OSTEOGENIC SYSTEMIC RESPONSE IN BONE MARROW STIMULATION

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ABSTRACT

Objective: To assess the influence of systemic osteogenic response caused by remote stimulation of bone marrow in a bone gap union. Method: 36 young adult rabbits were employed. The animals were randomly divided into 3 groups (A, B, C) and submitted to ostectomy of the right radius, removing 4mm of bone. The animals on Group A had their bone marrow stimulated by ablation on the left femur. Animals on Group B had their bone marrow stimulated by introducing a 1.5mm-thick Kirschner wire into the shaft of the left femur. The animals on Group C served as controls. X-ray images were taken on a weekly basis until the 4th post-surgical week, when the animals were sacrificed. Histomorphometric study of the bony callus formed at the ostectomy site was conducted. The x-ray images were evaluated in order to analyze the evolution of bone union at the ostectomy site. Results: The groups with remote bone marrow stimulation had a smaller number of bone cells as compared to the control group. On radiographic studies, no difference in terms of evolution of union was evident between the groups. Conclusion: Remote stimulation of bone marrow had an unfavorable influence on bone gap union in rabbits.

Keywords: *Rabbits/surgery. Bone marrow. Fracture healing. Bony callus.*

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INTRODUCTION

Fracture repair process involves local and systemic factors.[1-3] The systemic osteogenic response was observed after stimuli distant from the fracture focus, such as in bone marrow injuries[4-8] and/or blood leakage[9,10]. Tavares et al.[4] described a faster union in rabbits submitted to radius ostectomy associated to remote bone marrow stimulus, by placing a Kirschner wire at the femoral intramedullary space. One of the hypotheses that can justify this phenomenon is the action of growth factors, which are produced at the site of marrow regeneration and released on blood stream.[5,6] Other factors are released on blood stream through bone marrow regeneration, such as the 14 amino acid peptide – osteogenic growth peptide (OGP). The synthetic OGP with an identical structure as the original one has stimulated the proliferation and activation of alkaline phosphatase of in vitro osteoblastic cells and has increased bone mass in rats.[11-14]

Blood leakage is also directly correlated to the stimulation and activation of bone marrow. The production of erythropoietic cells may be associated to the production of osteogenic cells, by the correlation of its stem cells or by the release of factors affecting the differentiation and proliferation of stromal cell components of bone marow.[9] An example of this is the bone loss occurring from menopause, although its cause is usually attributed to hormonal changes on women, it is possible that repeated menstrual bleeding maintains the osteoblastic activity of bone marrow, contributing to skeletal mass maintenance.[10,15] Ilizarov et al.[16] concluded that the blood leakage amounting 1% of body weight in rabbits accelerated bone repair process of fibular osteotomies.

The objective of this study was to assess the influence of the osteogenic systemic response caused by remote stimulus to the bone marrow, by bone gap union.

METHOD

The experiment was carried out at the Sector of Experimental Surgery, Hospital do Servidor Público Estadual "Francisco Morato de Oliveira" in São Paulo, according to the rules established by the Brazilian College of Animal Experimentation (COBEA), bonded to the International Council for Laboratory Animal Science (ICLAS). Thirty six young adult white male New Zealand rabbits weighting 2.9 kg in average (2.5 to 3.3kg), randomly distributed into three groups (A, B and C) with 12 animals each. The anesthetic protocol consisted of intramuscular injection of a solution containing: ketamine hydrochloride (40mg/kg) and xylazin hydrochloride (5mg/kg).[17] At the moment of anesthesia, benzatine penicillin 40.000 UI/kg was administered intramuscularly. The rabbits did not present clinical signs of discomfort or pain during the surgical procedure.

The animals were placed on the surgical table at left lateral position, and partial trichotomy of the right forearm was provided. The venous depletion of the limb was made with an elastic band (Figure 1) using a garrote at arm's root.

