Eggshell membrane as a biodegradable bone regeneration inhibitor

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Abstract

The efficiency of chicken eggshell membranes combined with a minimally invasive small osteotomy procedure of the ulna to accomplish an efficient release of the radius so that it can continue to grow in an unstressed manner was tested in rabbits. Eggshell membranes were extracted from chicken eggs, rinsed, dried and sterilized with ethylene oxide for 24 h. For reactivity testing, four separate subcutaneous pockets were created in 10 rats in the paravertebral region by blunt dissection and eggshell membranes were implanted in two of them. After 1–16 weeks, the implants were retrieved with the surrounding soft tissues and submitted to histological examination. Subsequently, 10 rabbits were anaesthetized and a complete 0.5 mm wide osteotomy was performed in both the right and the left distal ulna. A piece of eggshell membranes was interposed in the osteotomy site of one ulna. The opposite osteotomized ulna was left as a negative control. The rabbits were injected with oxytetracycline at the time of surgery and this was repeated every 7 days for labelling new bone formation. After 1–16 weeks, ulnar osteotomized regions were histologically examined. After histological, fluorescence microcopy and radiological evaluation, we demonstrate here for the first time that eggshell membranes as interpositional material in rabbit osteotomized ulnar experiments acted as an active barrier against bone bridging. The degradation of the eggshell membrane, due to host reaction, appeared sufficiently late to cause the desirable delay of bone healing that is compatible with the time needed for a corrective response.

1. Introduction

Much effort has gone into making artificial bone grafts work better and more efficiently, especially in highly comminuted fractures or those with a large amount of bone loss, where the quality of the graft is often the determining factor in success (healing) or failure (non-union) of bone regeneration. Non-union current solutions include transfer of vascularized bone, distraction osteogenesis and the use of cancellous autografts, especially when defects are $\geq$4–5 cm (de Boer and Wood, 1989; May et al., 1989; Han et al., 1992).

Forelimb growth deformities are commonly a result of an irreversible injury to the growth plate of the physis, resulting in premature physeal closure. The distal radial and ulnar physes are responsible for most of the bone length, with the distal radial physis determining 60% of radial length and the distal ulnar physis determining 85% of ulnar length (Fox et al., 2006). The distal ulnar physis is most commonly injured, causing excessive radial procurvatum with external rotation and carpal valgus, potentially resulting in osteoarthritis in the carpal and elbow joints (Fox et al., 2006).

Young patients with open physes still have growth potential and can be treated with the excision of a section

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of bone (>2 cm) or ostectomy using an oscillating saw, Gigli wire or osteotome, and mallet ulnar osteotomies, until they reach skeletal maturity. The osteotomy will effectively release the radius so that it can continue to grow in an unstressed manner. However, the ulna normally reunites quickly after ulnar osteotomy, thus re-establishing the restraining force on the radius (Newton, 1974). To prevent early union, different procedures have been used, including the interposition of an autologous fat graft harvested from subcutaneous to avoid bone healing (Newton, 1974; Lau, 1977; Egger and Stoll, 1978; Vandewater et al.,1982; Piermatei and Flo,1997). Disadvantages in this procedure are: (a) the second incision that must be performed over healthy tissue in order to harvest the autologous fat graft; (b) the large piece of bone that must be removed, together with all of the periosteum at the ostectomy site; (c) the gap closing before patient reaches skeletal maturity, which often leads to the need for a second ostectomy (Vandewater et al., 1982).

