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Upgrowth of Tibial Heterotopic Vascularized Corticoperiosteal Flap in Dog compared with Rabbit

KEYWORDS: Heterotopic, corticoperiosteal, flap, vascularised.

C. Igna
Banat's University of Agricultural Sciences and Veterinary Medicine from Timioara, Faculty of Veterinary Medicine, Romania

L. Schüszler
Banat's University of Agricultural Sciences and Veterinary Medicine from Timioara, Faculty of Veterinary Medicine, Romania

A. Proteasa
Banat's University of Agricultural Sciences and Veterinary Medicine from Timioara, Faculty of Veterinary Medicine, Romania

A. Sala
Banat's University of Agricultural Sciences and Veterinary Medicine from Timioara, Faculty of Veterinary Medicine, Romania

R. Dasclu
Banat's University of Agricultural Sciences and Veterinary Medicine from Timioara, Faculty of Veterinary Medicine, Romania

ABSTRACT *Comparative investigation on capacity of vascularized corticoperiosteal tibial flap to produce heterotopic osteogenesis in dogs versus rabbits. Methods – Proximal tibial flap was taken and transferred in the popliteal muscle. The development of the flap and the new formed bone were evaluated by digital computer image - radioopacity, bone mineral density, osseous resorption and bone quality. Results - In rabbits the bone mineral density increased to values close to the reference value of normal tibial bone tissue. In dog, contrariwise, a marked decrease was observed. Histological sections in rabbit revealed that the regenerated tissue shows a high functional organization level and in the dog revealed the presence of the osseous resorption process. Clinical significance - The heterotopic flaps have different behavior in dog from rabbits. Dog's developmental model confirms the incapacity of the osteogenic reaction of the vascularized corticoperiosteal flaps in heterotopic transplantation.*

INTRODUCTION

Periosteum's role in tissue-engineered bone is widely recognized (Zhang et al., 2005; Zhu et al., 2006; Kaminski et al., 2009; Takeuchi et al., 2010) but the role of vascularized periosteum flap as a graft and the factors stimulating it into osteogenic activity remain obscure (Chen et al., 2009). The idea of using vascularized periosteal flaps in reconstructing bone defects is more than one hundred years old. Up to now, experimental and clinical results regarding their osteogenic capacity have been a subject of debate. In rats, the studies of ectopic bone formation in the groin and orthotopic bone formation in the femoral defect demonstrates that optimal bone formation requires four factors: bone morphogenetic protein (rh-BMP-2), a biodegradable matrix, osteoprogenitor cells, and blood supply (Kusumoto et al., 1997; Kusumoto et al., 1998; Vogelin et al., 2000; Vogelin et al., 2002).

Few articles recognized periosteum's osteogenic process without the dependence of stress stimulation or bony contact. Experimental study on rabbits reported that vascularized periosteal flaps presented strong osteogenic capacity in heterotopic conditions (Dailiana et al., 2002; Ortak et al., 2005; Chen et al., 2009).

The information above was the basis of our investigations in this study, in which we aimed to evaluate comparatively the quantity and quality of bone formed in heterotopic conditions from vascularized corticoperiosteal flaps in the dogs versus rabbits.

MATERIALS AND METHODS

In the present study 8 white New Zeeland mature rabbits were enrolled, weighing 2.5 to 3 kg (group A), and 8 adult (mixed breed) dogs weighing 22 to 25 kg (group B). The study was approved by the ethics committee of our institution (resuming European Directive 86/609/EEC). Under general anesthesia and aseptic conditions, on both hind limbs, a longitudinal skin incision was made on the medial surface of tibia. A portion of tibial diaphyseal periosteum (approximately of 5 mm x 7 mm in rabbits, and 20 mm x 30 mm in dogs) was dissected and elevated maintaining the contact of pedicle with the tibia at its proximal portion. A bone fragment, harvested with a saw, of 10/5/3 mm in rabbits, and of 20/30/5 mm in dogs (width/ length/ thickness) remains attached at the distal portion of the pedicle. The dissection of osteo-periosteal pedicle includes a vascular pedicle originating from saphenous artery and vein. Each flap consisted of four layers: cortical bone, periosteum, muscle, and fascia. The vascular patency was checked during specimen sampling. The elevated vascularized osteo-periosteal pedicle was transferred into the adjacent musculature in the pocket created in popliteal muscle.

The opposite limb served as control (reference values) for radiographic investigation and for bone mineral density (BMD) measurement.

After the surgical intervention, analgesia was provided by butorphanol (Butomidor, Richter Pharma) (0.2 mg/kg, subcutaneously q4h in the first 24 hours, and q12h in the following 48 hours).