All authors state no potential conflict of interests concerning this article.

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Figure 1 – *Venous depletion of the right anterior limb with elastic band.*

Following the antisepsis and asepsis of the whole limb, sterile drapes were placed and a straight incision of 2.5 cm in length on the torso-radial surface of the right forearm was made, from 1.5 cm of proximal distance to the radiocarpal joint. Dissection was made between the thumb's long abductor muscle and radial extensor of the carpus up to radius bone exposure. With a surgical marker, two marks were made on the radius for outlining the right place for ostectomy (Figure 2).

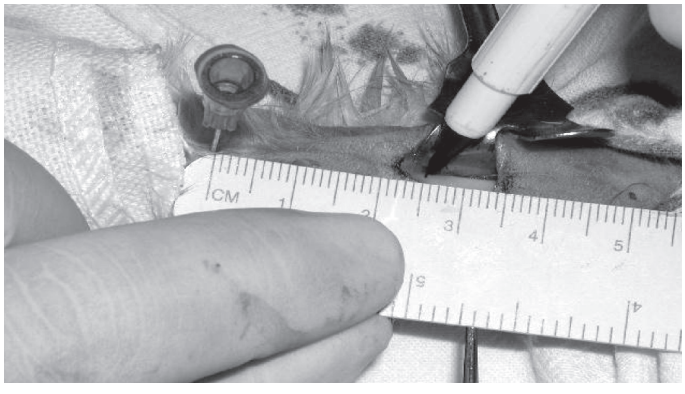

Figure 2 – *Marking of the ostectomy site on the radius of the animal's right anterior limb.*

Using a steel 0.5-mm thick mini saw, the ostectomy was performed removing a 4-mm cylinder of that bone, at 2.5 mm proximally to radiocarpal joint, protecting soft parts and the ulna, and irrigating the site with 5 ml saline solution (Figure 3).

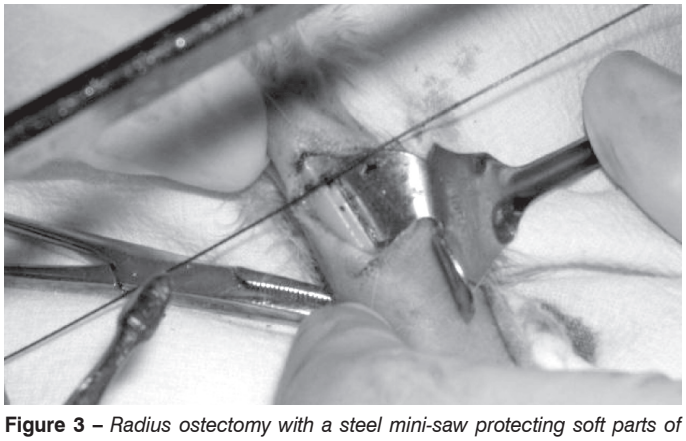

Figure 3 – *Radius ostectomy with a steel mini-saw protecting soft parts of animal's right anterior limb.*

Muscles and subcutaneous cell meshwork were approximated with absorbable polyglycolic acid suture (Vicryl 3.0®), intending to minimize postoperative bleedings and avoid muscular interposition on the bone gap created. The skin was sutured with non-absorbable monofilament nylon wire (Mononylon 4.0®). Radius stabilization was not necessary, due to its strong connection with the ulna by the interbone membrane.

In group A animals, using the same anesthetic procedure, bone marrow was stimulated by partially ablating it on left femur aspirating 3ml of the medullary canal content, using a 20-ml syringe and puncture needle for biopsy. The needle was positioned within the left femoral medullary canal through major trochanter up to distal femur, and aspiration was made as the needle was removed (Figure 4). The material collected from femoral medullary canal was discharged.