Type X collagen is a short-chain homotrimeric collagen expressed in the hypertrophic zone of growth plate calcifying cartilage, sternum and bone fracture repair (Capasso et al.,1982; Schmid and Conrad, 1982; Schmid and Linsenmayer, 1983; Gibson et al., 1984; Kielty et al., 1985; Solursh et al., 1986; Grant et al., 1987; Jacenko et al., 1991; Olsen et al., 2000; Shen, 2005). During endochondral ossification, chondrocytes undergo a sequence of proliferation, maturation and hypertrophy changes which are accompanied by changes in the synthesis and deposition of extracellular matrix components (Ortega et al., 2004). However, after two decades of research, the functional role of type X collagen remains to be elucidated. Although some authors support the notion that type X collagen is actively involved in regulating calcification (Capasso et al., 1984; Habuchi et al., 1985; Schmid and Linsenmayer, 1985a, 1985b; Leboy et al., 1988; Tacchetti et al., 1989; Kwan et al., 1991; Kirsch et al., 1992), other studies indicate both an inhibitory or a non-direct effect of type X collagen on mineralization (Poole and Pidoux, 1989; Poole et al., 1989; Lu Valle et al., 1992; Walker et al., 1995; Arias et al., 1997).

It has been previously shown that non-mineralized eggshell membranes (ESM) (Figure 1) are composed mainly of type X collagen (Arias et al., 1991a, 1991b; Fernandez et al., 2001) and that this collagen functions to inhibit cellular mineralization and thereby establishes boundaries which are protected from mineral deposition (Poole et al., 1989). Eggshell membranes are non-toxic to cells (Arias et al., 1997, 2007; Jia et al., 2008; Kim et al., 2007; Zadik, 2007) and, when tested in vivo, they fail to guide bone regeneration in cranial defects (Dupoirieux et al., 1999, 2001; Durmus et al., 2003). If eggshell membrane inhibits bone formation at the cellular level, then it is expected that the interposition of eggshell membranes in a small bone defect will interfere with normal bone healing.

To overcome the problems of harvesting fat grafts, osteotomizing a large piece of bone and trying to discourage bone healing, we here present evidence of the usefulness of chicken eggshell membranes combined with a minimally invasive small osteotomy procedure to delay bone formation, avoiding secondary surgical procedures.

2. Materials and methods

2.1. Eggshell membrane (ESM) collection and preparation

Eggshell membranes of White Leghorn hen eggs were obtained by opening the eggshell at the opposite pole to the air chamber, then emptying the albumen and yolk and thoroughly washed with distilled water several times. The emptied eggs were filled with 1% acetic acid solution for 10 min, then the membranes were manually extracted, rinsed and dried at 37 °C for 24 h and cut in sheets of 1 cm^2. The sheets were then individually packed and sterilized with ethylene oxide for 24 h at room temperature.

2.2. Rat subcutaneous *in vivo* implantation

All procedures were approved by the Committee of Animal Bioethics of the Faculty of Veterinary Sciences, University of Chile, which is in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS).

In order to assess the reaction to the eggshell membranes as a resorbable biomaterial for implant
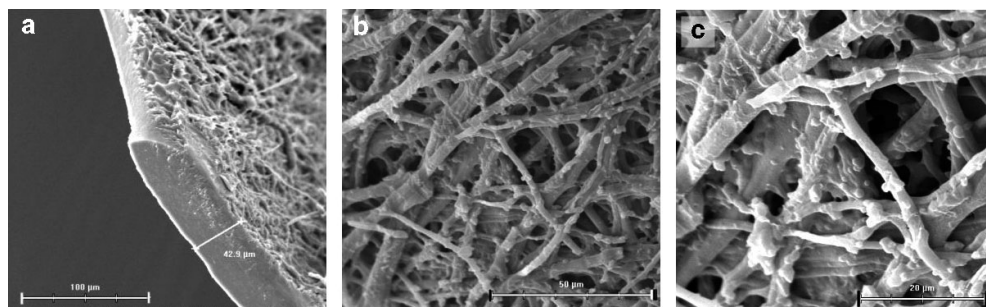

Figure 1. Scanning electron micrograph of (a) side view of a section of avian eggshell membranes and (b, c) top view of the same sections

application, pieces of the membrane were previously tested in rats according to ASTM F1983 (2003) and Upman *et al*. (2003). 10 Adult male Sprague–Dawley white rats, 200–300 g in weight, were divided into five groups of two individuals. The animals were anaesthetized by intraperitoneal (i.p.) injection of ketamina–diazepam mixture in doses of 75 mg and 5 mg/kg, respectively. Surgery was performed under sterile conditions.

