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Tissue response of defined collagen–elastin scaffolds in young and adult rats with special attention to calcification

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Abstract

Collagen–elastin scaffolds may be valuable biomaterials for tissue engineering because they combine tensile strength with elasticity. In this study, the tissue response to and the calcification of these scaffolds were evaluated. In particular, the hypothesis was tested that calcification, a common phenomenon in biomaterials, may be due to microfibrils within the elastic fibre, and that these microfibrils might generate a tissue response. Four scaffolds were subcutaneously implanted, viz. collagen, collagen + pure elastin, collagen + microfibril-containing, and collagen + pulverised elastic ligament (the source for elastin). Explants were evaluated at day 3, 7 and 21.

In young Sprague Dawley rats, collagen + ligament calcified substantially, whereas collagen + elastin (with and without microfibrils) calcified less, and collagen did not. Calcification started at elastic fibres. In both Sprague Dawley and Wistar adult rats, however, none of the scaffolds calcified. Mononuclear cell infiltration was prominent in young and adult Sprague Dawley rats. In adult Wistar rats, this infiltration was associated with the presence of microfibrils.

Degradation of scaffolds and new matrix formation were related with cellular influx and degree of vascularisation.

In conclusion, absence of microfibrils from the elastic fibre does not prevent calcification in young Sprague Dawley rats, but does reduce the tissue response in adult Wistar rats. Cellular response and calcification differs with age and strain and therefore the choice of animal model is of key importance in biomaterial evaluation.

Keywords: Biomaterial; Tissue engineering; Calcification; Tissue response; Biocompatibility

1. Introduction

Tissue engineering involves the development of innovative biomaterials to replace or repair damaged and defective tissue. Type I collagen and elastin are structural extracellular matrix proteins that are abundantly present in human tissues and form natural scaffolds in the body. Collagen provides tensile strength and is widely used as a biomaterial, e.g. in skin substitutes, vascular grafts, scaffolds for cartilage repair, and bone implants [1–3]. Elastin provides elasticity to tissues. This, together with its stability due to specific interchain crosslinks, makes elastin a desirable protein for tissue engineering [4]. Recently, it has been demonstrated that the elastin is capable of regulating proliferation, migration and differentiation of vascular smooth muscle cells, and that it reduces the vascular proliferative response to arterial injury in vivo [5]. This makes the application of elastin in tissue engineering even more significant. However, unlike type I collagen, elastin has found little use as a biomaterial [6–8]. One reason is that the elastin preparations have a strong tendency to calcify upon implantation [9]. Another is that the purification of elastin is complex [10]. Calcification may be due to microfibrillar components within the elastic fibre that are difficult to remove. A major component in this respect is fibrillin, which binds calcium due to its many calcium-binding EGF domains [11]. In dystrophic calcinosis cutis, mineralisation of the

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elastic fibre has been shown to start at microfibrils present within and surrounding the elastic fibre [12]. In general, calcification is a major, not well-understood problem in biomaterial application. Especially cardiovascular prosthetic implants such as bioprosthetic heart valves, aortic homografts and trileaflet polymeric valve prostheses are prone to calcify. Crosslinked cellular debris, collagen and elastin have been suggested as the onsets for calcification [13–15]. We previously described the preparation and biochemical, biomechanical, immunohistochemical and in vitro evaluation of collagen–elastin–glycosaminoglycan scaffolds [16]. In this study, we investigated whether purification of elastin, i.e. removal of microfibrils, can (partially) prevent calcification and may influence the tissue response such as cellular events and matrix remodelling. For this reason, we prepared four different scaffolds using purified collagen, purified elastin, partly purified elastin (i.e. with microfibrils) and elastic ligament (the source for elastin), and implanted these subcutaneously in rats. Different rat models were used to monitor variations between rat strain and age on the outcome of the experiment.

2. Materials and methods

2.1. Materials

Four different biomaterials were prepared: (1) purified type I collagen, (2) purified insoluble elastin, (3) partly purified insoluble elastin, still containing microfibrils and some collagen, and (4) pulverised ligamentum nuchae, the source for elastin. Insoluble type I collagen was purified from bovine achilles tendon using diluted acetic acid, NaCl, urea, and acetone extractions as described [17]. Insoluble elastin was isolated from equine ligamentum nuchae using extractions with NaCl, organic solvents, CNBr in formic acid, urea with diluted 2-mercaptoethanol, and digestion with trypsin, essentially as described, but with omission of the collagenase digestion [10]. Microfibril-containing insoluble elastin was prepared using only one NaCl extraction, organic solvents extractions and CNBr in formic acid treatment. Pulverised equine ligamentum nuchae was prepared using a Fritsch Pulverisette 19 with a 1 mm sieve (Idar-Oberstein, Germany).

Mouse anti-bovine elastin IgG (clone BA-4) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Rabbit anti-bovine type I collagen, rabbit anti-rat type I collagen, and rabbit anti-rat type III collagen IgGs were from Chemicon (Temecula, CA, USA). Goat anti-human type IV collagen IgG was from Southern Biotechnology Inc. (Birmingham, AL, USA). Alexa 488-labelled goat anti-mouse IgG, Alexa 488-labelled goat anti-rabbit IgG, and Alexa 488-labelled donkey anti-goat IgG were from Molecular Probes Europe (Leiden, The Netherlands).

2.2. Preparation of scaffolds

Four different scaffolds were prepared composed of purified collagen (COL), purified collagen + purified elastin in a 1:1 ratio (COL-EL), purified collagen + microfibril-containing elastin in a 1:1 ratio (COL-mfEL), and purified collagen + pulverised elastic ligament in a 1:1 ratio (COL–Ligament). Collagen was incorporated into all scaffolds, because we were unable to prepare coherent scaffolds of elastin only. Scaffolds were prepared by lyophilising an acidic 1% suspension of the various preparations. All scaffolds were chemically crosslinked for 4 h with 33 mM 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) and 6 mM *N*-hydroxysuccinimide (NHS) in 50 mM 2-morpholinoethane sulphonic acid (MES) (pH 5.5) in the presence of 40% (v/v) ethanol. After reaction, the scaffolds were washed in 0.1 M Na_2HPO_4 (pH 9.1), 1 M NaCl, 2 M NaCl, and demineralised water [16,17].