The development and density of osteo-periosteal pedicle was evaluated weekly, postoperatively during 16 weeks by digital computer image (C-arm, Siremobil Compact L (Siemens, Germany), using an image intensifier of 7 inches). On a period of 0 to 8 weeks X-ray investigations were made to a total of eight individuals from both groups, and between 10 to 16 weeks to four individuals from both groups. These investigations aimed to establish the presence, formation, emplacement and radiodensity of the flap. Under general anaesthesia each animal was exposed under fixed irradiated conditions (50 kV in voltage, 0.4 mAS current, 70 cm distance – for dogs and 47 kV in voltage, 0.3 mAS current, 70 cm distance – for rabbits) in two views (cranio-caudal and oblique). On the

Volume : 3 | Issue : 8 | Aug 2013 | ISSN - 2249-555X

computer screen image (saved in format JPG), radioopacity of the flap shadow - mean density of blackness - was measured using a computer system with ImageJ (ver. 1.44; http://rsbweb.nih.gov/ij/download.html). The area of flap shadow was measured in each case using the same computer system and averaged for four cases of each group.

Four animals from each group were killed (using an overdose of phenobarbital) at 8 and 16 weeks postoperatively providing a number of 16 specimens from each group (eight flaps of the corticoperiosteal transplant and eight control cortical tibial fragments – opposite limb). Specimens were fixed in 10% formalin and embedded in resin (methyl methacrylate – MMA - Tehnovit 9100 polymerization system Heraeus Kulzer GmbH & Co. KG. Germany).

Dual energy X-ray absorptiometry was used for BMD measurement. The osseous specimens embedded in resin (put down as a soft-tissue equivalent) have undergone bone density determination using a Hologic Delphy W Elite, USA, densitometer and the Hologic 11.1:7 program. For accurate results the measurements of bone density were performed twice for each sample in the longitudinal and vertical plan, rotating the piece in 1800 for the second measurement. The ultrahigh-resolution protocol was used for the analysis. As part of the quality control procedure, regarding the scanned surface, if there was a difference greater than 0.1 cm^2 between two scans, the samples scanning would be resumed.

For histological examination the specimens embedded in resin were cut with anular microtome (Leica SP 1600 - Leica Microsystems GmbH Wetzlar, Germany) into 30-µm sections, stained with hematoxylin and eosin (HE), and tricrome Masson. The following parameters were evaluated: osseous resorption and quality of bone. Histomorphometry was not performed because the purpose in this study was to define the variables for optimal bone formation, but histomorphometry has been used in a larger, more defined study of prefabrication of vascularized bone graft to heal a critical-size bone defects.

The results were analyzed using Student's t-test. Significance was set at $p<0.05$. Data are reported as mean ± SD.

RESULTS

(1) Radiological Examination

A radioopaque flap shadow was observed in all cases of both groups (Fig. 1-4). The flaps shadows in group A were wider in area and higher in radioopacity than those in group B. The results of computer-image-analysis are showed in Table 1.

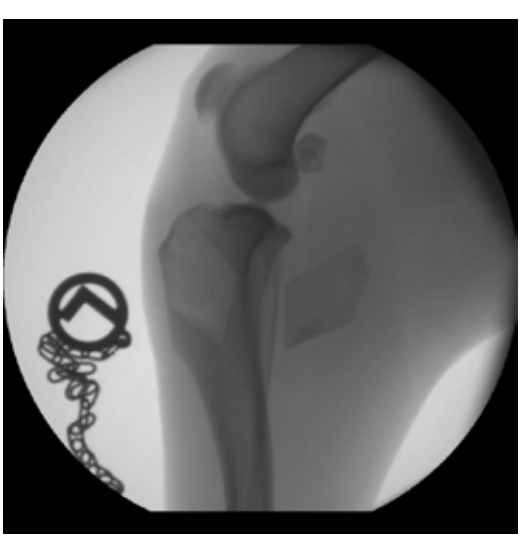

Figure 1. Computer screen image, dog postoperatory (group B, subject 5).

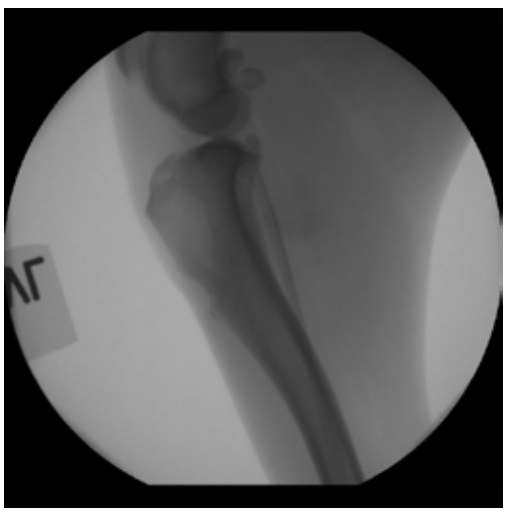

Figure 2. Computer screen image, dog at 16 weeks postoperatory (group B, subject 5).

