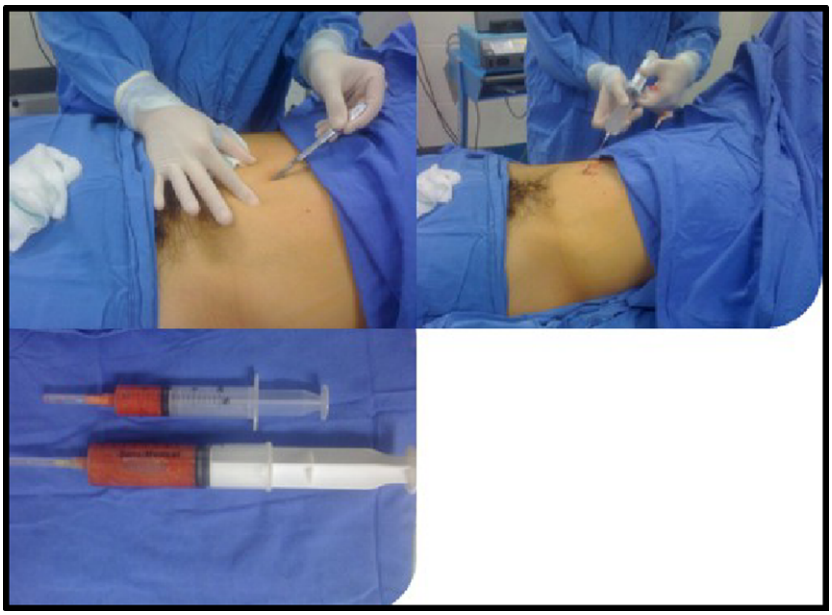

Fig. 1. Extraction of 50 cc of adipose tissue from the abdominal region, later sent to a cellular biotechnology laboratory to obtain stem cells.

The secondary outcome was to observe the development of complications after surgery as well as related events that could delay healing time. These included the following: development of infection (defined as an increase in local temperature, tenderness, leukocyte count >10 000 cells/μL, confirmed by Gram stain and blood culture) or bleeding after surgery (defined as inability to stop the haemorrhage using dressings), or surgical wound dehiscence (defined as subcutaneous tissue, bone or osteosynthesis material exposed through the skin).

Under general anaesthesia, intermaxillary fixation was performed with steel wire using Ivy cerclage on both sides of the mandible in the space between the molar and premolar teeth. A subangular Risdon-type incision was performed on the affected side, 2 cm below the mandibular angle, with previous application of local anaesthesia using xylocaine (2%) with epinephrine. The surgeon then proceeded to do a periostomy of the mandibular angle to reach the site of fracture, leaving enough space for placement of theminiplates. AMSCs were applied on both ends of the fracture in the SG, and later open reduction was carried out to obtain adequate occlusion. A compression titanium plate (DePuy Synthes Companies of Johnson & Johnson, West Chester, PA, USA) was placed on the inferior mandible border and another one on the superior alveolar region which was used as a tension plate.

Prophylactic antibiotics were used for all patients (cefotaxime 1 g, IV every 8 h), and they were hospitalized for 24 h for observation. All procedures were performed by the same surgeon with the same equipment and surgical material (Fig. 2).

Eight imaging studies were taken per patient, four panoramic radiographs and four CT scans, grouped in four pairs. Within each pair of images, the first pair corresponded to a healthy portion of bone (in the case of unilateral fractures, the image taken corresponded to the contralateral angle and in the case of bilateral fractures, the image taken corresponded to a non-fractured part of the mandible). The second pair of images corresponded to the site of fracture. The third pair corresponded to the repaired fracture (once the ORIF was done) 4 weeks after and the fourth pair corresponded to the repaired fracture at week 12 of the postoperative period. A number from 1 to 20 was randomly assigned to every patient, and the packages containing the four pairs of images were sent to an evaluator who had no knowledge of which images corresponded to each patient, the fracture or the time of evaluation. Data analysis was performed using Image Processing and Analysis IMAGE J VERSION 1.43 software (April 2010; http://imagej.net/Welcome). The panoramic radiography units were expressed in Voxels, and the CT units were expressed as Hounsfield Units.

A manual measure of 10 different points was made within the area of interest previously marked. Every set of points was divided into 10 more points to have 100 points per patient. At every 10 points, a value was obtained, giving a total of 10 values. Because the images were presented in pixels (bidimensional value), a conversion to Voxels was carried out (tridimensional value), obtaining three values per point (Fig. 3). The values obtained were interpreted as gray levels. This procedure was performed for every patient and for images from both panoramic radiography and CT scans.

