Osseous regeneration in the presence of oxidized cellulose and collagen

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Oxidized cellulose and collagen are two absorbable hemostatic scaffolding materials that are used widely in surgery. A histomorphological study was undertaken to determine the tissue response and extent of healing brought about by intraosseously implanting these two materials in the femur and tibia of sheep. There was no major difference in the rate of repair of the bone defects brought about by these two materials, with the bone defects being completely repaired by lamellar bone at 6–8 weeks. Therefore, our results suggest that, in most instances where collagen is presently used in surgical applications, it could be substituted by oxidized cellulose.
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1. Introduction

Hemorrhage is encountered to a greater or lesser degree in all surgical procedures. In most oral surgical procedures, compression to the treated area may be sufficient to stop the bleeding. If bleeding persists, or occurs over a large area, and especially if it originates from cancellous bone, it may be necessary to use local hemostatic agents [1–4]. The insertion of absorbable spongy hemostatic materials into bony cavities not only induces hemostasis, but also act as scaffolding for granulation tissue [5]. When using any absorbable material to treat bone defects, its immediate effect on tissues and long-term behavior during bone healing must be considered.

For the last decade, collagen has been used extensively for its properties as a biomaterial for such purposes as soft tissue augmentation, as a bone substitute, as a hemostatic agent, and as a wound dressing for skin lesions. Its excellent biocompatibility together with biological characteristics, such as biodegradability and weak antigenicity, have made collagen the primary resource in medical applications [6]. It has therefore been used routinely in surgery [1, 4, 7–10]. However, there are a number of reports where the use of collagen grafts in the form of cadaveric dura mater has caused transfer of infectious diseases such as CJD [11–14]. Furthermore, patients generally dislike surgical implantation of products derived from animal tissues. Commercially available collagen is extracted from animal carcasses [15]. These materials have undergone extensive purification and sterilization procedures. However, with prions, most sterilization processes are not completely successful [16–19].

We felt it was appropriate to reexamine the properties of a widely used hemostatic agent, oxidized regenerated cellulose (polyanhydroglucuronic acid) [20–22], on the healing of bony defects. This paper reports on the tissue response to oxidized cellulose and collagen intraosseously implanted in the femur and tibia of adult sheep and assesses the healing of the bony defects brought about by these two materials under normal functional conditions.

2. Materials and methods

2.1. Material

Oxidized cellulose (as pellets) and collagen (insoluble type 1, bovine achilles tendon) were obtained from Sigma. Collagen suspensions were prepared by incubation of 0.8 g insoluble type I collagen in 50 ml 0.5 M acetic acid (pH 2.5) at 4 °C for 16 h and homogenization after the addition of 50 ml ice-cold distilled water in a Waring blender [23]. The collagen suspension was filtered using a Falcon nylon cell strainer 100 μm (Becton Dickinson, USA) and deaerated under vacuum to remove entrapped air bubbles. Finally the collagen was poured into polystyrene culture dishes, frozen at − 80 °C and lyophilized, resulting in porous collagen matrices of 3 mm thickness. These matrices were washed extensively in distilled water to remove any residual acetic acid, and lyophilized. The resulting fleeces were cut into small pieces (approximately 5 mm × 5 mm).

The test materials (a pellet of oxidized cellulose or a small piece of collagen fleece) were individually contained in sealed plastic packets and sterilized by

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gamma radiation (2.8 Mrads, Schering-Plough Animal Health Limited, Wellington).

2.2. Experimental procedure

Eight adult female Romney cross sheep (body weight approximately 60 kg) were used in this study. The experimental procedure described below was approved by the Animal Ethics Committee, University of Otago.

