The Effect of Bone Marrow Mesenchymal Stem Cells Application on Distracted Bone Quality during Rapid Rate of Distraction Osteogenesis

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Abstract

Distraction osteogenesis (DO) bone regenerate usually suffers from an inferior quality especially with rapid rate. This study was conducted to investigate the effect of mesenchymal stem cells (MSCs) application on different rates of distraction bone quality. Twenty-four goats were divided into group A with standard DO and group B with rapid distraction osteogenesis (RDO) both aided by MSCs. Group C with standard DO and group (D) with RDO were controls. Kruskal–Wallis test and Conover's post hoc analysis was used to evaluate significance ($p = 0.05$). Histomorphometry showed a strongly significant (SS) increase ($p = 0.00036$) in trabecular bone (TB) in group A (TB = 174.7 μ m, SD = 33.5) and group B (TB = 166.8 μ m, SD = 14) compared with group C (TB = 115.4 μ m, SD = 19.6) and group D (TB = 86.1 μ m, SD = 9.3). There was SS decrease ($p = 0.00093$) in osteoid percentage (OP) in group A (OP = 13.4%, SD = 2) and group B (OP = 11.5%, SD = 6.5) compared with group C (OP = 27.3, SD = 3.5) and group D (OP = 26.2%, SD = 2.6). Energy dispersive X-ray showed a nonsignificant increase ($p = 0.11$) in calcification ($\text{Ca}^{2+}\%$) in group A ($\text{Ca}^{2+}\% = 17.6\%$, SD = 4.9) and group B ($\text{Ca}^{2+}\% = 17.6\%$, SD = 4.3) compared with group C ($\text{Ca}^{2+}\% = 14.2\%$, SD = 6.7) and group D ($\text{Ca}^{2+}\% = 11.5\%$, SD = 2.4). MSCs application improved microscopic bone quality during standard DO and RDO. However, macroscopic bone quality improvement still needs further investigation.

Keywords

- ▶ rapid rate distraction osteogenesis
- ▶ mesenchymal stem cells
- ▶ distraction osteogenesis

Distraction osteogenesis (DO) is a surgical technique used to regenerate bone. McCarthy et al were the first to perform clinical trial for mandibular distraction in 1992.¹

Rapid rate distraction osteogenesis (RDO) was proposed to decrease the distraction time to avoid consequences of the long distraction procedures. Djasim et al² reviewed variable DO rates ranging from 1 to 4 mm/day. According to Djasim et al,² the use of rapid distraction is debatable and not always

recommended and they recommended a rate of 1 mm/day as the ideal rate and even recommended splitting it to half mm every 12 hours.

Tee and Sun in their review found that the addition of mesenchymal stem cells (MSCs) improved bone quality and quantity.³ Kitoh et al were the first to use bone marrow concentrate (BMAC) clinically as a source for MSCs in distraction gap to decrease time needed for healing of the distracted bone.⁴

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The hypothesis of this study is the assumption that RDO decreases bone quality.⁵ This study aimed to improve RDO bone quality by the addition of MSCs to the distracted bone.

Materials and Methods

This article is a modification of a study published in 2016 to evaluate the effect of the application of MSCs on standard rate of DO bone regeneration⁶; the previous article was published by the same authors of this article. The current study is an independent extension and expansion of the previous study.⁶ The standard DO results of the previous study were included and used as a control for this study. This study focuses on the application of MSCs during RDO.

Animal Grouping and Housing

This study was conducted in respect to the guidelines of the research ethical committee of the Faculty of Dentistry of Ain Shams University. This research got the ethical approval before the beginning of the experiment.

Twenty-four goats, *Capra aegagrus hircus*, of average weight of 10 to 15 kg were included in the study. The goats were between 2 and 3 months old,⁷ and were of same sex (female goats). The goats were divided into four groups: groups A, B, C, and D and each group consisted of six goats. These goats were assigned in each group using shuffled deck of cards (simple randomization technique).⁶

Group A had a standard distraction rate of 1 mm/day and adjunctive MSCs (injected in inert physiological saline carrier) were injected in the distraction gap; group A is considered as a positive control. Group B had a rapid distraction rate of 2 mm/day and adjunctive MSCs (injected in inert physiological saline carrier) were injected in the distraction gap. Saline was injected in groups C and D (control groups) because they were considered as negative control.

The study design necessitates the use of three control groups, as two factors affected the quality of the distracted bone: the increase in rate usually decreases bone quality and the addition of MSCs is added to improve bone quality. Group C presented the classic unassisted normal rate distraction, group D presented the rapid rate unassisted distraction, and group A presented the classic normal rate distraction assisted by MSCs.