The bone intensity and density were compared between the SG and CG, and the patient's recovery time was defined as the time lapsed from the surgery to their resumption of normal daily activities.

Statistical analysis by SPSS statistical software (version 20 for Windows; IBM Corp., Armonk, NY, USA) was performed using raw numbers, proportions, means and mean standard error. For the independent samples, if normal distribution results were obtained, the Student's *t*-test was applied and if they were abnormal, the Mann–Whitney *U*-test was used. All $P < 0.05$ were considered significant.

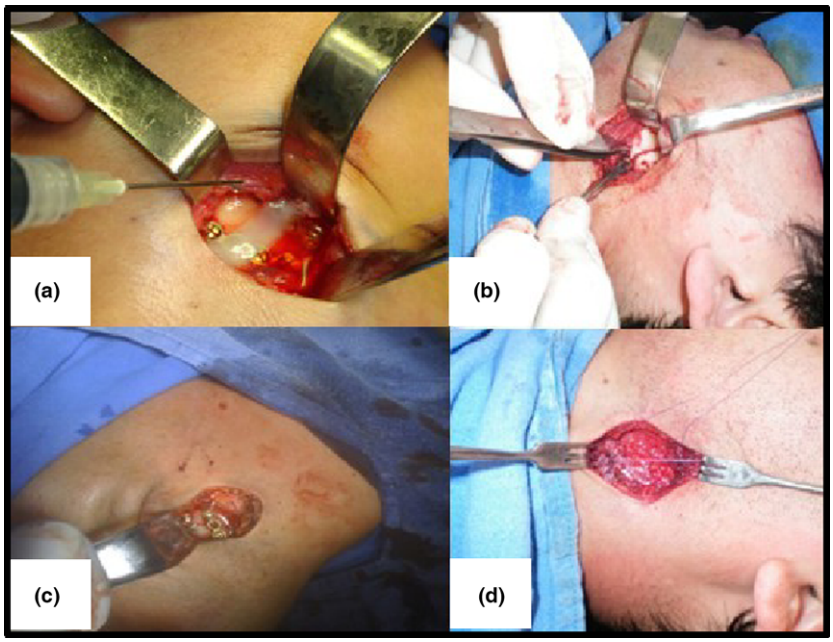

Fig. 2. (a) Application of autologous mesenchymal stem cells (AMSCs) on the fracture line according to the randomized selection of the patients. (b–d) open reduction and internal fixation (ORIF) of the mandible fractures.

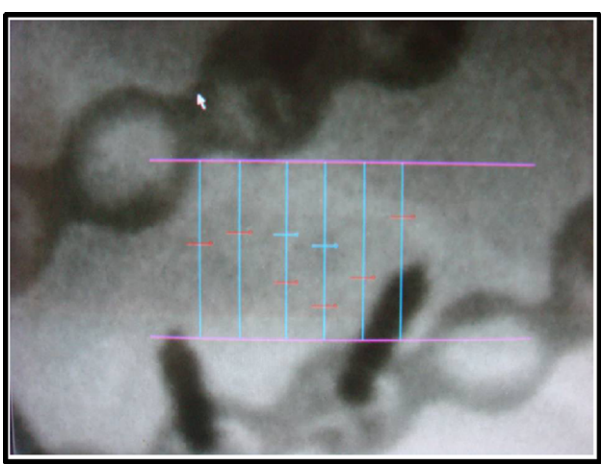

Fig. 3. Bone density analysis, using Software Image Processing and Analysis IMAGE J (Version 1, 43, April 2010).

Table 1. Patient's general characteristics, injury localization and associated fractures

	Study group	Control group	*P* value
Number of patients	10	10	
Age (mean ± SD)	31.2 ± 6.2	29.7 ± 7.2	0.9
Fracture localization			
Right mandible angle	2	3	
Left mandible angle	6	6	1.0
Associated fractures			
Op. mandible angle	2	1	
Zygomatic complex	1	2	
Orbit floor	1	2	
Nasal bones	1	0	
Opposite condyle	1	0	
Mandible symphysis	0	1	
Zygomatic arch	1	0	
Total	6	7	0.91

Op, opposite; SD, standard deviation.

Results

Twenty patients were included and they were divided into two groups, 10 patients in each. All patients were male. The mean age in the SG was 31.2 ± 6.2 years and in the CG was 29.7 ± 7.2 years ($P = 0.9$).