Each animal was anesthetized by intravenous thiopentone 20 mg/kg and maintained with halothane 2–4%. The hind limbs were shaven and the skin disinfected with iodine and alcohol. A surface marking for the femur was made and a skin incision, 8–10 cm length, was made along the distal two-thirds of this line. The fascia lata was exposed, was undermined through a small incision, and incised parallel to the hamstring muscles. The groove between the biceps femoris and the vastus lateralis muscles was identified, and these two muscles separated by blunt dissection. The surface of the midshaft and distal part of the femur was exposed. The periosteum at the selected sites was incised and elevated as two-sided flaps. Two cylindrical bone defects of 4 mm diameter and 3 mm depth were drilled at each of these sites with a surgical tungsten-carbide round burr using a low-speed, high torque surgical micromotor drill with saline irrigation. The defects were filled with implants of the test materials which prior to insertion were immersed in sterile saline for 10–20 s, and the periosteum replaced. The fascia lata and subcutaneous tissue were repaired in layers. A long-acting local anesthetic solution (''Merkcain'' 5 ml) was injected in close proximity to each of the surgical sites to control postoperative pain. The skin was closed with resorbable suture material using horizontal mattress sutures.

In a similar manner the anteromedial surface of the midshaft and proximal part of the tibia was exposed and pairs of cylindrical bone defects made at each site. They were filled with implants of the test materials that had been immersed briefly in sterile saline. This procedure was repeated for the femur and tibia of the other hind limb. The allocation of test materials to the surgical sites was made randomly. Long-acting tetracycline was given subcutaneously preoperatively, and at 24 h postoperatively.

The washing of the oxidized cellulose pellets in saline prior to insertion in the bone was performed to remove any surface acidic residues produced in the manufacture of this material which could cause local tissue toxicity.

Radiographs of the surgical sites in the femur and tibia of each hind limb were taken immediately postoperatively. The sheep were closely monitored and when conscious were placed in holding pens. They were kept for 4 days at the Department of Laboratory Animal Sciences, University of Otago, before being taken back to the farm. The animals were monitored at first at 12-h intervals, and then daily.

At selected time points the sheep were euthanized by intravenous injection of barbiturate 100 mg/kg. The two hind limbs were excized, radiographed, and defleshed and biopsy specimens of each femur and tibia were taken to include the surgical sites. These were fixed in 10% formalin in neutral phosphate buffer and decalcified using ethylenediaminetetraacetic acid. Wax embedded sections were cut at right angles to the original longitudinal axis of the bone defects, and stained with hematoxylin and eosin, Mallory's trichrome, and with silver stain for reticular fibers.

2.3. Histomorphology

2.3.1. Qualitative and semi-quantitative studies

Examination of the stained sections was performed by an experienced clinical histopathologist (F. Teixeira) who reported on the inflammatory response, formation of the granulation tissue/fibrous tissue capsule around the implant, and changes occurring in the implant including fragmentation and penetration by osteoid tissue.

Several morphological features were best described semi-quantitatively. These included:

1. Density of the inflammatory cell infiltrate, the cell types and their distribution with regard to the implant.
2. Fragmentation/resorption of the material.
3. Osteoblast hyperplasia in the bony walls of the cavity.

and were graded from zero (absent), + (mild), + + (moderate), and + + + (intense).

2.3.2. Quantitative studies

A representative section of each specimen was examined using an Olympus AX 70 microscope with a Plan Apo × 1.25 objective, under bright field observation. Images were captured using a high resolution Panasonic digital video camera, and processed on a Power Macintosh 8500/120 computer.

Quantitative measurements were made using the public domain program NIH Image v.1.62 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). The following parameters were measured:

1. Area of the implant, to quantitate its breakdown and elimination.
2. Area of shell of new bone deposited at the surface of the original surgically-prepared cavity, measuring separately the woven bone and the lamellar bone.
3. Maximum thickness of the granulation tissue sheath around the implant; the type of granulation tissue (early or late) was specified.
4. Percentage of the original cavity obliterated by laying down of new bone, and that occupied by the implant.