2.3. Characterisation of elastin-containing preparations and scaffolds

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (*SDS-PAGE*): Purified elastin, microfibril-containing elastin, and pulverised ligamentum nuchae preparations were incubated at 95°C for 10 min under reducing conditions (5% (v/v) 2-mercaptoethanol) and analysed on a 10% (w/v) gel. Non-elastin components will penetrate the gel and are visualised by a Coomassie Brilliant Blue solution (0.1% (w/v)) [18].

Transmission electron microscopy (*TEM*): TEM was used to analyse the presence/absence of microfibrils and other (extra)cellular components. Purified elastin, microfibril-containing elastin, and pulverised ligamentum nuchae preparations were embedded in 1.5% (w/v) agarose, fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C, and post fixed with 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h. After a rinsing period of 3 h with 0.1 M phosphate buffer (pH 7.4), the samples were dehydrated in an ascending series of ethanols and embedded in Epon 812. Ultrathin sections (60 nm) were picked up on formvar-coated grids, post stained with lead citrate and uranyl acetate, and examined in a JEOL 1010 electron microscope.

Scanning electron microscopy (*SEM*): SEM was used to analyse the morphology of the scaffolds. Scaffolds were mounted on stubs and sputtered with an ultrathin layer of gold in a Polaron E5100 SEM coating system. The specimens were studied with a JEOL JSM-6310 SEM apparatus with an accelerating voltage of 15 kV.

Amine group content: The amine group content of scaffolds was determined spectrophotometrically after reaction with 2,4,6-trinitrobenzene sulfonic acid [19].

2.4. Implantation of scaffolds

NIH guidelines for the care and use of laboratory animals (NIH publication 85-23 Rev. 1985) were observed. The study was approved by the Ethical Committee of the University of Nijmegen. Sprague-Dawley rats (male, 21 days old and 3 months old) and Wistar rats (male, 3 months old) were purchased from Harlan (Zeist, The Netherlands). Young rats were housed per 3, and adult rats per 2. The animals were fed pelleted diet (RMH-B 10 mm) and water ad libitum.

Scaffolds were washed in 70% (v/v) ethanol (4×30 min) and sterile phosphate-buffered saline (PBS) (pH 7.2) (8×30 min) at 22°C. Rats were anaesthetised with isoflurane. After disinfection, subcutaneous pockets were made to the right and left of two midline incisions on the back. Punches ($\varnothing$ 6 mm) of the scaffolds were implanted in the pockets at a distance of about 1 cm from the incisions (4 implants/rat). For each implantation time, two rats received two punches of the same scaffold on one side of the midline incision. Implants with surrounding tissue were harvested from Sprague Dawley rats at 3, 7 and 21 days after implantation, from Wistar rats at 21 days only.

2.5. Processing of explants

Immediately after explantation, scaffolds were divided in two halves. One half was processed for conventional light microscopy, the other half for immunohistochemistry.

2.5.1. Conventional histology

Scaffolds and surrounding tissue were fixed in 4% (v/v) formaldehyde in phosphate buffer (pH 7.2) immediately after explantation for at least 24 h at 4°C, and embedded in paraffin. Consecutive sections of 5 μm were mounted onto glass slides, dewaxed in xylol and hydrated through a graded series of ethanol. Sections were routinely haematoxylin–eosin stained. Elastin and collagen were stained according to Elastin Van Gieson (EVG), and calcium deposits were visualised by Von Kossa staining [20]. The sections of the explants were semi-quantitatively scored independently by at least two experienced investigators.

2.5.2. Immunohistochemistry

Immunofluorescence microscopy was used to study the biodegradation of scaffold collagen and elastin, and the formation of new collagens by the host. Scaffolds were frozen in liquid nitrogen. Cryosections were mounted onto glass slides. After blocking with 1% (w/v) bovine serum albumin (BSA) in PBS, sections were incubated with mouse anti-bovine elastin (1:1000), rabbit anti-bovine type I collagen, rabbit anti-rat type I collagen, rabbit anti-rat type III collagen (all 1:100), or goat anti-human type IV collagen (1:50) for 90 min, washed with PBS, followed by a 1 h incubation with Alexa 488-labelled secondary antibodies (1:100). All antibodies were diluted in PBS containing 1% (w/v) BSA. Sections were washed and mounted in mowiol. The antibody to bovine elastin also reacts with equine elastin; the antibody to human type IV collagen cross-reacts with rat type IV collagen; the antibody to bovine type I collagen does not substantially cross-react with rat type I collagen or vice versa.

3. Results

3.1. Characteristics of elastin preparations

Elastin preparations were studied using SDS-PAGE and TEM. SDS-PAGE of the elastin preparations indicated no contamination with other proteins in purified elastin, whereas microfibril-containing elastin and pulverised elastic ligament contained a large number of additional proteins (Fig. 1A). TEM indicated that the purified elastin fibres were free of microfibrils and no other structural elements could be detected (Fig. 1B). In microfibril-containing elastin, microfibrils could be easily observed as well as some residual collagen fibrils, but without any other contaminations (Fig. 1C). Pulverised elastic ligament contained all tissue elements, including damaged cells (Fig. 1D).

3.2. Characteristics of scaffolds

The 3D morphology of the scaffolds was analysed with SEM (Fig. 2). Collagen was present as fine fibrillar and sheet-like structures, whereas elastin was observed as thick fibres. Pulverised ligament showed bundles of elastic fibres, since the collagenous meshwork between individual elastic fibres was not removed.

The amine group content was measured to investigate the extent of EDC/NHS crosslinking. In all scaffolds, 35–40% of amine groups were used in crosslinking. Collagen possesses a larger amount of amine groups than elastin, which explains the absolute values for the different preparations (Table 1).

3.3. Calcification of scaffolds

All animals remained in good condition and no infectious problems occurred. Macroscopically, a thin capsule was observed around the scaffolds at explantation. In Table 2 an overview of calcification behaviour and cellular response is given.