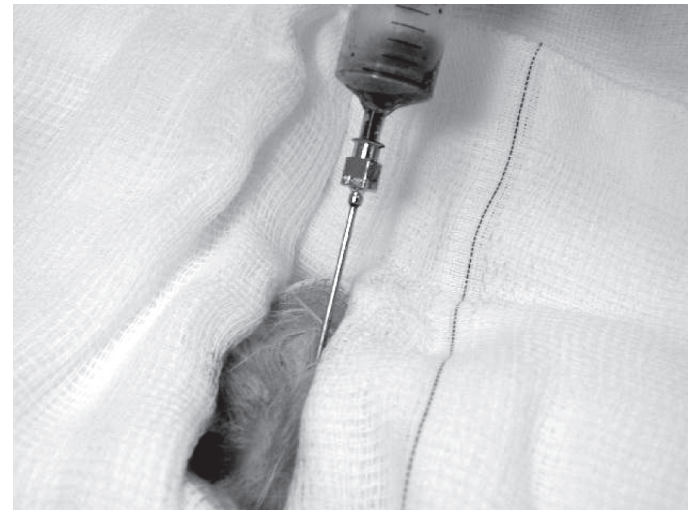

Figure 4 – *Bone marrow ablation by aspirating animal's femur.*

On group B, also using the same anesthetic procedure, bone marrow was stimulated by introducing a 1.5-mm thick Kirschner wire within the left femoral medullary canal. The wire was percutaneously inserted through major trochanter towards the shaft, where it was buried (Figure 5). The wire burial site was confirmed by X-ray images at anteroposterior and lateral planes.

On group C, which served as a control, no remote bone marrow stimulation was provided.

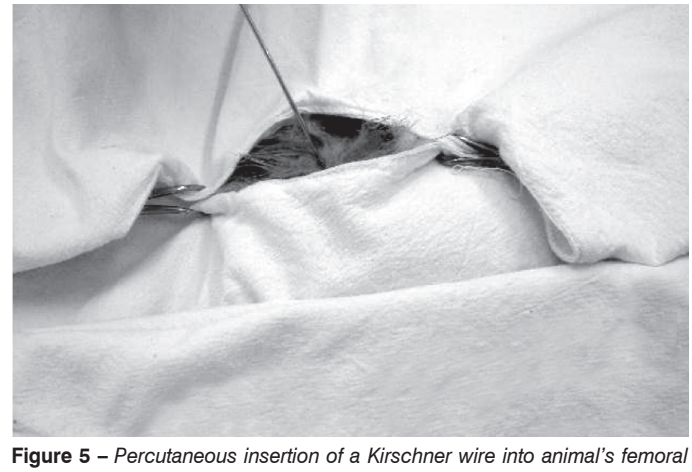

Figure 5 – *Percutaneous insertion of a Kirschner wire into animal's femoral medullary canal.*

At early postoperative period, X-ray images were taken at anteroposterior and lateral plane of the right forearm (groups A, B and C) (Figure 6) and left femur (group B) (Figure 7). X-ray images of the forearm were repeated at 7-day intervals until the 21st day with an apparatus of 36kV, 0,025mAs and 100mA. The film (Kodak®), model T-MAT G/RA of 18x24cm was positioned at a distance of 30cm from the X-ray equipment's ampoule.

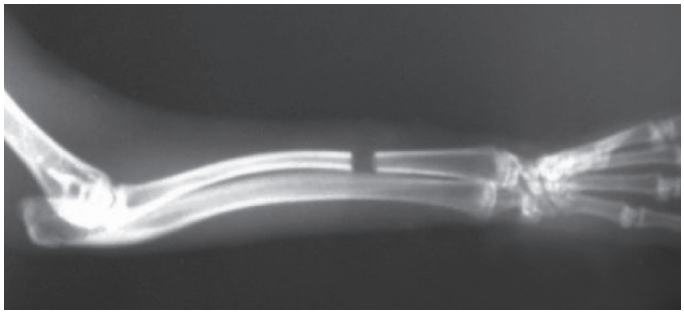

Figure 6 – *Lateral X-ray image of the operated forearm showing radius ostectomy.*