3. Results

3.1. Surgical procedure

All of the sheep recovered from the general anesthesia relatively quickly and the skin incisions were healthy, with no signs of leakage, and all were returned to the farm. All of the animals except two remained well and were free of complications. They stayed at the farm until the planned time of euthanasia which was to be 2, 6, 12,

24 weeks. However it was necessary to euthanize one of the sheep at 10 days because it was found to be lame. A radiograph showed a spiral fracture of the left femur which was probably caused by the bone defects being made too close together. As a result of this, a minimum separation of 1.5 cm for the bone defects was used in subsequent surgeries. A second sheep was euthanized at 21 days as it had developed a large discharging abscess-like lesion of the right lateral thigh over the midshaft of the right femur (no infection was found in subsequent tests). The remaining six sheep were euthanized at the chosen times of 6 weeks (2 sheep), 8 weeks (1 sheep), 12 weeks (2 sheep), and 24 weeks (1 sheep).

3.2. Histomorphology

3.2.1. Reaction to oxidized cellulose

At 10 days (Fig. 1), the implant was located inside a 5.4-mm diameter cavity in the dense cortical bone. The distance from the border of the cavity to the overlying periosteum was 1 mm, and a thin sinus perforated this thin roof. Inside the cavity, and occupying 73% of it, there was an acellular, reticulated, densely eosinophilic material, which was surrounded by fibrin, extravasated red blood cells and some neutrophils. The periosteum showed the presence of granulation tissue, constituted by proliferated fibroblasts and newly formed blood vessels, and cells differentiating into osteoblasts, that were depositing osteoid in the first step towards formation of new bone. This granulation tissue entered through the sinus towards the cavity and formed new trabeculae of woven bone, surrounded by numerous osteoblasts. The cavity, however, still kept its original size, and there was no new bone formation inside it.

At 3 weeks (Fig. 2(a)), the bony cavity was filled with granulation tissue, constituted by fibroblasts and thin-walled blood vessels. There was extensive infiltration by newly-formed trabeculae of woven bone, which obliterated the peripheral 22% of the original cavity. No part of the repaired bone had yet evolved towards a lamellar structure. There were no recognizable remains of the implant material, but at the core of the cavity there were empty spaces that measured up to 0.5 mm in diameter, and which were surrounded by a scarce inflammatory infiltrate, constituted of macrophages, some foreign-body giant cells, lymphocytes and plasma cells. Underneath the periosteum there was an area of new bone formation, above the level of the cortical defect.

At 6 weeks (Fig. 3(a)), the cavity was completely obliterated by remodeled lamellar bone, and could only be differentiated from the surrounding dense cortical bone because the bone in the cavity appeared as radially oriented trabeculae. Around these trabeculae, and in the surrounding cortical bone, osteoblasts were intensely hyperplastic. A basophilic seam line clearly delimited the boundaries of the original cavity. Between the new bony trabeculae there was mature connective tissue, heavily infiltrated by macrophages with abundant hemosiderin in their cytoplasm, and some foreign-body giant cells. There was no infiltration of inflammatory cells besides those mentioned, and no remaining woven bone.

At 8 weeks (Fig. 4(a)), it was even more difficult to detect the boundaries of the original cavity, because many of the new bony trabeculae had been replaced by thick cortical bone. Some radially-oriented trabeculae