Through 1 cm incisions, four separate subcutaneous pockets were created in the paravertebral region by blunt dissection in each rat. 0.5 cm^2 pieces of sterilized membrane were implanted in either the symmetrical left or right pockets at the level of the shoulder, and another piece of membrane was implanted in the opposite pocket at the level of the thigh. The other pockets were left empty as a negative control. Wound closure was accomplished by a simple discontinuous skin pattern (4-0 nylon).

The animals were euthanized at 1, 2, 4, 8 and 16 weeks and the implants were retrieved with the surrounding soft tissue. The specimens were then fixed in a 10% formaldehyde solution and embedded in paraffin. Serial cross-sections (5 μm) were made through the implant and stained with haematoxylin & eosin (H&E). Mallory stain was also used for fibrillar collagen staining. Each specimen was examined by light microscopy for histological evaluation of inflammation, fibrosis and osteogenesis.
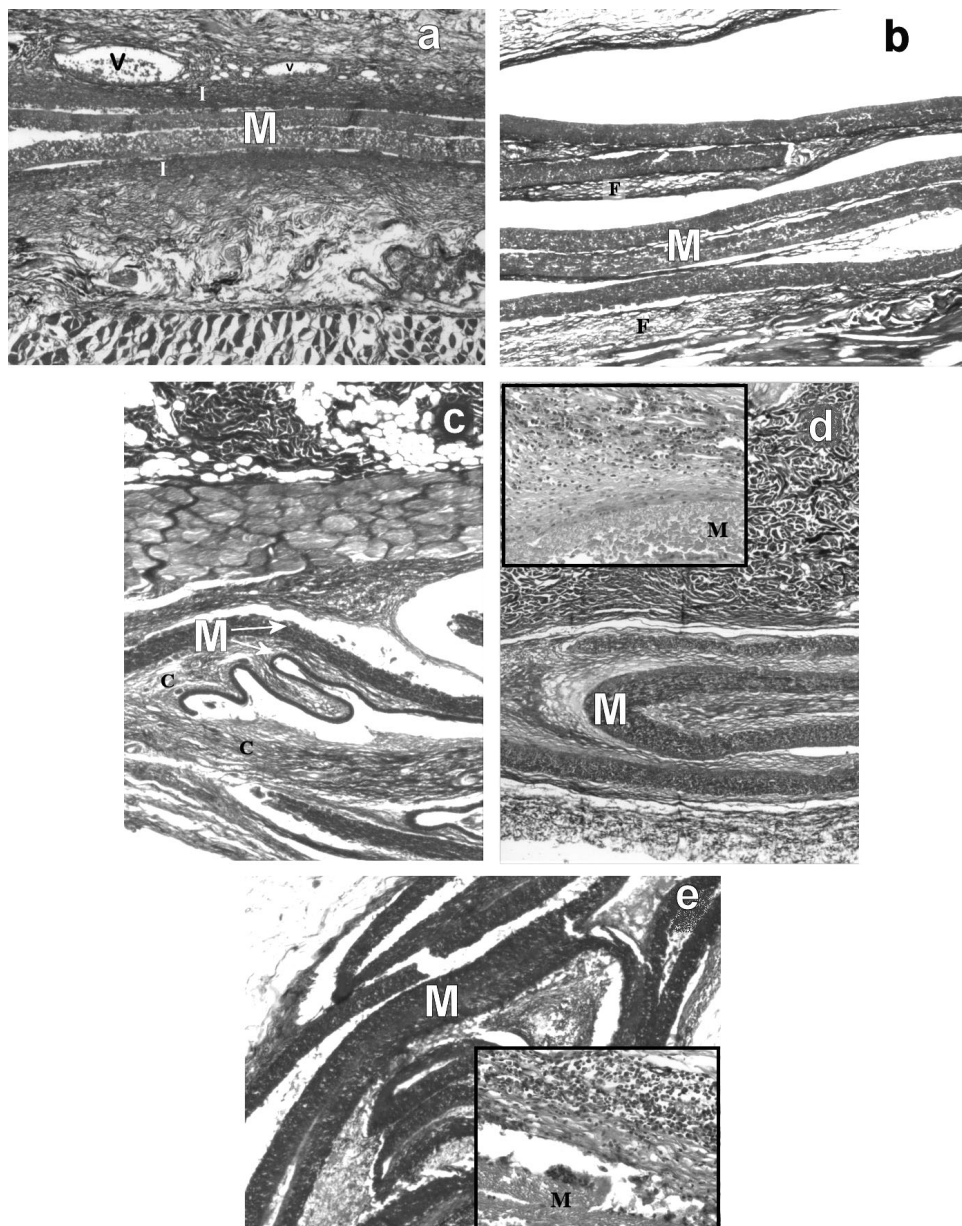