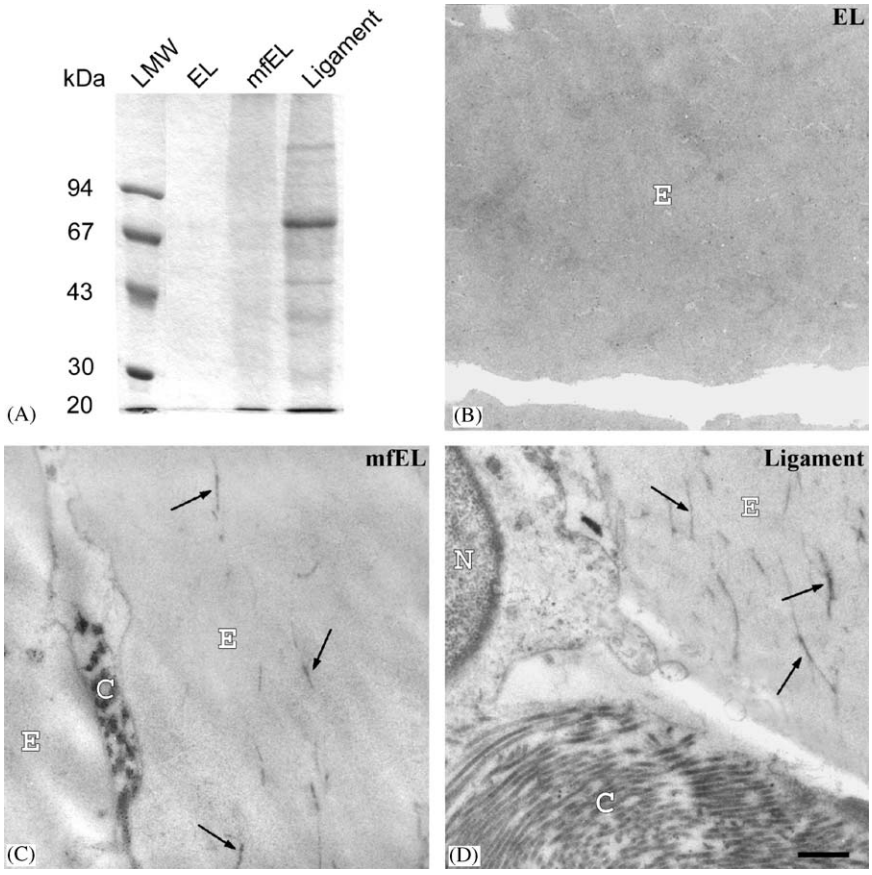

Fig. 1. (A) SDS-PAGE gel of highly purified elastin (EL; 0.5 mg), microfibril-containing elastin (mfEL; 0.5 mg) and pulverised ligament (0.1 mg). Only soluble contaminants and/or elastin breakdown products will enter the gel; insoluble elastin, which represents the vast majority of the preparations, does not. (B–D) Transmission electron micrographs of highly purified elastin (B), partly purified microfibril-containing elastin (C), and pulverised elastic ligament (D). C = collagen; E = elastin; N = nucleus of fibroblast; arrows indicate microfibrils. Bar is 0.5 μm.

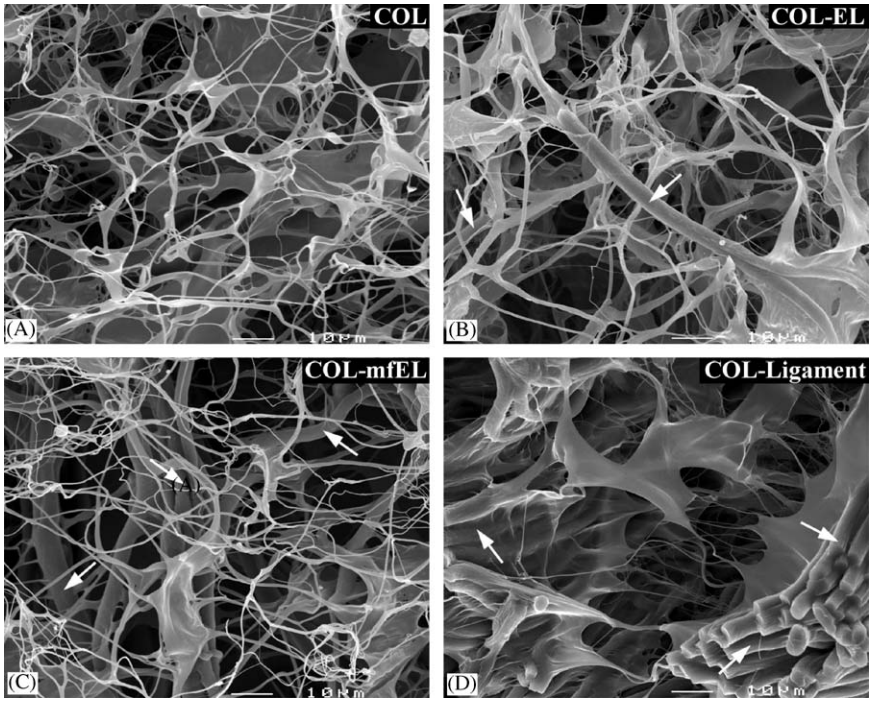

Fig. 2. Scanning electron micrographs of the crosslinked scaffolds. (A) COL, (B) COL-EL, (C), COL-mfEL, and (D) COL-ligament. Collagen is present as thin fibrils and sheets, elastin as thick fibres. Arrows indicate elastic fibres. Bar is 10 μm.

Table 1
Amine group content of various scaffolds

Scaffold	Crosslinked with EDC/NHS	Amine group content (nmol/mg)
COL	–	294±1
COL	+	191±10
COL-EL	–	155±8
COL-EL	+	98±8
COL-mfEL	–	163±11
COL-mfEL	+	97±17
COL-ligament	–	232±7
COL-ligament	+	137±10

COL: scaffold composed of collagen only; COL-EL: scaffold composed of equal amounts of collagen and highly purified elastin; COL-mfEL: scaffold composed of equal amounts of collagen and microfibril-containing elastin; COL-ligament: scaffold composed of equal amounts of collagen and pulverised ligamentum nuchae. Results are mean±SD of three individual experiments.