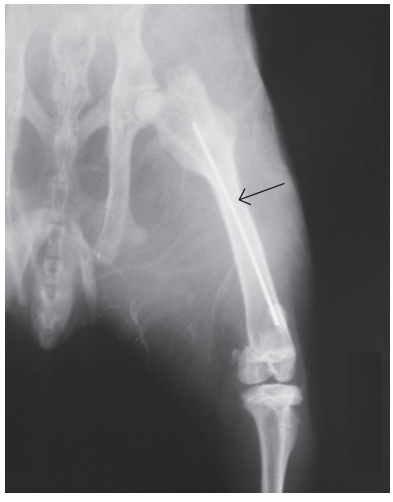

Figure 7 – *Anteroposterior X-ray image of the operated femur showing an intramedullary Kirschner wire (arrow).*

Animals experiencing intraoperative complications were excluded from the study: one rabbit from group A (concomitant ulnar fracture) and one rabbit from group B (incomplete radius ostectomy).
Postoperatively, the animals were lodged in individual cages, where they received commercially available ration and water *ad libitum*. All rabbits were sacrificed at the end of the 4th postoperative week with intravenous sodium pentobarbital at a lethal dose of 200mg/kg.[18] Then, the forearm of each animal was submitted to X-ray imaging at anteroposterior and lateral planes (Figure 8).

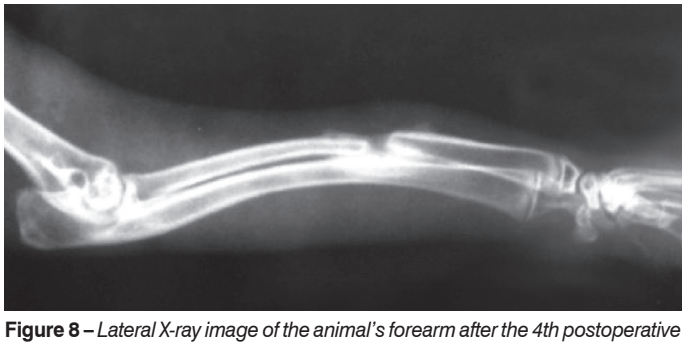

Figure 8 – *Lateral X-ray image of the animal's forearm after the 4th postoperative week. Note the bony callus on the radius.*

Dissection of animals' forearm, and radius resection in conjunction with the ulna proceeded (Figure 9).

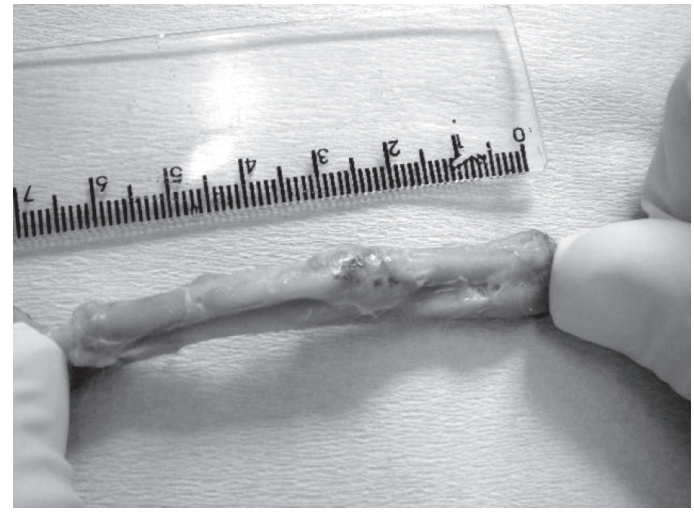

Figure 9 – *Dissected forearm of a sacrificed animal, with concurrent radius and ulna resection.*

After being stored in containers with 10% buffered formol and properly identified, the ostectomy region was submitted to histomorphometric analysis.