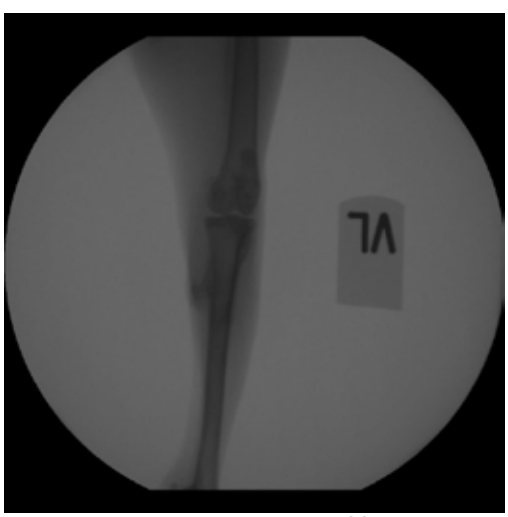

Figure 3. Computer screen image, rabbit postoperatory (group A, subject 7).

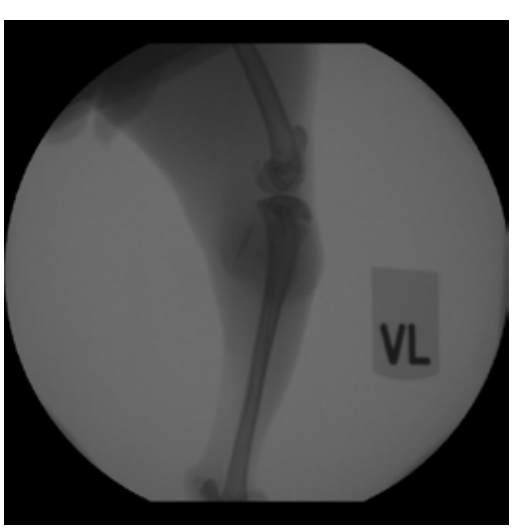

Figure 4. Computer screen image, rabbit at 16 weeks

Volume : 3 | Issue : 8 | Aug 2013 | ISSN - 2249-555X

postoperatory (group A, subject 7).

Table 1. Comparison of Group A and Group B

	Group value	Time (weeks)	Rabbits Group A		Dogs Group B	
			Mean	SD	Mean	SD
Radioopacity of the flap shadow[a]	n=8	0	62.55[b]	1.33	96.57[b]	1.25
		2	58.81[b]	1.11	103.66[b]	1.02
		4	54.04[b]	0.73	107.37[b]	0.95
		6	52.11[b]	0.68	110.30[b]	1.02
		8	50.87[b]	0.66	115.63[b]	1.23
	n=4	10	49.49[b]	0.81	120.01[b]	0.39
		12	48.68[b]	0.76	129.58[b]	0.83
		14	48.00[b]	0.52	157.95[b]	0.73
		16	47.03[b]	0.61	162.12[b]	0.80
Area of the flap shadow (mm^2)	n=8	0	33.44[b]	0.73	683.37[b]	3.81
		2	55.44[b]	1.51	608.12[b]	2.03
		4	85.62[b]	1.10	511.87[b]	2.03
		6	92.91[b]	1.27	501.75[b]	1.83
		8	99.36[b]	1.19	484.00[b]	2.39
	n=4	10	102.40[b]	1.49	458.25[b]	1.25
		12	113.83[b]	1.59	422.50[b]	1.29
		14	122.30[b]	2.83	388.50[b]	1.29
		16	127.60[b]	0.870	300.75[b]	1.25

[a]Referring density data white - 0; black - 256; air – 248; dogs: tibia - 92.6; calf muscle - 202.2; rabbits: tibia – 78.5; calf muscle - 143

[b]p <0.001 significantly different between group A and group B at measurements made at 0, 2, 4, 6, 8, 10, 12, 14 and 16 weeks.

(2) BMD Examination

There were observed variations in BMD of the vascularized corticoperiosteal flap in both groups (Table 2). In group A, at 16 weeks postintervention (p.i), the BMD increased to values close to the reference value of normal tibial bone tissue. The statistical differences between the density values at 8 and 16 weeks are insignificant. In group B, contrariwise, a marked decrease (statistical significant) of BMD was observed.