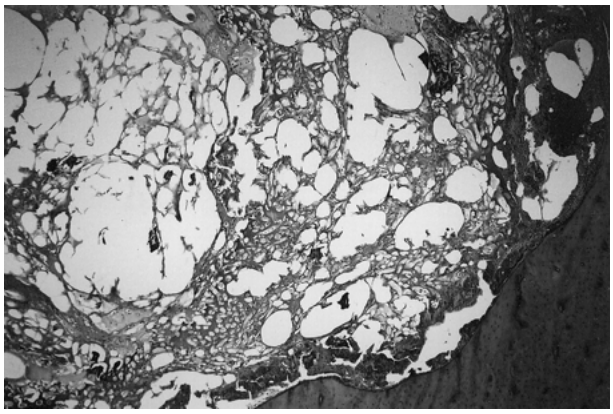
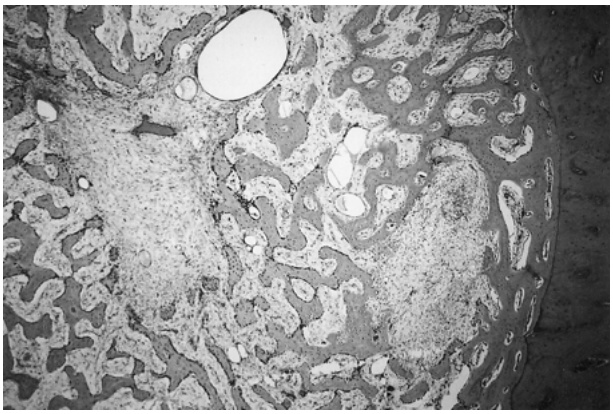
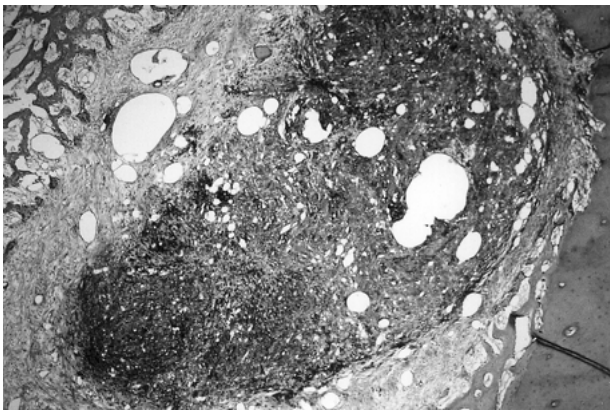


Figure 1 At 10 days: (a) Oxidized cellulose. On the right bottom corner is the border of the cavity drilled in the bone. The cavity itself is filled with an acellular, reticulated material, which is separated from the walls of the cavity by a thin layer of blood clot. There is no bone repair at this stage. All the sections in Figs. 1 to 5 were stained with hematoxylin and eosin, and all the photographs were printed at a final magnification of × 45.

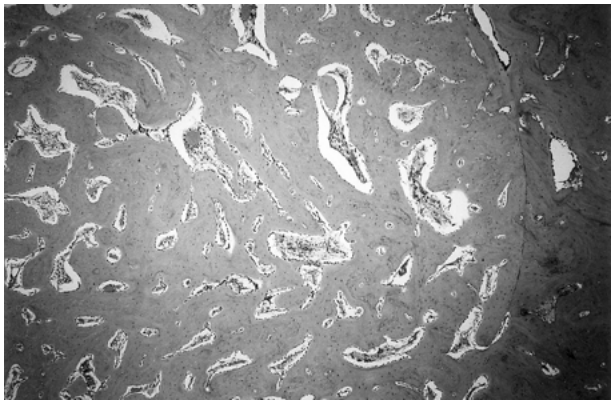


(a)



(b)

Figure 2 At 3 weeks: (a) Oxidized cellulose. The implanted material has been completely reabsorbed, and in its place there are few empty looking spaces (top center). The cavity is extensively infiltrated by newly-formed trabeculae of woven bone, surrounded by numerous proliferated osteoblasts. (b) Collagen sponge. The cavity is filled by tissue disposed in three concentrical layers: a peripheral layer of few and thin trabeculae of woven bone, an intermediate layer of granulation tissue, and a central area, densely infiltrated by macrophages and foreign-body giant cells. Comparison with Figure 2(a), shows that repair seems to be slower, and inflammation more intense, when collagen sponge is used.