HISTOMORPHOMETRIC STUDY

Decalcification of pieces was carried out with 7% nitric acid and included into paraffin. 3μm-thick histological sections were made at coronal direction with a rotational microtome on paraffin blocks containing bony callus samples, which were stained by using Masson's trichrome technique for histomorphometric analysis. The qualification of the various kinds of bony callus tissues was carried out in an image analyzer system (Kontron Electronic 300®).
For each animal, cartilaginous, bony and fibrous tissue areas were summed up, obtaining the total bony callus area. The area fraction of each kind of tissue was obtained by the relation between the relevant tissue area fraction divided by bony callus area (Figure 10).

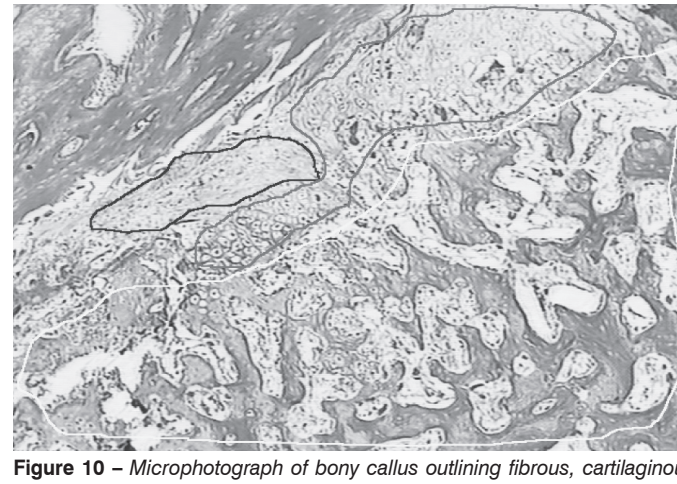

Figure 10 – *Microphotograph of bony callus outlining fibrous, cartilaginous and bony tissues.*

X-RAY STUDY

The weekly X-ray images taken were assessed by the X-ray scoring system described by Lane and Sandhu[19] for evaluating the evolution of bone union at ostectomy site by an orthopaedic doctor blinded to study data (Table 1).

Table 1 – *X-ray score according to Lane and Sandhu19*

		Scores
Bone formation		
	No evidence of bone formation	0
	Bone formation (25% of the gap)	1
	Bone formation (50% of the gap)	2
	Bone formation (75% of the gap)	3
	Bone formation (100% of the gap)	4
Union		
	With complete fracture trace	0
	With incomplete fracture trace	2
	Absence of fracture trace	4
Remodeling		
	No evidence of remodeling	0
	Intramedullary remodeling	2
	Cortical remodeling	4

STATISTICAL ANALYSIS

As summary measures, mean, median and standard deviation, minimum and maximum values were used to show variability.[20] On the histomorphometric study, variance analysis (ANOVA) was employed. For the application of this test, groups homogeneity was examined by Levene's test. In the presence of significant difference between groups, multiple comparisons were provided (comparisons between groups – pair to pair) by Bonferroni's test in order to identify groups with differences between each other. For the X-ray scoring by Lane and Sandhu[19] the non-parametric test of Repeated Measures for Ordinal Data was used.[20,21] By this technique, group effects (comparison between the three groups), moment effects (comparisons between weeks) and interaction effects (distinct behaviors as a function of other variables) were evaluated. For all tests, the null hypothesis rejection level was 0.05 (95% significance level) according to biological studies standards.

RESULTS

The percentages of fibrosis, cartilage and bone cells found on the histomorphometric study of bony callus on animals from groups A, B and C are listed on Tables 2, 3 and 4.