Table 2. Comparative variations in bone mineral density (g/cm^2) of the vascularized corticoperiosteal flap in rabbit versus dog at 8 and 16 weeks after intervention

Flap sample	Subject	Rabbits Group A (n=8)				Dogs Group B (n=8)			
		BMD	Mean	SD	% of reference values[a]	BMD	Mean	SD	% of reference values[a]
at 8 weeks p.i.	1	0.673	0.684[b]	0.015	97.9	0.801	0.830[b]	0.027	93.0
	2	0.692				0.823			
	3	0.702				0.830			
	4	0.671				0.867			
at 16 weeks p.i.	5	0.684	0.690[b]	0.008	98.9	0.602	0.556[b]	0.030	62.3
	6	0.682				0.538			
	7	0.692				0.543			
	8	0.700				0.544			
Total			0.687	0.011	98.4		0.693	0.148	77.6

[a] reference values = normal tibial bone tissue (dog: BMD = 0.892 g/cm^2 rabbit: BMD = 0.698 g/cm^2)

[b]p <0.001 significantly different between group A and group B in measurements made at 8 weeks, between group A and group B in measurements made at 16 weeks, and in group B between measurements made at 8 and 16 weeks .

(3) Histological Examination

In rabbits (group A), the histological exam highlights the new bone formation. The exam of the sample of corticoperiosteal fragment revealed a higher growing bone activity and also a concentrated calcification of the examined tissue.

Histological sections in rabbit showed that the regenerated tissue describes a high functional organization level.

The histological exam of corticoperiosteal flap tissue in group B revealed the presence of the osseous resorption process with large empty spaces without cells in the area where specific compact bone architecture was maintained. All these aspects corroborated with radiological observations correspond to a degenerative process of detached compact bone.

The osteoblasts, which are indicators of new osteogenesis, were in greater number in group A than in group B. There was no chondrogenesis or no inflammation in or outside the flaps of both groups.

DISCUSSION

The data that we obtained show that the ectopic implanted corticoperiosteal flaps have different behavior in rabbit than in dog. Therefore Chen's (2009) and Dailiana's (2002) studies regarding rabbit are confirmed. By comparison, the results of the experiments on dogs reveal a different behavior than in rodents, mouse (Zhang et al., 2005), rat (Kusumoto et al., 1998; Vogelin et al., 2000), and rabbit (Dailiana et al., 2002; Ortak et al., 2005; Chen et al., 2009).

The results obtained in this study in the dog contradict with the data from literature in the human patients which mention that periosteal flaps that include a bone fragment and have vascular support can generate bone tissue at the implantation site (Sauerbier et al., 2007; Kaminski et al., 2009). In some experimental cases, the transplants were in contact with deficiency areas of bone substratum – maxilla, mandible, tibia, iliac spine. The contradiction is relative, our study investigating the heterotopic transplantation (muscular).

Other results that show osteogenic potential of ectopic transplanted periosteal flaps were reported by Vögelin (2000 and 2002), in studies on rats that include the following conditions (factors) for optimal bone formation: osteoinductive bone morphogenetic protein (BMP), a biodegradable matrix, osteoprogenitor cells, and blood supply (Vogelin et al., 2000; Vogelin et al., 2002).

The studies of Kusumoto (1998), showed that ectopic osteoinduction occurred in the rat latissimus dorsi muscle flap and depended upon the dose of BMP, which suggests that osteogenesis is possible even in the absence of osteoprogenitor cells BMP addition (Kusumoto et al., 1998).

Vascularisation maintenance, an important factor of the periosteal osteogenetic capacity assurance (Fernandez-Tresguerres et al., 2006), doesn't seem to be the main trump in dog. The deficit of the circulant progenitor cells, which may be the origin of up to 50% of the osteocytes that are present in an ectopic (Otsuru et al., 2008) regenerated osseous tissue, does not explain the osteogenic reaction incapacity of the free vascularized corticoperiosteal flaps in heterotopic transplantation in the dogs.

The lack of inflammatory phenomena, on the other hand, can explain only partially the limited osteogenic capacity. The influx of undifferentiated cells and hereafter their proliferative phase and differentiation is initiated by inflammatory process (Probst & Spiegel, 1997).

Our study in the dog brings data to sustain the hypothesis that osteogenic capacity activation of the osteoprogenitor

Volume : 3 | Issue : 8 | Aug 2013 | ISSN - 2249-555X

cells contained by corticoperiosteal flaps occurs only in the condition of direct informational signals' existence from the contact with a structural bone defect and/or by addition of bone morphogenetic protein. This developmental model represents the confirmation of osteogenic reaction incapacity of the free vascularized corticoperiosteal flaps in heterotopic transplantation in the dogs.

It will be to explain away the contribution of flap to osteogenesis and the importance of mechanical stress of the transplanted flap into osseous defect.

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