Figure 2. Subcutaneously implanted avian eggshell membrane (M) stained with Mallory after (a) 1, (b) 2, (c) 4, (d) 8 and (e) 16 weeks of implantation (×40). V, angiogenic process; I, inflammatory response: F, fibrotic process; C, infiltrative collagen tissue. Giant cells, infiltrative lymphocytes, macrophages and plasmocytes along with haemosiderin deposits stained with H&E are shown in the insert to (d) (×400). H&E staining shows lymphocyte infiltration and giant cells around and between the fibres of the implanted membrane, evidencing the degradation of collagen fibres [insert to (e)] (×400)

2.3. Rabbit *in vivo* ostectomy and implantation of eggshell membrane

In order to assess the effect of eggshell membranes as a surgical implant to effect bone healing (ASTM F981, 2004), 10 adult male New Zealand White rabbits, 2–3 months old and 3–3.5 kg in weight, divided into five groups of two individuals, were used. The animals were then anaesthetized via isoflurane inhalation and operated on under sterile conditions.

The left or right ulnas were approached through 1–1.5 cm incisions in a caudo-lateral manner over the distal one-third of the ulna and the lateral digital extensor muscle separated from the extensor carpi ulnaris to expose the distal ulna. After elevating the surrounding musculature to isolate the ulna just proximal to the physis, a complete 0.5 mm wide osteotomy was performed using an oscillating saw. Then a 1 cm^2 piece of ESM was fold in two and interposed into the osteotomy site. The fixation of the implant was accomplished by two simple discontinuous sutures (4-0 nylon) of the ESM against the periosteum of the cranial and caudal face of the proximal fragment. The opposite ulna was only osteotomized and left for natural healing as a negative control.

Wound closure was accomplished by a simple discontinuous subcutaneous pattern (4-0 polyglactin 910) with a simple discontinuous skin pattern (4-0 nylon) and ketoprofen (1 mg/kg every 24 h for 3 days) was administered as analgesic/anti-inflamatory treatment. The rabbits were injected with 25 mg/kg oxytetracycline at time 0 and this was repeated every 7 days in order to fluorescently mark the newly deposited bone in the healing osteotomy.

The animals were euthanized at 1, 2, 4, 8 and 16 weeks and X-rays were taken. Implanted and negative ulnas were retrieved with the surrounding soft tissues and cut into two saggital halves. One half was fixed in a 10% formaldehyde solution, decalcified with 10% formic acid and embedded in paraffin. Serial sagittal sections (5 µm) were made through the implant and were stained with H&E and Mallory stains to be examined by light microscopy for histological evaluation of inflammation, fibrosis and osteogenesis. The other half was fixed in Alfac fixative solution (8.5 ml 80% ethanol, 1 ml 40% formaldehyde and 0.5 ml glacial acetic acid), dehydrated with 95% ethanol and embedded in Lowicryl. Sagittal 1 mm sections across the long axis were obtained using an Isomed Diamond Blade Low Speed Saw (Buehler) and reduced to a final thickness of 300 µm by wet grinding on a Knuth-Rotor grinding machine, then mounted on slides to be examined by fluorescent light microscopy for neo-osteogenesis.