Table 2 – *Percentage of fibrous, cartilage and bone cells found on the histomorphometric study of bony callus of the group A animals' samples*

Animal	Fibrosis (%)	Cartilage (%)	Bone (%)
1	2.5	14.5	83
2	8	17	75
3	2	19	79
4	15	29	56
5	5	30	65
6	0	20	80
7	4	17	79
8	5	15	80
9	10	15	75
10	16	14	70
11	4	20	76

Table 3 – *Percentage of fibrous, cartilage and bone cells found on the histomorphometric study of bony callus of the group B animals' samples*

Animal	Fibrosis (%)	Cartilage (%)	Bone (%)
1	0.5	2	97.5
2	0.6	9.2	90.2
3	8.5	17.5	74
4	3	14	83
5	2	26	72
6	8.5	4	87.5
7	3	10	87
8	3	26	71
9	16	15	69
10	5	25	70
11	1.4	22	76.6

Table 4 – *Percentage of fibrous, cartilage and bone cells found on the histomorphometric study of bony callus of the group C animals' samples*

Animal	Fibrosis (%)	Cartilage (%)	Bone (%)
1	16.5	13.5	70
2	3	12	85
3	0	0	100
4	0.05	0.05	99.9
5	0	11	89
6	3	8	89
7	2	18.5	79.5
8	1.5	19.5	79
9	10	15	75
10	3	15	82
11	5	0.5	94.5
12	3.5	12.5	84

Comparison of the percentage of fibrous cells

No difference was found for the percentage of fibrous cells between the three groups (p=0.455) (Table 5).

Table 5 – *Percentage measurements for fibrous cells on groups A, B and C*

		Group		
		A	B	C
% Fibrosis	Mean	6.5	4.7	4.0
	Median	5.0	3.0	3.0
	Standard Deviation	5.2	4.7	4.8
	Minimum	0	0.5	0
	Maximum	16	16	16.5

p-value = 0.455

Comparison of the percentage of cartilaginous cells

A statistically significant difference was found for mean percentages of cartilaginous cells between the different groups (p=0.022) (Table 6).

Table 6 – *Percentage measurements for cartilaginous cells on groups A, B and C*

		Group		
		A	B	C
% Cartilage	Mean	19.1	15.5	10.5
	Median	17.0	15.0	12.3
	Standard Deviation	5.6	8.6	6.9
	Minimum	14	2	0
	Maximum	30	26	19.5

p-value = 0.022

On Table 7, multiple comparisons of two by two groups are presented in order to identify the groups presenting differences between each other. The results found that group A showed a significantly higher percentage (p=0.02) of cartilage cells compared to group C (control) (Table 7, Figure 11).

Table 7 – *Multiple comparisons between animal groups A, B and C for the presence of cartilaginous cells*

Group	Comparative group	Descriptive level (p-value)
A	B	0.730
A	C	0.020*
B	C	0.299

* = Significant

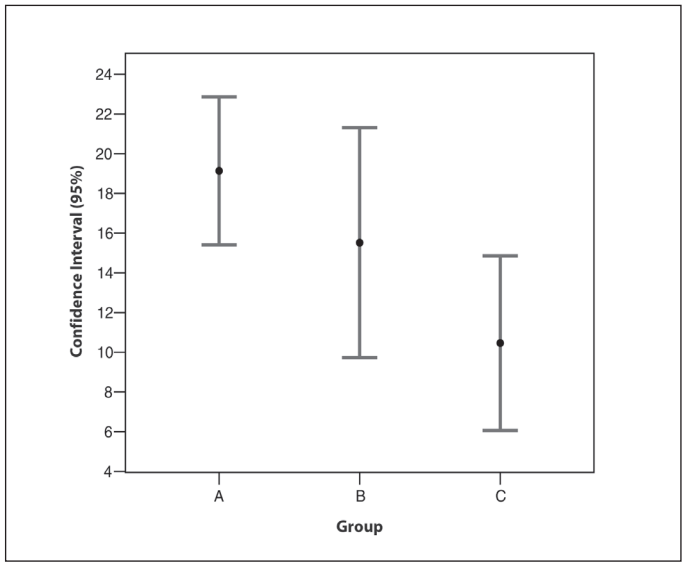

Figure 11 – *Mean percentage of cartilage cells and confidence intervals for the different animal groups.*

Comparison of the percentage of bone cells

A statistically significant difference (p=0.02) was found for the mean values of bone cells percentage between the different groups (Table 8).