Young Sprague Dawley rats: COL did not calcify in young Sprague Dawley rats except one (out of 4) at day 21 as was assessed by Von Kossa staining. At day 3, some calcification could be observed in COL-ligament where it was present within the elastic fibres. Calcification did not seem to be dependent on local cell infiltration, since calcification developed in cell-free as well as cell-dense areas. All other scaffolds showed no calcification at this time point. At day 7, all of the composite scaffolds (COL-EL, COL-mfEL, COL-ligament) showed calcification to some extent. COL-ligament showed intensive calcification at elastic fibres only. At day 21, calcification of COL-EL, COL-mfEL, and COL-ligament had increased (Fig. 3). COL-EL and COL-mfEL calcified to about the same extent. In COL-ligament, calcification had extended to collagen.

Adult Sprague Dawley rats and *Adult Wistar rats:* All scaffolds implanted in adult rats did not show any calcification up to day 21.

3.4. Tissue response to scaffolds

Figs. 4 and 5 show some representative examples of the tissue response to scaffolds in young and adult Sprague Dawley rats and adult Wistar rats at different time points (see Table 2).

3.4.1. Young Sprague Dawley rats

At day 3, several macrophages and some polymorphic nuclear granulocytes (PMNs) were present, mostly at the periphery of the scaffold. More PMNs were present near elastic fibres than near collagen, especially in COL-ligament and COL-mfEL. At day 7, a fibrous capsule was observed around the scaffolds. Some capillaries were located in the capsule and at the periphery of the scaffolds. At this time, PMNs were only sporadically present, while early events of mononuclear cell infiltrations were found. Mononuclear cells include phagocytic cells like active monocyte-derived macrophages, monocytes as well as non-phagocytic cells such as lymphocytes, plasma cells and mast cells. Foreign body giant cells (fused macrophages with multiple nuclei) were encountered in variable amount. At day 21, capillaries were present in between the fibres of the scaffolds. Phagocytic cells dominated the scaffolds, while the amount of non-phagocytic cells increased at the periphery, especially near COL-ligament and COL-mfEL (Fig. 4).

3.4.2. Adult Sprague Dawley rats

At day 3, all implants were encapsulated with a fibrous capsule. Some PMNs had infiltrated mainly COL-mfEL and COL-ligament (Fig. 5A and B), while more phagocytic cells infiltrated COL and COL-EL. At day 7, PMNs were only sporadically found. Increased fibrous encapsulation was commonly present with capillaries both intracapsularly and at the periphery of the scaffolds. Phagocytic cells accumulated both at the periphery and to less extent in the scaffold. Most of these cells were present in COL-ligament, less in COL-mfEL and COL-EL, and only at the periphery of COL. (Fig. 5C and D). At day 21, capillaries were found throughout COL-ligament, COL-mfEL and COL-EL and these scaffolds were almost completely infiltrated with phagocytic cells, but to less extent in COL.

3.4.3. Adult Wistar rats

Scaffolds from adult Wistar rats were only explanted at day 21 (Fig. 5E and F). At this time, a fibrous capsule was formed around all scaffolds. In general, the tissue response of adult Wistar rats to implanted scaffolds was distinctly milder compared to Sprague Dawley rats. Few cells had infiltrated the scaffolds. COL and COL-EL showed few mononuclear and giant cells at the periphery. In COL-mfEL and COL-ligament, these cells had infiltrated deeper into the scaffolds.

3.5. Vascularisation of the scaffolds

Young Sprague Dawley rats: Few small blood vessels, visualised by immunostaining for type IV collagen present in endothelial basement membranes, were found at day 7 at the periphery of the scaffolds. At day 21, some blood vessels were found at the periphery of COL, while other scaffolds showed more vascularisation at that time, both at the periphery and within the scaffolds.

Adult Sprague Dawley rats: Vascularisation increased over time. In general, COL-EL, COL-mfEL and COL-ligament contained more blood vessels than COL. At day 7, vascularisation of COL-EL, COL-mfEL and COL-ligament was mainly in the capsule and at the periphery of the scaffolds; at day 21 it was also within the scaffold (Fig. 6A and Fig. 6B).

Table 2
General overview of calcification and cellular events after subcutaneous implantation of the scaffolds in rats

Scaffold	Rat strain and age	Implantation time (days)	Calcification	PMNs	Phagocytic and giant cells[a]	Non-phagocytic cells	Blood vessels[b]
COL	Sprague Dawley	3	−	sp	±	−	−
	3 weeks	7	−	sp	+±	±	±
		21	−[c]	−	++±	+	+
COL-EL	Sprague Dawley	3	−	±	+	−	−
	3 weeks	7	±	sp	+±	±	+
		21	+	−	+++	+±	++
COL-mfEL	Sprague Dawley	3	−	+	+	−	−
	3 weeks	7	±	sp	+	±	+
		21	+	−	++	++	+±
COL-ligament	Sprague Dawley	3	sp	+	±	−	±
	3 weeks	7	+±	sp	±	+±	+
		21	+++	−	+±	++±	+±
COL	Sprague Dawley	3	−	sp	±	sp	−
	3 months	7	−	±	±	+±	±
		21	−	sp	++	±	+
COL-EL	Sprague Dawley	3	−	sp	±	sp	sp
	3 months	7	−	sp	+±	±	+±
		21	−	sp	+++	+	++
COL-mfEL	Sprague Dawley	3	−	±	sp	sp	sp
	3 months	7	−	sp	++±	+	+
		21	−	sp	+++	+	+±
COL-ligament	Sprague Dawley	3	−	+	sp	sp	±
	3 months	7	−	sp	++++	+	+
		21	−	sp	+++	+	+±
COL	Wistar						
	3 months	21	−	−	±	−	−
COL-EL	Wistar						
	3 months	21	−	−	+	−	sp
COL-mfEL	Wistar						
	3 months	21	−	−	+±	+	±
COL-ligament	Wistar						
	3 months	21	−	−	+	+	±

COL: crosslinked collagen scaffold; COL-EL: crosslinked scaffold composed of equal amounts of collagen and highly purified elastin; COL-mfEL: crosslinked scaffold composed of equal amounts of collagen and microfibril-containing elastin; COL-ligament: crosslinked scaffold composed of equal amounts of collagen and pulverised ligamentum nuchae. PMNs = polymorphic nuclear cells, i.e. granulocytes; phagocytic cells include monocyte-derived macrophages and monocytes; non-phagocytic cells include lymphocytes, plasma cells and mast cells.
Events were scored ranging from sporadic (sp) to severe (++++). −: not present.