Surgical Protocol

The submandibular area was prepared and shaved prior to the procedure. Intravenous ketamine and propofol were administered to anesthetize the goats. A submandibular incision was followed by an osteotomy performed by fissure bur of no. 1 size using a low speed hand piece associated with copious saline irrigation. The distractor was fixed at both osteotomy ends by 2.0 mm self-tapping mini screws. A skin puncture in the lip region allowed the exit of the distractor activation screw (→ Fig. 1).⁶

Distraction Protocol

Distractors were kept in the state of latency for 5 days in all groups. Active distraction of the goats followed 5 days of

Fig. 1 Distractor fixed in place.

latency in a rate of 1 mm/day for 10 days to create a distraction gap of 10 mm in groups A and C. Active distraction of the goats followed latency at a rate of 2 mm/day for 5 days to create a distraction gap of 10 mm in groups B and D.

Three million MSCs were injected in groups A and B using 18-gauge needles in the distracted gap on two doses (each dose had 1.5 million MSCs) at days 10 and 20 of consolidation to improve bone quality of the distracted bone. All groups had 30 days of consolidation following the active distraction.⁶

Stem Cell Preparation (Ficoll Protocol)

MSCs were prepared to be injected in groups A and B. Bone marrow aspirated from iliac crest during the surgical procedure was diluted by Dulbecco's Phosphate Buffered Saline (PBS; Biochrom AG, Germany) in a ratio of 4:1. The dilute was titrated and suspended over BIOCILL (Biochrom AG), separating solution and then centrifuged at 2,000 revolutions per minute for 30 minutes.⁵ Extraction of the buffy layer which is rich with undifferentiated MSCs was done by pipette. Centrifugation was performed twice to ensure high concentrate of undifferentiated MSCs. The MSCs were allowed to multiply in an incubator for 2 weeks suspended in bovine serum and high streptomycin–penicillin concentrate at 37°C. One and a half million MSCs counted using an inverted microscope were injected twice (at day 10 and day 20 of consolidation) in every goat in the study groups A and B. The cells were extracted from three goats and multiplied and injected in all other goats, as theoretically the MSCs do not induce immune reaction in same species.⁸ The MSCs were injected in the soft bony callus slowly under pressure to avoid inducing pain; no local anesthesia was added to avoid any disturbance or effect on the cells and the procedure did not require sedation or general anesthesia of the goats.

Killing (Euthanasia Procedure)

All the goats of all the groups (regardless of rate or addition of MSCs) were killed after 30 days of consolidation after the end of active distraction using overdose thiopental sodium, and their dead bodies were incinerated.

Sampling

Samples were extracted carefully after soft-tissue dissection after the killing of all animals after 30 days of consolidation. After radiographic assessment was done, smaller sections were made and preserved in formalin 10% solution prior to energy dispersive X-ray (EDX) assessment and histological assessment.

Assessment

Assessment of the bone quality was done histologically, as histological assessment is considered the gold standard in bone quality assessment.⁹ Sections were stained by hematoxylin and eosin (H&E) and Masson's trichrome (MT). The stained samples were evaluated histomorphometrically.⁶ After decalcification, bony samples were sectioned longitudinally. Each histological section was divided into three zones: proximal, middle, and distal zones. The average of histomorphometric results for each section was calculated and tabulated.

Histological assessment was assisted by two radiological methods (cone beam computed tomography [CBCT] and EDX) to confirm results. Percentage of calcium ion was assessed by EDX, and an X-ray technique was used to identify element composition in a sample to evaluate mineralization and calcium content. CBCT results were excluded and removed from the current study because the concordance correlation coefficient between examiners was 0.5, indicating poor agreement between examiners.

Statistical Evaluation

Results were tabulated and evaluated statistically by the Kruskal–Wallis test (one-way ANOVA on ranks because of inclusion of different variables in the study and Conover's post hoc analysis) and *p*-value was set at 0.05. Eta-squared test recommended by Cohen was used to measure the effective size of sample. According to Cohen, if the Eta-square value is 0.02, the sample is small; if the value is 0.13, the sample size is medium, and if the value is bigger than 0.26, the sample size is large.¹⁰

Results

The results of the study are presented in the following subsections.

Histomorphometric Evaluation

Histomorphometric assessment of H&E-stained sections showed a strongly significant (SS) increase ($p = 0.00036$) in trabecular bone (TB) in group A (TB = 174.7 μ m, standard deviation [SD] = 33.5) and group B (TB = 166.8 μ m, SD = 14) compared with group C (TB = 115.4 μ m, SD = 19.6) and group D (TB = 86.1 μ m, SD = 9.3). The Eta-square value regarding this test was 0.8 indicating that the sample size is large enough to give statistical data ($\blacktriangleright$ Fig. 2a–d and $\blacktriangleright$ Fig. 3; $\blacktriangleright$ Table 1).