Table 8 – *Percentage measurements for bony cells on groups A, B and C.*

		Groups		
		A	B	C
% Bone	Mean	74,4	79,8	85,6
	Median	76,0	76,6	84,5
	Standard Deviation	7,9	9,7	9,4
	Minimum	56	69	70
	Maximum	83	97,5	100

p-value = 0.02

Table 9 shows the multiple comparisons of paired groups. The results reveal that the animals from group A presented a significantly lower percentage (p=0.017) of bone cells than animals from group C (control) (Table 9, Figure 12).

Table 9 – *Multiple comparisons between animal groups A, B and C for the presence of bone cells.*

Group	Comparative Group	Descriptive level (p-value)
A	B	0.505
A	C	0.017*
B	C	0.408

* = Significant

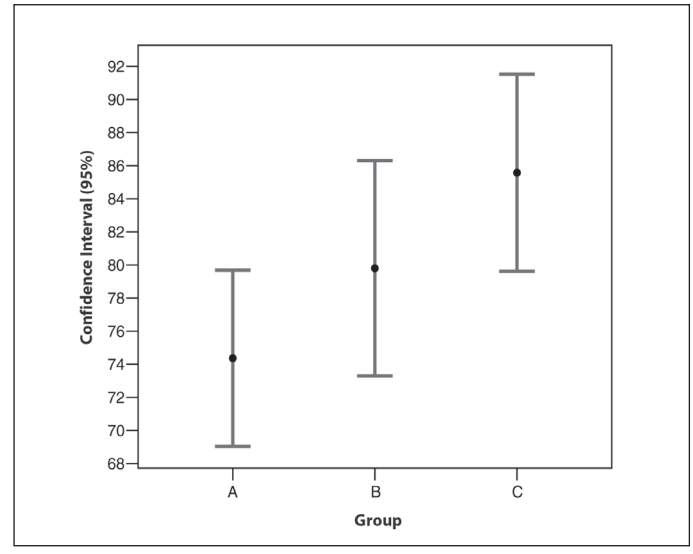

Figure 12 – *Mean percentage of bone cells and confidence intervals for the different animal groups*

In the X-ray study, differences between weeks were found, independently of the studied groups, not comparing groups for each week or between weeks for each group, because no interaction effect was found (p=0.098). In this test, no difference was found between groups, independently of the week (p=0.359). (Table 10)

Table 10 – *Repeated measurements for ordinal data for Groups A, B and C in weeks 1, 2, 3 and 4.*

	result	Group					
		A		B		C	
		N	%	N	%	N	%
Week 1	0	9	81.8%	10	90.9%	10	83.3%
	1	2	18.2%	1	9.1%	2	16.7%
Week 2	0			2	18.2%		
	1	9	81.8%	4	36.4%	5	41.7%
	2	2	18.2%	3	27.3%	5	41.7%
	3			2	18.2%	2	16.7%
Week 3	1					1	8.3%
	2	5	45.5%	3	27.3%	4	33.3%
	3	6	55.5%	4	36.4%	4	33.3%
	4			4	36.4%	3	25.0%
Week 4	3	3	27.3%	2	18.2%	2	16.7%
	4	7	63.6%	6	54.5%	6	50.0%
	6	1	9.1%	3	27.3%	4	33.3%

Interaction effect: p-value= 0.098
Group effect (difference between groups): p-value= 0.359
Moment effect (difference between weeks): p-value < 0.001

Table 11 shows the results of multiple comparisons between weeks, where a significant difference ($p<0.001$) is found between all weeks.