[a] Absolute numbers of non-phagocytic cells were lower than of phagocytic and giant cells, e.g. ++ for phagocytic and giant cells refers to more cells than ++ for non-phagocytic cells.

[b] Blood vessels were assessed by immunostaining for rat type IV collagen, present in vascular basement membranes.

[c] One out of four implanted collagen scaffolds showed moderate calcification at this time point.

Adult Wistar rats: Few blood vessels were only found at the periphery of COL-mfEL and COL-ligament.

3.6. Scaffold degradation and new matrix synthesis

Table 3 gives an overview of the immunofluorescence staining for scaffold components and for newly formed extracellular matrix molecules at different time points.

3.6.1. Scaffold degradation

Young Sprague Dawley rats: Conventional histology showed small amounts of degraded collagen which were locally visible from day 7 on at the periphery. At day 21, degraded collagen was also found deeper within the scaffolds, but especially centrally a large amount of intact scaffold collagen remained present. Immunostaining for bovine type I collagen supported these findings. At day 21, the presence of thin and fragmented collagen structures were most profound at the scaffold periphery. Locally, fragmented elastin in COL-EL, COL-mfEL and COL-ligament was found from day 7 on. At day 21, the total amount of elastic fibres was decreased and more fragmented elastic fibres were observed, suggesting resorption and degradation of the fibres. Both calcified elastin/elastic fibres and non-calcified elastin/elastic fibres were found within macrophages and giant cells. Immunofluorescence results showed that staining for elastin gave the best signal for COL-EL, most likely due

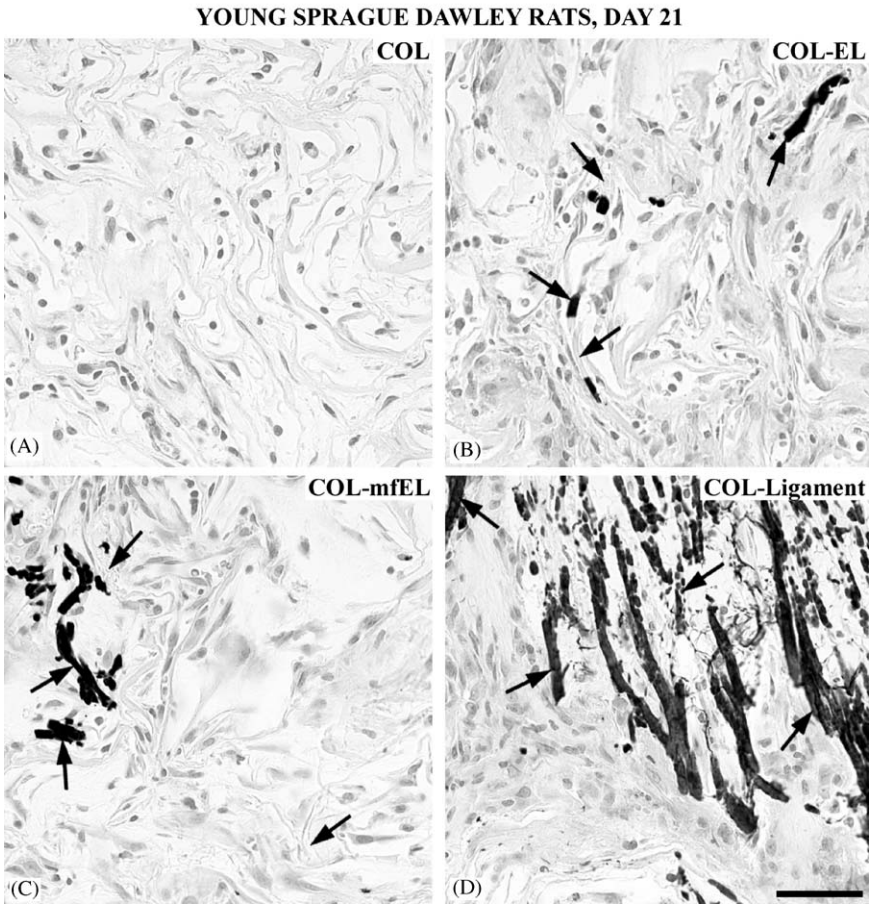

Fig. 3. Light microscopical evaluation of the calcification of scaffolds, 21 days after implantation in young Sprague Dawley rats. (A) COL, (B) COL-EL, (C), COL-mfEL, and (D) COL-ligament. Calcium deposits have an intense black colour due to Von Kossa staining. Arrows indicate elastic fibres. Bar is 50 μm.

to unmasking of the epitope during purification. Calcified elastin could also be visualised with the antibody. The fragments of elastin (smaller than elastic fibres) were found in all elastin-containing scaffolds at day 21.

Adult Sprague Dawley rats: Degraded collagen was visible at the periphery at day 7. Fragmented elastin (both EL and mfEL) was visible locally from day 7 on. Especially in COL-ligament scaffolds, severely fragmented elastic fibres were present at day 21, suggesting fastest degradation of these scaffolds.

Adult Wistar rats: At day 21, a large amount of scaffold collagen and elastin was still present in adult Wistar rats, implying the lowest degradation rate of scaffolds in all rat models used. Only locally, degraded collagen fibrils and elastic fibres were observed at the periphery for all scaffolds.