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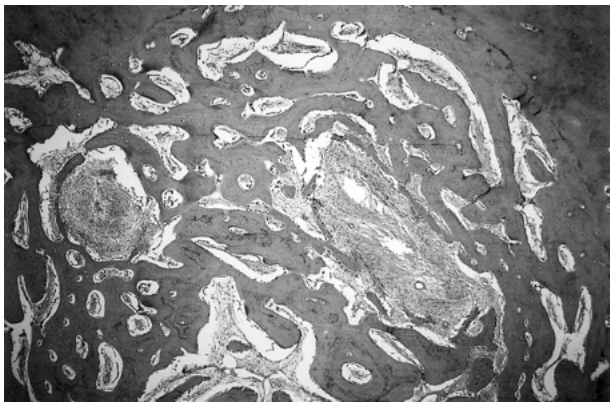
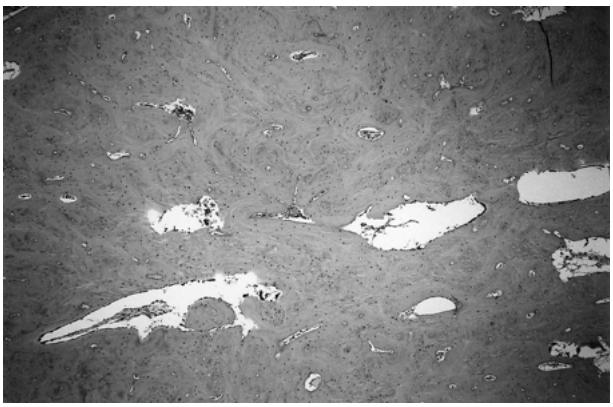


(b)

Figure 3 At 6 weeks: (a) Oxidized cellulose. The cavity is completely obliterated by repaired lamellar bone. A cement line (right) clearly separates the newly formed bone from the original border of the cavity. (b) Collagen sponge. Approximately 80% of the cavity has been repaired by thin trabeculae of lamellar bone. At the same stage, Oxidized cellulose-treated animals showed complete repair of the defect.



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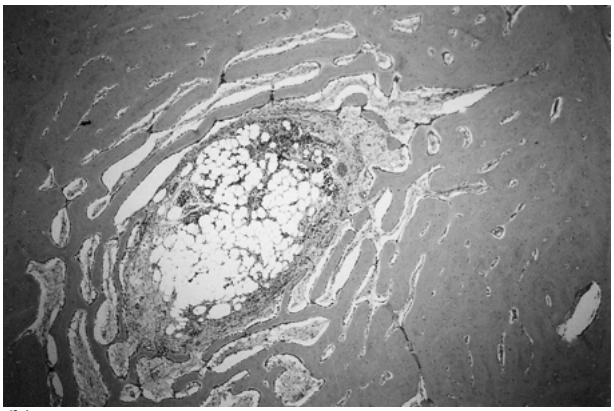


(b)

Figure 4 At 8 weeks: (a) Oxidized cellulose. The thickness of the trabeculae of new lamellar bone is increased, and the fibrovascular spaces are few and small, so that the new bone is quite compact. (b) Collagen sponge. Repair is not yet complete, and a central area of connective tissue and inflammatory infiltrate is still present. The peripheral trabeculae are starting to thicken.

lined with very abundant osteoblasts still remained. In the mature connective tissue located between the trabeculae, there were still occasional foreign-body giant cell and some hemosiderin-loaded macrophages.

At 12 weeks (Fig. 5(a)), dense lamellar bone had completely filled the cavity, and the only indications about its previous state were the larger and more numerous connective-vascular spaces, as compared with the surrounding cortex. These cavities were lined by numerous osteoblasts, which seemed to be building Haversian systems of higher order. By 24 weeks, the sizes of the connective-vascular spaces were even smaller, as the thickness of the trabeculae increased.

3.2.2. Reaction to collagen sponge

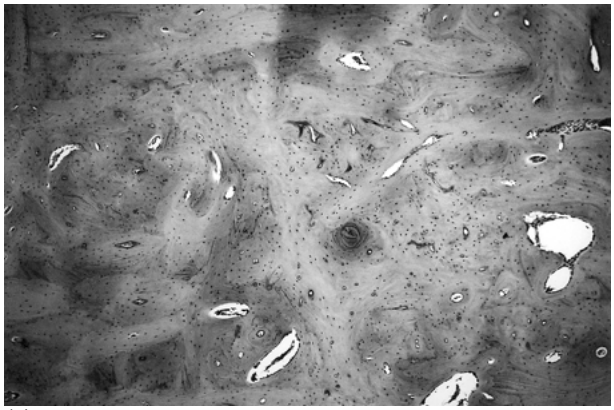
At 10 days, the collagen implant was not recovered as this animal had suffered a spiral fracture of the left femur where the implant had been inserted. No other collagen implants had been inserted in this animal.