3. Results

3.1. Histological results in rat subcutaneous

In the first week of implantation there is a fibroblastic response that can be evidenced by the encapsulation of the intact eggshell membrane by about a
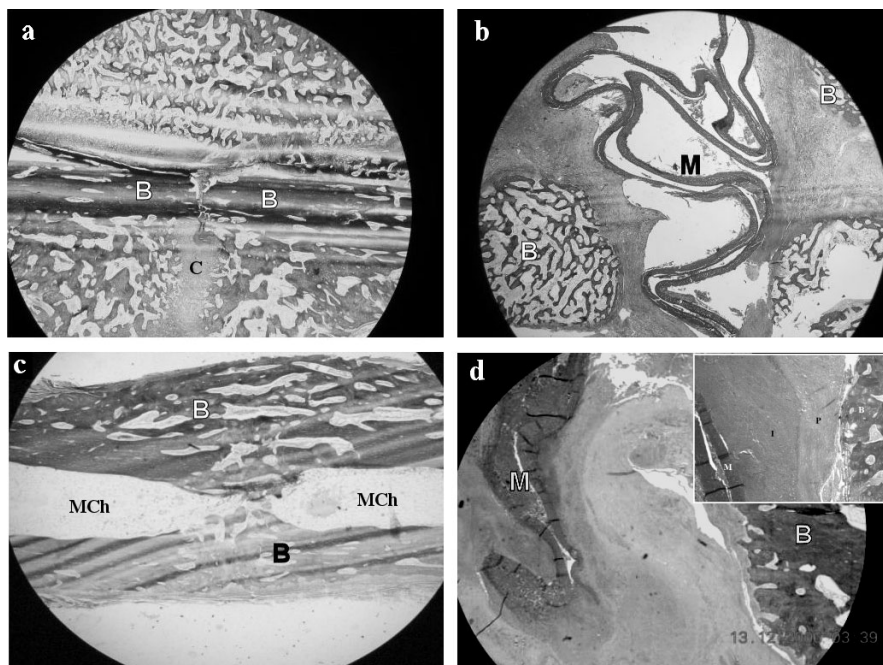

Figure 3. Mallory-stained decalcified bone sections without eggshell membrane after 4 (a) and 16 (c) weeks of interposition and with eggshell membrane (M) interposed between osteotomized bone ends (B) after 4 (b) and 16 (d) weeks. Controls without eggshell membrane after 4 (a) and 16 (c) weeks of osteotomy (×40). Notice bone callus with small remains of cartilage tissue (C) and medullary channel (MCh). Eggshell membrane starting degradation process (M), inflammatory tissue (I) and rounded bone end (B) surrounded by periosteum (P) are shown in insert to (d) (×400)

dozen layers of fibroblasts together with an increase in the angiogenic process. There is also a chronic inflammatory response predominantly with lymphocytes and macrophages (Figure 2a). By the second week there is more of a fibrotic process, with abundant presence of collagen connective tissue and fewer fibroblasts, although the eggshell membranes are still intact (Figure 2b).

Separation of the inner and the outer layers of the eggshell membrane by the presence of dense infiltrative collagen tissue occurs during the fourth week of implantation. There is also an increasing presence of lymphocytes and macrophages, along with giant cells (Figure 2c). After 8 weeks of implantation, there is an increase in the occurrence of fibrosis (Figure 2d), and giant cells, infiltrative lymphocytes, macrophages and plasmocytes, along with hemosiderin deposits (Figure 2d insert).

An important subcutaneous degradation of the eggshell membrane was observed at 16 weeks of implantation (Figure 2e), accompanied by an excessive lymphocyte infiltration and giant cells around and between the fibres of the implanted membrane, evidencing the degradation of collagen fibres (Figure 2e insert).

3.2. Histological results in rabbit ulna

Between 1 and 4 weeks after interposition, the eggshell membrane remained intact and inhibited the regeneration of the bone lesion (Figure 3b), while the control osteotomy showed a noticeable and structured bone callus with small remains of cartilage tissue in the centre of the osteotomy (Figure 3a).