Table 11 – *Multiple comparisons between weeks.*

Group	Comparative Group	Descriptive level (p-value)
week 1	week 2	< 0.001*
week 1	week 3	< 0.001*
week 1	week 4	< 0.001*
week 2	week 3	< 0.001*
week 2	week 4	< 0.001*
week 3	week 4	< 0.001*

* = Significant

DISCUSSION

The study of bone union and growth factors influencing it are increasing, intending to accelerate the recovery of fractured patients. Some authors have been conducting experiments in an attempt to isolate substances produced on bodies, which would supposedly be correlated to bone formation.[8-14,22]

In this experiment, three groups of animals were used: group A, submitted to ostectomy and to bone marrow remote stimulation by ablation, in order to assess the systemic osteogenic response after an intensive bone marrow regeneration process; group B, submitted to ostectomy and to bone marrow remote stimulation by placing a Kirschner wire inside the bone marrow in order to assess the systemic osteogenic response after an intensive bone marrow regeneration process , and; group C – control group – which was used for comparison. On the three groups, we performed blood depletion and used a garrote on the operated limb with an elastic band in order to reduce bleeding.

Bone marrow regeneration process can lead to release of osteogenic factors on blood stream[4-8], but we don't think this is enough to influence remote bone gap union. The indirect stimulation by isolated bleeding, independently from any skeletal trauma or bone marrow injury, can also be associated to a marked osteogenesis. Even a small blood leakage can lead to a moderate erythropoiesis stimulation and resultant activation of bone marrow.[9,10] One can speculate that the offset hematopoiesis occurs in response to bleeding, and, in certain conditions, is mediated by increased osteogenesis and potential bone marrow stromal cells activities. Lippiello et al.[9] stated that blood leakage is associated to release of circulatory factor, osteopoietin according to this author, which would activate the differentiation and proliferation of stromal cell components of the bone marrow. Lucas et al.[10], defended that blood leakage shows an identical magnitude to medullary bone trauma, in terms of bone formation gain (number of osteoblasts, apposition mineral rate, alkaline phosphatase, growth peptide and osteocalcin). As far as we are concerned, no similar experiments have been conducted using garrote on the operated limb for minimizing bleeding. We used blood depletion and garrote on the root of the operated limb to homogenize groups and separately assess remote bone marrow stimulus, without the bias caused by a variable blood leakage.

The results of the present study found a higher number of bone cells at the end of union process on animals belonging to group C (control) when compared to the others. When ostectomy associated with reduced bleeding was performed during surgical procedure, the remote stimulation of bone marrow did not generate enough osteogenic response to stimulate bone gap union, in fact, it delayed cell maturation of the bony callus revealed by a larger number of cartilaginous cells on animals from group A.

In the experiment conducted by Gazit et al.[6], bone marrow injury did not show any increased production of osteogenic factors when its regeneration was inhibited by silicone insertion. This increase was only found in cases where an intensive marrow regeneration occurred. We found that placing a Kirschner wire away from fracture focus (group B) has not generated systemic osteogenic response as well to interfere on union process, probably because wire placement did not demand an intensive bone marrow regeneration, differing from the results found in literature.[4,7] It is possible that the reduced intraoperative bleeding might have influenced these results.

The difference of the results reported here as compared to literature may be explained as follows: a) the existence of few studies addressing this kind of experiment; b) the reduced number of experiments using rabbits as models; c) the use of difference methodological criteria in each study; d) the reduced intraoperative bleeding by the use of a garrote.

Further studies are warranted to confirm the findings of the present experiment, and thus to provide a better understanding about the systemic mechanisms that may influence fractures union.

CONCLUSION

Under the conditions of this experiment and due to the results achieved, we conclude that the bone marrow remote stimulation associated to a reduced intraoperative bleeding has unfavorably influenced bone gaps union in rabbits.

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