At 3 weeks (Fig. 2(b)), the bony cavity was still quite evident, and was filled by three different concentric layers. The first layer, adjacent to the margins of the defect, was composed of thin trabeculae of newly formed woven bone, surrounded by very numerous osteoblasts. This layer occupied the peripheral 45% of the original cavity. No lamellar bone was recognizable at this stage. The middle layer showed granulation tissue, with thin-walled, newly formed blood vessels and plump fibroblasts. The central core of the cavity was filled with granular basophilic material, which was being extensively phagocytosed by numerous macrophages and some foreign-body giant cells. Some small empty-looking areas were present in this central core, and were surrounded by the same cell types.

At 6 weeks (Fig. 3(b)), the three layers described previously were still present, but the middle and inner layers constituted approximately 20% of the lumen of the original cavity. The layer adjacent to the margins of the defect was now very thick, and composed of trabeculae of lamellar bone, surrounded by numerous osteoblasts. The middle layer was composed of mature connective tissue, which was breached by thin trabeculae of woven bone. The central core contained empty-looking spaces, surrounded by numerous macrophages and foreign-body giant cells. The implanted material seemed to have been completely phagocytosed and could not be recognized.

At 8 weeks (Fig. 4(b)), most of the original cavity had been repaired by newly formed trabeculae of lamellar bone. Osteoblasts surrounding these trabeculae were still numerous, and the peripheral trabeculae were much thicker than at 6 weeks. The middle layer of connective tissue was extensively crossed by thin trabeculae of woven bone. The core of the cavity still showed numerous macrophages and foreign-body type giant cells.

At 12 weeks (Fig. 5(b)), the borders of the original cavity were difficult to identify, and the cavity was filled with thick trabeculae of lamellar bone. The only



(a)

(b)

Figure 5 At 12 weeks: (a) Oxidized cellulose. Dense lamellar bone has replaced the cavity. Osteoblasts are still numerous. At all stages, the inflammatory infiltrate elicited by oxidized cellulose was of mild intensity. (b) Collagen sponge. Repair is complete and the borders of the original cavity are difficult to identify. The bony trabeculae are much thicker, and osteoblasts seem to be still active.

indication of the surgical site was the presence of more numerous and larger fibrovascular spaces in the repaired bone, as compared to the surrounding cortical bone. These spaces were lined by proliferated osteoblasts, and, by 24 weeks, they were still larger than those seen at the same time in lesions healed with oxidized cellulose.

3.2.3. Semi-quantitative and quantitative studies

Semi-quantitative evaluations are shown in Table I. There seemed to be faster resorption of oxidized cellulose than collagen. With oxidized cellulose, there was a mild neutrophilic infiltrate at 10 days, which was replaced by a moderate lympho-histiocytic infiltrate by 3 weeks. By 6 weeks, only some hemosiderin-laden macrophages were seen in the connective tissue between the bone trabeculae. In contrast, collagen elicited a very marked inflammatory infiltrate, which persisted for several weeks. Osteoblast hyperplasia in the pre-existing, surrounding bone was most evident at 6 weeks, but tended to decrease after complete obliteration of the original cavity.

Table II shows the progression of repair in osseous wounds which were implanted with either material. There was slight variation between the sizes of the original drilled cavities. Hence, the data are expressed as the percentage of the original cavity that, in the process of repair, was filled by granulation or connective tissue, the implant material, or new bone. At 6 and 8 weeks, the lamellae of woven bone were very thin and small with irregular contours in animals treated with collagen. There were no measurements at 10 days owing to loss of the bone in which the collagen had been implanted.

4. Discussion

The ideal material to fill surgical cavities in bone should promote hemostasis, be resorbable, not interfere with the healing process, be readily obtainable and inexpensive. Collagen has been used for this purpose. However as it is derived from animal carcasses, there exists the possibility, although small, of transmitting infectious agents such as prions. Also patient sensitivity to insertion of materials derived from animal carcasses has to be taken into consideration. As oxidized cellulose is manufactured from plant material, these concerns do not apply and it is of low cost.