Histomorphometric assessment of MT-stained sections showed a SS decrease ($p = 0.0009$) in osteoid percentage (OP) in group A (OP = 13.4%, SD = 2) and group B (OP = 11.5%, SD = 6.5) compared with group C (OP = 27.3, SD = 3.5) and group D (OP = 26.2%, SD = 2.6). The Eta-square value regarding this test was 0.8 indicating that the sample size is large enough to give statistical data ($\blacktriangleright$ Fig. 4a–d and $\blacktriangleright$ Fig. 5; $\blacktriangleright$ Table 1).

Evaluation of Radiographic Calcium Ion Concentration by EDX

EDX showed a nonsignificant (NS) increase ($p = 0.11$) in calcification in group A ($\text{Ca}^{2+}\%$ = 17.6%, SD = 4.9) and group B ($\text{Ca}^{2+}\%$ = 17.6%, SD = 4.3) compared with group C ($\text{Ca}^{2+}\%$ = 14.2%, SD = 6.7) and group D ($\text{Ca}^{2+}\%$ = 11.5%, SD = 2.4). The Eta-square value regarding this test was 0.25, indicating

Fig. 2 (a–d) Histological section at $\times 10$ of H&E-stained samples showing bone trabeculae in group a, b, c and d, respectively.

Fig. 3 Bar chart showing means of trabecular bone thickness.

that the sample size is of acceptable medium strength to give statistical data (► **Fig. 6**; ► **Table 2**).

Discussion

A wide range of DO rates was applied and reported previously in the literature.¹¹ Rates applied on experimental small animals such as rats and rabbits reached 4 mm, while large animals' rates varied between 1, 1.5, and 2 mm/day.^{11–13} Those studies conducted on the rapid distraction rate of 2 mm/day were highly debatable; RDO was associated usually with inferior bone quality compared with the normal DO.¹⁴

One of the main drawbacks of DO is the long duration taken through the procedure. Patients are at risk of pain, fracture, infection, and discomfort. In contrast to long bones, the craniofacial bones have unique characteristics such as abundant blood supply and rapid rate of bone healing. Hence,

a rapid distraction rate might be a possible alternative to the normal distraction rate. Rapid distraction rate will shorten the distraction period reducing the associated complications of the DO procedure.¹⁵

The current research idea is based on the previously mentioned facts: the diversity of rates of distraction in literature and on the need to decrease the time of distraction to decrease complications. However, the literature showed that rapid distraction usually decreases the bone quality. Therefore, the aim of this study was to assess the effect of MSCs on bone quality of the rapidly distracted bone.

The distraction protocol of this study was based on two systematic reviews published by and Djasim et al² and Swennen et al.¹⁶ The standard distraction rate of groups A and C was set at 1 mm/day and the rapid distraction rate was set at 2 mm/day in agreement with several studies that advocated this rate.^{17–23}

In a meta-analysis, Tee and Sun³ found that only 19 articles were published in literature concerned with the evaluation of the effect of MSCs application and tissue culture on the distracted bone. Most of the studies were conducted on small animals such as rodents; few studies were conducted only on large mammals. Siegel and Mooney,²⁴ O'Loughlin et al,²⁵ and Mills and Simpson²⁶ claimed that bones of higher-order mammals and nonhuman primates mimic bones of humans more effectively than the rodents.

The systematic review performed by Tee and Sun mentioned only four studies that were conducted on MSCs applications during DO performed on large animals. Krocsek et al²⁷ conducted a study on minipigs, Castro-Govea et al²⁸ on dogs, Sun et al²⁹ on dogs, and Aykan et al³⁰ on goats. All the previously mentioned studies conducted on large animals were concerned with normal DO,^{27–30} not the RDO. Hence, to the best of our knowledge, there is a deficiency in literature concerned with the assessment of the effect of MSCs application during RDO in large animals.

Table 1 Difference in trabecular bone thickness and osteoid bone percentage statistical data (Kruskal–Wallis test [one-way ANOVA on ranks] and post hoc; *p*-value is set at 0.05)

	Group A		Group B		Group C		Group D	
Histological stain	H&E (TB)	MT (OP)	H&E (TB)	MT (OP)	H&E (TB)	MT (OP)	H&E (TB)	MT (OP)
Sample 1	215.3	11	150.3	6.4	86.3	23.7	77.3	22.7
Sample 2	194.7	12.9	157.7	24	101.7	25	78.7	29.7
Sample 3	135.7	16.9	169	8.7	112	25	79	26
Sample 4	152.7	13.9	172.7	9.3	127.7	29.3	87	28
Sample 5	203	12	190.3	7.7	141	33	97.7	23.7
Sample 6	147	13.7	160.7	12.7	124	27.7	97	27
Mean	174.7	13.4	166.8	11.5	115.4	27.3	86.1	26.2
SD	33.5	2	14	6.5	19.6	3.5	9.3	2.6
Average rank	18.50	8.17	18.33	5.33	9.17	18.75	4.00	17.75
Statistical significance <i>p</i> < 0.05	C, D	C, D	C, D	C, D	A, B, D	A, B	A, B, C	A, B

Abbreviations: H&E, hematoxylin and eosin; MT, Masson's trichrome; NS, nonsignificant; OP, osteoid percentage; SD, standard deviation; TB, trabecular bone.