The cause of injury was physical aggression in 16 patients, car accident in two patients and injury by fall in two patients. Five right mandible condyle fractures were found (two in SG and three in CG). Twelve fractures were left-sided (six in each group). An isolated mandible condyle fracture was found only in seven patients, three patients in SG and four in CG. The remaining 13 patients had associated facial fractures (seven in SG and six in CG) (Table 1).

In the CG, panoramic radiography analysis showed 30.29% lower gray levels than normal bone. At week 4 and week 12 after surgery, the respective gray levels were lower by 22.48% and 15.97% compared to normal bone. The results of the CT analysis also showed 31.60%, 17.88% and 7.73% lower gray levels in the pre-operative period, at weeks 4 and 12, respectively.

In the SG, panoramic radiography analysis showed 30.81% lower gray levels than normal bone. At week 4, there were 12.61% fewer gray levels while at week 12, the gray levels were 24.42% higher compared to normal bone. The values obtained from the CT images were also positive compared to normal bone.

Comparing the differences between groups, the SG at week 4 presented an average of 14.904 gray levels more than the CG, representing a 13.69% higher intensity using panoramic radiography. At week 4, in CT images, the SG presented an average of 23.23 gray levels more than the CG, which represents a 18.93% higher value. At week 12, the difference was 2.4 times higher using panoramic radiography, with 51.72 gray

levels more than the CG, which represented a 33.68% higher value. Using CT at week 12, the SG had 53.38 gray levels more than the CG which represented a 32.36% higher value.

In the CG, the bone quality did not reach similarities with normal bone either at week 4 or week 12. On the other hand, the bone quality was similar at week 4 using the CT images and higher at week 12 using both panoramic radiography and CT images. The data are presented in Table 2.

The SG patients had higher gray levels using CT images at week 4 than normal bone (123.0 ± 4.5 vs 121.2 ± 8.5). At week 12, the levels obtained using CT images (165.4 ± 4.2) were 36.48% higher compared to normal bone. This was clinically manifested with less pain and reaching an earlier return to their daily activities.

No harm or unintended effects occurred to the patients. The only complication that developed was a local infection in one patient in the SG, which was treated with a 1-week course of antibiotics, with no implications on the imaging results.

Discussion

Tissue engineering and cell therapy using AMSCs have raised the possibility of implanting living tissue for bone reconstruction. The present study supports the feasibility for the use of AMSCs in the treatment of bone defects, especially in mandibular fractures.

The use of biomaterials for the treatment of bone defects has been widely accepted, although often finding the biomaterials with the right properties is not a simple process. Biomaterials should ideally possess properties such as mechanical strength, biodegradability, support and stem cell differentiation with regard to mimicking bone-forming components to elicit specific cellular responses to provide an ideal environment for bone formation. To date, no synthetic or biological scaffolds fulfil all these criteria as they can be influenced by the surrounding microenvironments or produce immunological reactions that may culminate in rejection (21). The present study supports the use of AMSCs as a viable, non-synthetic material, with non-immunological response as an alternative for the treatment of bone defects.

Table 2. Results of radiographic and tomographic evaluation

	Study Group $n = 10$	Control Group $n = 10$	*P* value
Panoramic radiography[1]			
Normal bone	123.4 ± 4.1	121.3 ± 3.2	0.19
Site of fracture	85.4 ± 2.2	84.5 ± 2.0	0.35
Week 4	108.8 ± 3.4	93.9 ± 2.6	0.000
Week 12	153.5 ± 1.8	101.8 ± 4.8	0.000
CT[2]			
Normal bone	121.2 ± 8.5	121.4 ± 7.7	0.95
Site of fracture	84.2 ± 1.0	83.0 ± 3.8	0.42
Week 4	123.0 ± 4.5	99.7 ± 5.7	0.000
Week 12	165.4 ± 4.2	112.9 ± 2.0	0.000

CT, computed tomography.
[1]Voxels.
[2]Hounsfield units.

One of the most important outcomes of the present study was the ability to attain AMSCs using a minimally invasive procedure from adipose tissue which demonstrated a great level of efficiency and effectiveness, achieving an ossification rate 2.4 times higher than the conventional treatment for mandibular fractures. Adipose tissue-derived stem cells demonstrate several advantages over those obtained from bone marrow (which are still considered the golden standard even if the procedure to obtain them may require a second surgical procedure with associated pain and potential complications). In summary, advantages of adipose tissue-derived AMSCs include a less invasive harvesting procedure, a higher number of stem cell progenitors from an equivalent amount of tissue harvested, increased proliferation and differentiation capacities, and better angiogenic and osteogenic properties *in vivo* (21). These advantages of adipose tissue-derived AMSCs are also of relevance, considering the various approaches proposed with other cell sources; for instance, Stanovici et al. (22) suggested that cell therapies based on bone marrow or *ex vivo* expanded mesenchymal stromal stem cells may serve as an alternative to autologous bone grafting.