3.6.2. Matrix synthesis

Young Sprague Dawley rats: Newly formed rat type I collagen was generally found in the fibrous capsule surrounding the implant and aligning with the collagen fibrils in the scaffold. At day 21, young Sprague Dawley rats showed more staining for type I collagen in elastin-containing scaffolds than in collagen scaffolds. From day 7 on, type III collagen could be clearly observed in the fibrous capsule and at the periphery of the scaffolds. At day 21, type III collagen was present throughout the scaffolds, aligning with the collagenous and elastinous scaffold components. Staining for type III collagen in COL was less intense than in the other scaffolds.

Adult Sprague Dawley rats: In adult Sprague Dawley rats, staining for rat type I and III collagen was similar to staining in young Sprague Dawley rats. Whereas at day 7 type III collagen was mainly present in the capsule and the outer part of the scaffolds, at day 21 it was found throughout the scaffold (Fig. 6C and D).

Adult Wistar rats: At day 21, minor amounts of new matrix collagens were observed in scaffolds implanted in adult Wistar rats. Type I collagen was sporadically found in all scaffolds, more type III collagen was found in COL-mfEL and COL-ligament scaffolds than in COL and COL-EL.

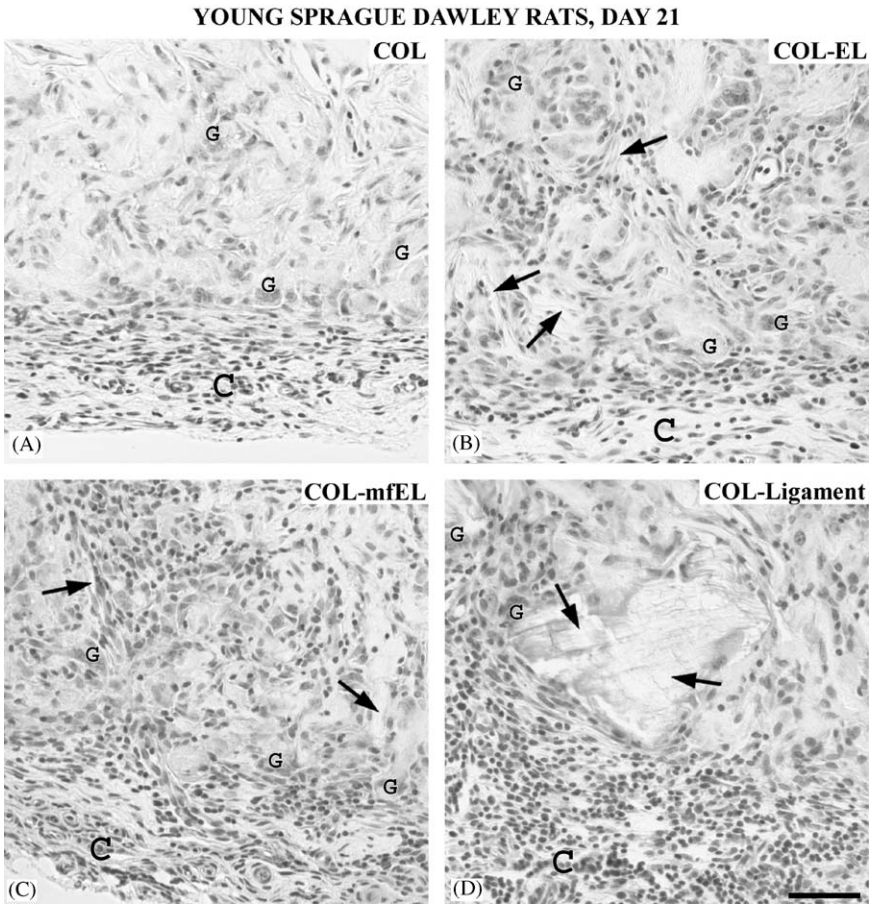

Fig. 4. Light microscopical overview of the cellular response to scaffolds at day 21 in young Sprague Dawley rats. (A) COL, (B) COL-EL, (C), COL-mfEL, and (D) COL-ligament. Note the increased amount of mononuclear cell infiltration, especially in COL-Ligament scaffolds. Sections were HE stained. C = capsule; G = giant cell; arrows indicate elastic fibres. Bar is 50 μm.

4. Discussion

Tissue response to new biomaterials has to be evaluated before their use in tissue engineering for clinical purpose. Here, we studied the tissue response and calcification of subcutaneous implanted porous biomaterials composed of either collagen only (COL), collagen + highly purified insoluble elastin in a 1:1 ratio (COL-EL), collagen + microfibril-containing (i.e. partly purified) elastin in a 1:1 ratio (COL-mfEL), and collagen + pulverised ligament in a 1:1 ratio (COL-ligament). Young and adult Sprague Dawley rats and adult Wistar rats were used as animal models.

The juvenile rat is widely accepted as a sensitive model to evaluate calcification [9,13,21,22]. In our experiment, calcification only occurred when elastin-containing scaffolds were implanted in young Sprague Dawley rats (3 weeks old at implantation), but no calcification was found when scaffolds were implanted in adult Sprague Dawley or Wistar rats (3 months old). Calcification of implanted biomaterials depends on many variables. One of them is the animal's age. Nimni et al. already found that after 2 weeks skin collagen subcutaneously implanted in young Long Evans rats (1 month old) calcified much more than in older ones (8 months old) [23]. Another parameter for calcification may be the purity of the material. In our experiment, collagen scaffolds did not give rise to calcification in young rats (only 1 out of 12 showed calcification). Using a purified acellular intestinal collagen layer, Abraham et al. did not find calcification either [24]. With less-defined collagen preparations, calcium deposits are formed indicating that contaminants may trigger calcification [14,23,25]. This partially applies to elastin as well. Calcification started within the elastic fibre [15]. Calcification was seen from day 3 for COL-ligament scaffolds, and from day 7 for COL-mfEL and COL-EL scaffolds. Although absence of microfibrils from the elastic fibre did not result in a decrease of calcification, calcification was more severe if an impure preparation (i.e. pulverised ligament) was used. Our hypothesis that calcification may be partly due to the presence of microfibrils within the elastic fibre could be rejected in this study. Another calcification variable might be