The present study has examined the properties of oxidized cellulose and compared these with collagen when implanted in surgically-made defects in the femur and tibia of the hind limbs of adult sheep. This exposed the implants to normal functional loads which are relatively high in these limbs, and differed from a previous study in the rabbit where the two materials were implanted in the mandible [4]. The sheep is a recommended animal model for long-term implantation studies in bone [24]. Our results indicated that both

TABLE I Semi-quantitative histological assessment

Time	Test material	Inflammation	Resorption of test material	Osteoblast hyperplasia
10 days	Oxycell	+ (N)	+	0
3 weeks	Oxycell	+ + (M,L)	+ +	+ +
	Coll	+ + + (M,L)	+ +	+
6 weeks	Oxycell	+ (M)	+ + +	+ + +
	Coll	+ + (M,L)	+ +	+ + +
8 weeks	Oxycell	+ (M)	+ + +	+ +
	Coll	+ + (M,L)	+ +	+ + +
12 weeks	Oxycell	0	+ + +	+ +
	Coll	+ (M)	+ + +	+ +
24 weeks	Oxycell	0	+ + +	+ +
	Coll	0	+ + +	+ +

Oxycell = oxidized cellulose; Coll = collagen sponge; N = neutrophils; M = macrophages; L = lymphocytes; + = mild; + + = moderate; + + + = intense.

TABLE II Tissues and materials obliterating the original cavity, expressed as percentages by volume

Time	Test material	GT/CT	Implant	New bone	
				Lamellar	Woven
10 days	Oxycell	0	73.4*	0	0
3 weeks	Oxycell	77.7	0	0	22.3
	Coll	54.9	0	0	45.1
6 weeks	Oxycell	0	0	100	0
	Coll†	18.9/6.6	0/3.0	81.1/91.4	N.M.
8 weeks	Oxycell	0	0	100	0
	Coll	4.0	0.9	95.1	N.M.
12 weeks	Oxycell†	0/0	0/0	100/100	0/0
	Coll†	0/0	0/0	100/100	0/0

Oxycell = oxidized cellulose; Coll = collagen sponge; GT = granulation tissue; CT = connective tissue; N.M. = not measured (very small amount only).
*Within the cavity there was the implant material together with a thin layer of blood clot.
†2 animals in this category.

materials, oxidized cellulose and collagen, were effective in the healing of bone defects. The rate of repair of the bone defect brought about by oxidized cellulose is at least as fast as that obtained with collagen, and the defects were completely replaced by lamellar bone at 6–8 weeks.

The results of this study are not in agreement with those of Lattes and Frantz [25] who reported that oxidized cellulose interfered with the normal healing process of bone. Also the neurotoxicity due to diffusion of acidic products from the oxidized cellulose reported by Nagamatsu and Low [26] and Nagamatsu *et al*. [27] is not applicable to the present study as this material was not implanted in close proximity to large peripheral nerves. It is noteworthy that the oxidized cellulose used in our study was obtained from Sigma and was of research grade and high purity. Surgicel, a commercial brand of oxidized cellulose manufactured by Johnson & Johnson, is the type of oxidized cellulose used in surgery [28]. This is the material that has been used in most of the previous studies with oxidized cellulose.

In conclusion, we suggest that in instances where collagen is presently used in surgical applications, it could be substituted by oxidized cellulose. Oxidized cellulose is cheaper to manufacture, has been used in surgery for several decades, and is derived from plant material. However, the lower physical properties of oxidized cellulose [28] and the high tensile strength of collagen [29, 30] need to be taken into consideration when surgical choices are being made. In future work it may be possible to reduce the acidity of oxidized cellulose by controlling its degree of oxidation, and improve its physical properties by combining it with other biocompatible materials, e.g. hyaluronic acid [31] or hydroxyapatite [32].

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