Fig. 4 (a–d) Histological section at $\times 10$ magnification of Masson's trichrome (MT)-stained samples showing osteoid immature bone in green and mature bone in red in group a, b, c and d, respectively.

The assessment of H&E samples showed that there was a significant increase ($p = 0.00036$) in the TB thickness in study groups enhanced by MSCs (groups A and B) compared with control groups (groups C and D), which is in agreement with Qi et al.⁵ The TB-thickness improvement is attributed to the addition of the MSCs.

Histomorphometrical assessment of the MT samples showed a statistical significant decrease ($p = 0.00093$) in the percentage of the osteoid bone in study groups enhanced by MSCs (groups A and B) compared with the control groups (groups C and D). This decrease in the osteoid bone per-

centage indicates more rapid bone maturation associated with MSCs application, which is in agreement with Song et al.³¹

CBCT is considered to be a reliable method in the assessment of bone quality and comparable even to micro-CT,³² multislice CT,³³ and dual-energy X-ray.³⁴ However, in this study, there was poor reliability between examiners. The authors attribute this finding to the human error in reproducibility of bone evaluation points on CBCT. The poor agreement between examiners was documented by Pauwels et al.,³⁵ which showed that the inter-examiner reliability of CBCT results is poor and not reliable.

Fig. 5 Bar chart showing difference in osteoid bone percentage between groups.

Fig. 6 Bar chart showing mean of calcium ion concentration percentage of groups.

Table 2 Difference in calcium ion percentage statistical data (Kruskal–Wallis test [one-way ANOVA on ranks] and post hoc; *p*-value is set at 0.05)

	Group A	Group B	Group C	Group D
Radiographic assessment	Ca ²⁺ %	Ca ²⁺ %	Ca ²⁺ %	Ca ²⁺ %
Sample 1	23	19.58	20.31	9.45
Sample 2	21.6	9.8	15.8	10.02
Sample 3	20.18	16.1	19.86	11.5
Sample 4	15.7	19.2	5.3	13.56
Sample 5	15.02	18.83	6.4	9.45
Sample 6	10	22.3	17.34	15.2
Mean	17.6	17.6	14.2	11.5
SD	4.9	4.3	6.7	2.4
Average rank	15.67	15.50	11.83	7.00
Statistical significance <i>p</i> < 0.05	NS	NS	NS	NS

Abbreviations: NS, nonsignificant; SD, standard deviation.

Busse et al³⁶ considered EDX to be a reliable method for the assessment of bone quality. Rachmiel et al³⁷ used EDX to assess the newly formed distracted bone. Evaluation of Ca²⁺ ion concentration in specimens was done using EDX to validate and support the CBCT results. In this study, there was an increase in the bone Ca²⁺ ion concentration in groups A and B assisted by the addition of MSCs, indicating better ossification and more rapid healing in MSCs-assisted groups. EDX and CBCT findings were correlated confirming the increase in calcification. However, the increase was not statistically significant (*p* = 0.11), which is in agreement with Tai et al.³⁸ Tai et al evaluated bone healing enhanced by MSCs in an experimental study. The nonsignificant increase may be attributed to early killing of the animals (the bone is newly formed; not fully calcified yet).

Histological assessment is considered the gold standard for assessing bone because it analyzes the bone microstructure.⁹ In conclusion, this study showed that the RDO rate (2 mm/day) had a negative effect on bone quality, which is in agreement with Djasim et al,² since the trabecular bone thickness of RDO bone regenerate was significantly less compared with standard distraction rate.

However, the addition of MSCs application was capable of improving the bone quality of the distracted bone generated in RDO at microscopic level. There was a strong correlation between the results, since the increase in the size of the calcified trabeculae was associated with a decrease in the osteoid bone percentage and an increase in the calcium level content in the sample.

Recommendations

This study recommends an immune histochemical assessment of the MSCs before their injection to ensure their nature. The aim of immune histochemical addition is to

ensure that the MSCs are the effective factor in the improvement of bone microstructure.

This study recommends further evaluation of the associated soft tissue such as muscle and nerves generated by RDO which may not be fixed by MSCs.

Conflict of Interest

All authors disclose no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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