Between 8 and 16 weeks of implantation the eggshell membrane was still interposed between the two bone ends and gradually started to be degraded. At 16 weeks after surgery the bone ends showed a rounded and vascularized appearance, without bridging between them and surrounded by periosteum (Figure 3d), while the control ulna showed continuity of the cortical bone with an abundant bone matrix in a remodelling process with medullary channel formation (Figure 3c).

3.3. Radiographic results in rabbit ulna

Between the first and the fourth week, the control and implanted ulnas of the rabbits behaved in a similar way, showing a moderate periosteal reaction with the fracture line still visible (Figure 4c–h). Between the eighth and sixteenth weeks, the control ulnae showed a progressive bridging of the osteotomized bone ends (Figure 4i), ending in a complete loss of the fracture line (Figure 4k). The remodelling process also started to appear. On the other hand, the implanted ulnae showed a lack of bridging of the osteotomized bone ends by the eighth week of implantation (Figure 4j) and was only partially bridged by the sixteenth week (Figure 4l).

Figure 4. Mediolateral radiographic results of control osteotomies (c, e, g, i and k) and experimental osteotomies with interposed eggshell membrane (d, f, h, j and l) at 1, 2, 4, 8 and 16 weeks after surgery, respectively. An untreated rabbit ulna (a) and a time 0 interposed eggshell membrane osteotomy (b) are also shown. White arrows show osteotomy sites

3.4. Fluorescence results in rabbit ulna

From the first week of implantation and throughout the experiment, control osteotomies showed an active new bone formation from the medullary marrow cavity and subperiosteal space, evidenced by a high fluorescent reaction (Figure 5a, c, e, g).

On the other hand, in the implanted ulnae, the eggshell membrane showed to be an active barrier between the osteotomized ends. From the first to the sixteenth week of implantation new bone is formed at both sides of the membranes but without bridging between the cortical bone ends (Figure 5b, d, f, h).

4. Discussion

In this study, chicken eggshell membrane was shown to be a good interpositional material to be used in rabbit ulnar experimental osteotomy. Eggshell membranes (ESM), like fat grafts, discouraged vascularization, thus inhibiting osteoblast infiltration and proliferation, which
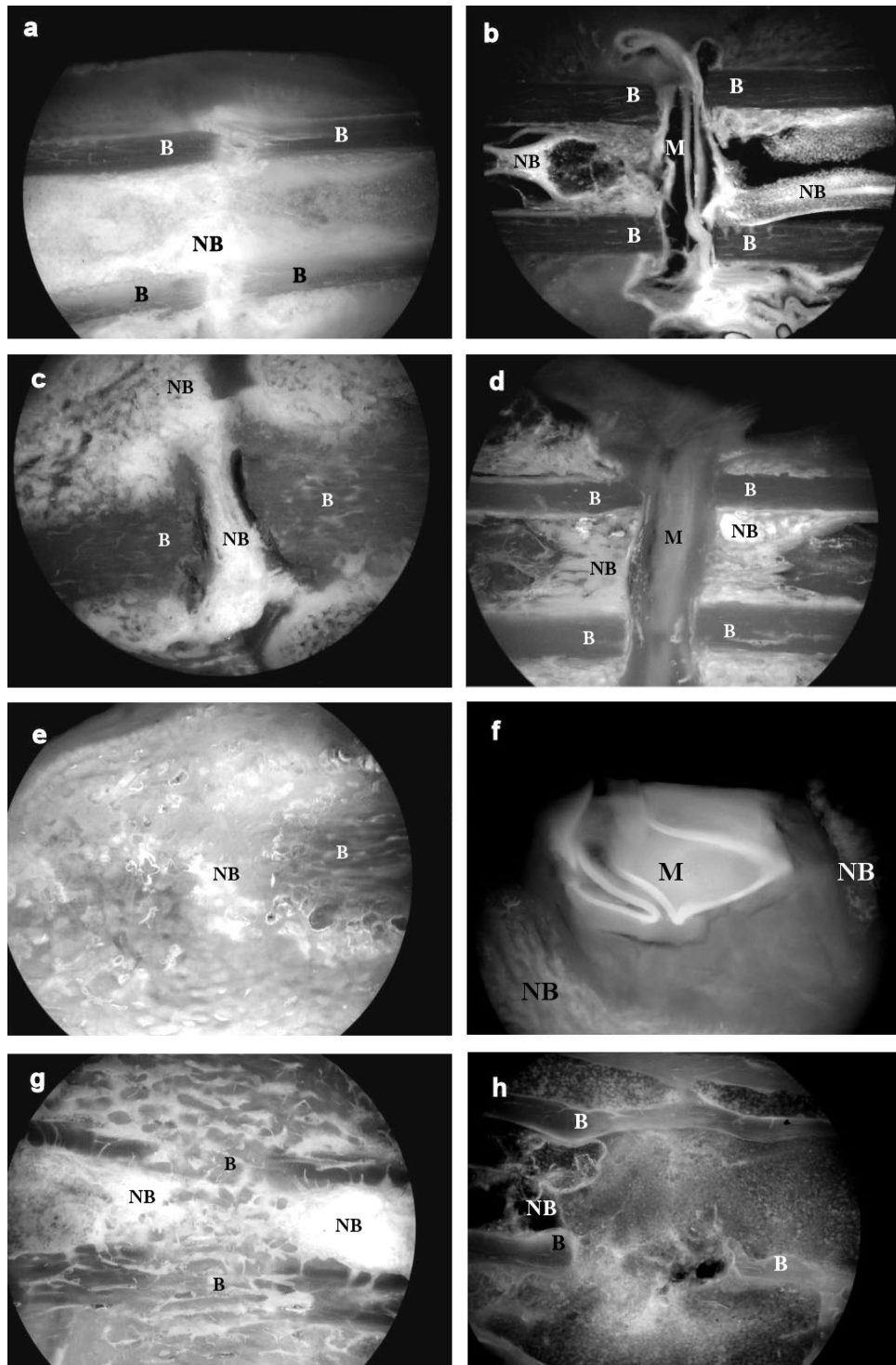