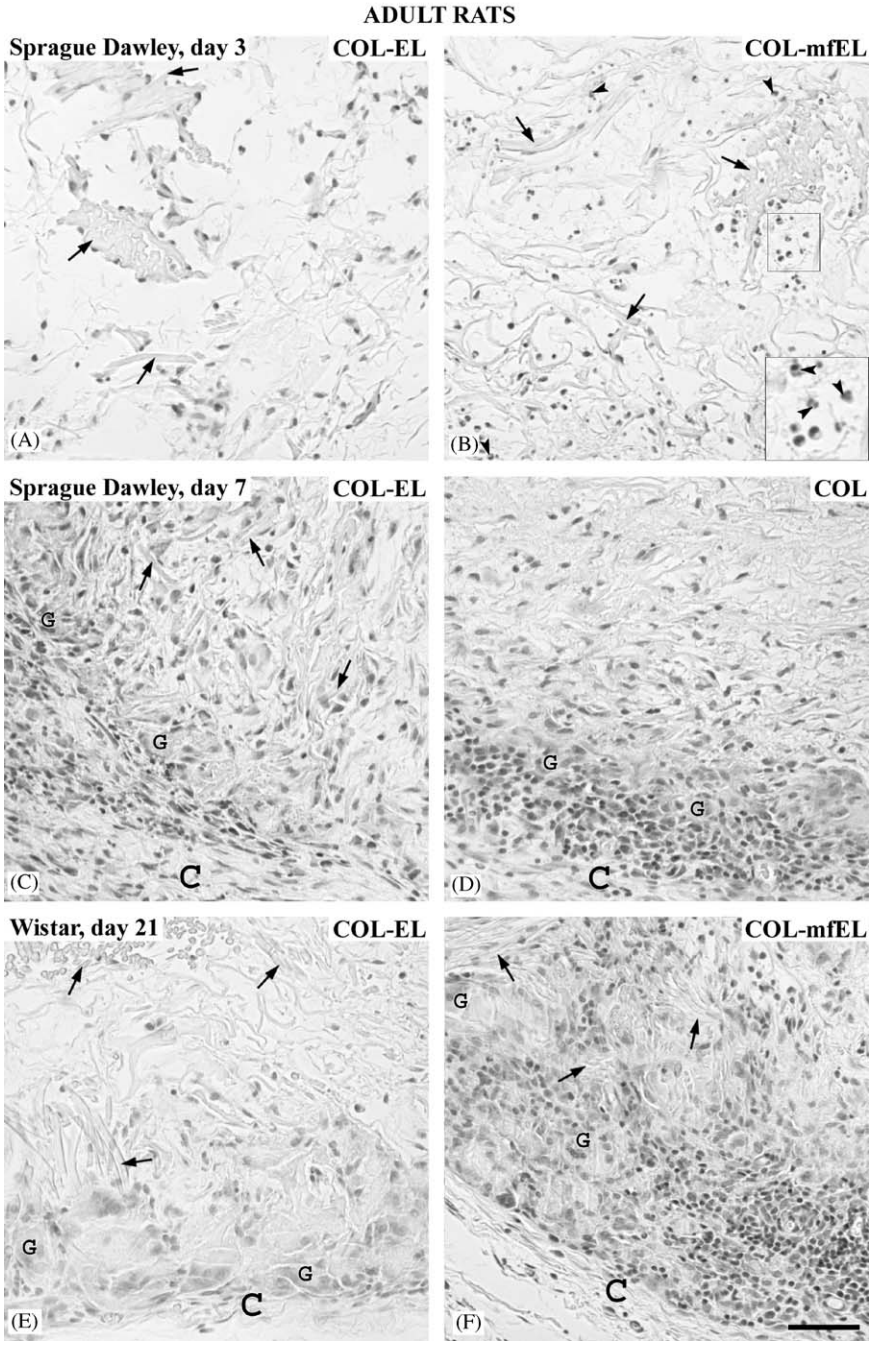

Fig. 5. Light microscopical evaluation of the cellular response to scaffolds implanted in adult Sprague Dawley rats (A–D) and adult Wistar rats (E–F). (A) COL-EL and (B) COL-mfEL 3 days after implantation. Note that more PMN's (neutrophiles) are present in COL-mfEL than in COL-EL. (C) COL-EL and (D) COL 7 days after implantation. In COL, an increase of mononuclear cells, especially non-phagocytic cells, was found at the periphery of the scaffold. (E) COL-EL and (F) COL-mfEL 21 days after implantation. In COL-mfEL, locally an increase of mononuclear cells was found. Sections were HE stained. C = capsule; G = giant cell; arrow heads indicate neutrophiles; arrows indicate elastic fibres. Bar is 50 μm.

imputed to the intactness of the elastic fibre. Bailey et al. recently found that fragmented and damaged aortic elastic fibres are associated with more severe calcification than intact ones [26]. Our purified elastic fibres are still intact, though. Calcification may be genetically controlled by molecules that actively inhibit calcification and may occur passively when these inhibitors are absent [27]. Other possible solutions to prevent calcification are aluminium chloride treatment [9], ethanol/EDTA treatment [28], and attachment of glycosaminoglycans [29,30].

This study underlines that young Sprague Dawley rats are a sensitive model to study calcification. Apart from that, an accompanying strong tissue response was