Padha et al. (23) evaluated the percutaneous application of bone marrow, in 50 post-trauma cases with failure or late bone union, considered as failure in bone regeneration after a minimum of 3 months. They found that 46 cases had successful bone union, while four had failed. The Padha et al. (23) study was conducted in patients with fractures with consolidation failure and not in patients who had fractures that had a normal healing process. In contrast, the present study excluded those patients who had fractures of more than 10 days of evolution. It should be noted that the ideal time of application of AMSCs to get their maximum beneficial effect has yet to be defined. The present study suggests that their maximum beneficial effect is obtained with their immediate application rather than waiting until there is consolidation failure.

Different opinions have been proposed about the method of application of AMSCs. Hernigou et al. (24) evaluated percutaneous autologous bone marrow (which contains AMSCs) grafting in 60 patients with non-infected atrophic non-unions of the tibia, in which 53 patients achieved bone union with an 88.3% fusion rate. On the other hand, a review by Qin et al. (25) found that application of AMSCs directly to a biomaterial scaffold was effective in the treatment of large bone defects. A recent study by Tawonsawatruk et al. (26) tested human mesenchymal stem cells obtained from bone marrow and adipose tissue in a rat model. At 8 weeks, 80% of the animals in the cell treatment group showed evidence of bone healing compared to only 14% of those in the control group. Bone healing was confirmed by radiographic parameters and by histopathological analysis. Tawonsawatruk's results demonstrated that application without scaffold could be effective. Although many studies suggest that the application should be performed using scaffolds, this is

a time- and resource-consuming procedure that may also be associated with comorbidities. As a standardized method of application has not yet been established (even when a scaffold seems justified), direct application was chosen for the present study.

A study by Quarto et al. (27) described the use of bone marrow stromal cells to treat three patients with large bone defects. In all three patients, radiographs and CT scans revealed abundant callous formation along the implants and good integration at the interfaces with the host bones by the second month after surgery. Clough et al. (28) reported that a composite prepared with osteogenically enhanced MSCs and their extracellular matrix had an unprecedented capacity for the repair of critical bone defects of murine femora, and that the attachment to the extracellular matrix by these AMSCs stimulated the production of osteogenic and angiogenic factors. These and other results have supported the implantation of AMSCs in the treatment of large bone defects in humans. In the present study, although large bone defects were not treated, similar results were obtained as those reported by the Quarto and Clough studies. For the evaluation of bone regeneration, a non-invasive method based on imaging studies (CT scan and radiography), using a punctuation scale in grey levels to evaluate the ossification rate was chosen. At week 4, the SG presented an average of 14.904 gray levels more than the CG, representing a 13.69% higher intensity using panoramic radiography while in the CT images, the SG presented an average of 23.23 gray levels more than the CG, which altogether represents a 18.93% higher value. Meanwhile, the most compelling evidence was found at week 12 using CT, where the SG had 53.38 gray levels more than the CG which represented a 32.36% higher value.

Bone regeneration is frequently delayed in patients with active smoking or alcoholism, and there is an involution of bone marrow in these patients. Therefore, there is a reduction in the number of progenitor cells (29). This can be a disadvantage for the application of AMSCs in some patients and is an important factor in the diminishment of beneficial results obtained from an autologous transplant. Due to the information available from previous reports, patients with chronic degenerative diseases, active smoking, collagen disorders or patients with signs of infection were excluded from this study.

Park et al. (30) reported one case of a patient with mandibular reconstruction with autologous human bone marrow mesenchymal stem cells and autogenous bone graft. The patient recovered masticatory function and did not require microanastomosis to provide blood supply to the grafted bone. As in the present study, this case showed favourable results with AMSCs but future studies need to be conducted in larger populations to confirm the benefits of this treatment option.

Conclusion

The present results support the effectiveness of AMSCs application in the treatment of mandibular fractures to improve bone regeneration. CT images at week 12 following AMSCs application showed a 36.48% higher ossification rate. This was also clinically manifested with less pain and reaching an earlier return of the patients to their daily activities. An advantage of taking AMSCs directly from the patient's adipose tissue is the minimally invasive nature of the procedure associated with more than 90% viability of the cells that translates into an improvement and acceleration of bone healing and a faster recovery time.

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Conflict of interest

The authors confirm that they have no conflict of interest.

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