Figure 5. Wet-ground bone section showing fluorescent new bone (NB) in rabbit ulna without eggshell membrane interposition after 2 (a), 4 (c), 8 (e) and 16 (g) weeks and with eggshell membrane (M) interposed between osteotomized bone ends after 2 (b), 4 (d), 8 (f) and 16 (h) weeks. Notice that new bone is formed at both sides of the eggshell membranes (M) but not inside or through them. Old cortical bone is also noticeable (B) (×40)

are necessary characteristics for good osteoconduction. We do not know whether the eggshell membrane effect is due to the described mineralization inhibitory effect of the type X collagen they contain, or whether the inflammatory reaction associated with the eggshell membrane material may also contribute to delayed bone healing. In fact, we have previously shown that experimental culture of osteoblasts on eggshell membrane does not mineralize it unless the terminal ends of type X collagen are removed (Arias *et al.*, 1997). Whatever the mechanism was, this study shows that, instead of a partial ulnar osteotomy and complete periostum removal, minimally invasive surgery with a small osteotomy and ESM interposition is a plausible way to accomplish non-union at least for 16 weeks in rabbit ulnae.

In addition, a secondary surgery site is not needed to obtain ESM as it is for harvesting fat grafts. Moreover, ESM can be obtained in large quantities and is inexpensive, sterilizable with ethylene oxide and easily stored after freeze–dry liophilization. It is also important to note that splint protection was not needed in order to keep the implant in place between the two bone ends.

Finally, there have been many attempts to develop bone atrophic or traumatic non-union animal models for studying bone healing (Kokubu *et al.*, 2003; Viateau *et al.*, 2006; Bodde *et al.*, 2008; Russell *et al.*, 2007). In this regard, the interposition of eggshell membranes between the two ends of a sectioned bone would be interesting not only for the treatment of premature physeal closure, but also because it could be an experimental procedure for developing a non-union model in rabbits.

Acknowledgements

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