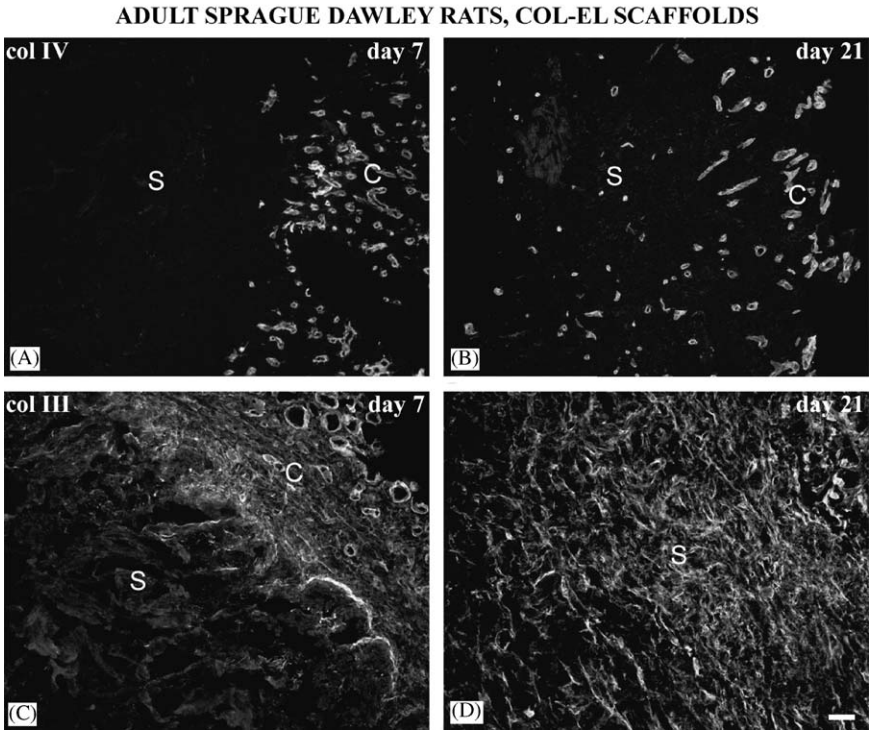

Fig. 6. Immunostaining of COL-EL scaffolds for types IV and III collagen as indicators of vascularisation and matrix production after subcutaneous implantation in adult Sprague Dawley rats. (A) COL-EL after 7 and (B) after 21 days immunostained for type IV collagen. At day 7, type IV collagen staining is mainly present at the periphery, at day 21 in the whole scaffold. (C) COL-EL after 7 days and (D) after 21 days immunostained for type III collagen. At day 7, type III collagen staining is mainly present at the scaffold periphery, at day 21 it is found throughout the scaffold. C = capsule; S = scaffold. Bar is 50 μm.

characteristic of these rats. Major cellular infiltrations were found in COL-EL, COL-mfEL and COL-ligament scaffolds at day 21. Van Wachem et al. observed a huge infiltration of lymphocytes, including numerous plasma cells, after subcutaneous implantation of porcine heart valve cusps and walls into young Sprague Dawley rats [31]. However, in our scaffolds, we found substantially less mononuclear cells in both young and adult Sprague Dawley rats. Adult Wistar rats showed an even milder tissue response to the implanted scaffolds. Elastin without microfibrils resulted in less infiltration of mononuclear cells compared to microfibril-containing elastin. For tissue engineering purposes, fibres of highly purified elastin are thus preferred to microfibril-containing elastic fibres.

Scaffolds showed to be biodegradable by all hosts, as expected, but none of the scaffolds was fully resorbed at day 21. Degradation was associated with phagocytic cells, and calcified elastic fibres were found in macrophages and to a certain extent in giant cells. More degradation was found in Sprague Dawley rats, which was underlined by the higher numbers of phagocytic cells in these scaffolds. Minor collagen degradation of crosslinked collagen scaffolds took place in Wistar rats, which was comparable with results described by Pieper et al. [32]. In this rat strain, elastin was only locally degraded. Collagen–elastin scaffolds may thus function as a temporary elastic matrix here [16]. Type I and type III collagens were made by all hosts. Generally, with a more extensive tissue response, new matrix formation and vascularisation occurred both earlier in time and to a larger extent. This means that in both young and adult Sprague Dawley rats more new matrix components were produced than in Wistar rats. In most cases, the presence of elastin in the scaffold was associated with a higher degree of vascularisation.

5. Conclusions

Elastin-containing scaffolds did not calcify in adult rats. In young rats, however, calcification of scaffolds containing elastin or elastic fibres was observed and removal of microfibrils from the elastic fibre did not prevent or reduce this. Absence of microfibrils reduced the tissue response in adult Wistar rats. Tissue response to and calcification of scaffolds differed with age, strain and purity of the materials and therefore the choice of animal model is of key importance when evaluating biomaterials for their capacity to function as a temporary matrix for tissue engineering and matrix remodelling.

Table 3
Overview of immunostaining results of explants for scaffold and rat extracellular matrix components

Scaffold	Rat strain and age	Implantation time (days)	Scaffold collagen	Scaffold elastin	Rat type I collagen	Rat type III collagen
COL	Sprague Dawley	3	++	–	sp	–
	3 weeks	7	++	–	±	±
		21	+±	–	±	+
COL-EL	Sprague Dawley	3	++	++	sp	–
	3 weeks	7	++	++	±	+
		21	+	+	+	++
COL-mfEL	Sprague Dawley	3	++	++	sp	–
	3 weeks	7	++	++	±	+
		21	+	+	+	++
COL-ligament	Sprague Dawley	3	++	++	sp	±
	3 weeks	7	++	+±	+	+
		21	+	±	+±	++
COL	Sprague Dawley	3	++	–	sp	–
	3 months	7	++	–	±	+
		21	+±	–	±	+
COL-EL	Sprague Dawley	3	++	++	sp	sp
	3 months	7	+±	+±	±	+
		21	+	+	+	++
COL-mfEL	Sprague Dawley	3	++	++	sp	sp
	3 months	7	+±	+±	±	+
		21	+	+	+±	++
COL-ligament	Sprague Dawley	3	++	++	sp	±
	3 months	7	+	+	+	+
		21	±	±	+±	++
COL	Wistar					
	3 months	21	++	–	sp	±
COL-EL	Wistar					
	3 months	21	++	++	sp	±
COL-mfEL	Wistar					
	3 months	21	++	++	sp	+
COL-ligament	Wistar					
	3 months	21	++	++	sp	+

COL: crosslinked collagen scaffold; COL-EL: crosslinked scaffold composed of equal amounts of collagen and highly purified elastin; COL-mfEL: crosslinked scaffold composed of equal amounts of collagen and microfibril-containing elastin; COL-ligament: crosslinked scaffold composed of equal amounts of collagen and pulverised ligamentum nuchae.
Events were scored ranging from sporadic (sp) to abundant (++). -: not present.

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