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REGULAR ARTICLE

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Microscopical study of experimental wound healing in *Notothenia coriiceps* (Cabeçuda) at 0°C

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Abstract *Notothenia coriiceps* (Cabeçuda) is an Antarctic benthic fish frequently found with lesions in the tegument caused by seal predation. We have investigated epidermal repair in these animals by means of a microscopic study of experimental wound healing at 0°C. At 24–48 h after wound induction, mucous exudate and necrotic lining cells covered the wound. At 7–14 days, an epidermal "tongue" could be discerned, folded at the tip, with intercellular oedema between the tip and the wound border. After 23–30 days, the wound was completely closed and the migrating epidermis, with intercellular oedema, was reduced. By 45–90 days, melanocytes progressively increased in the epidermis but no scales were formed. The inflammatory infiltrate was mainly composed of neutrophils after 7 days, at which time they were mostly replaced by macrophages; lymphocytes and plasma cells were also present. The border epidermis slid towards the centre, folding at the tip and finally fusing to form a diaphragm. The cells of the epidermis began to multiply only after complete closure of the wound. The lack of scale formation on induced and naturally found wounds, even after 90 days, suggests that different mechanisms in wound repair occur at 0°C from those in fish from temperate and tropical environment. This is the first report of successful wound repair at polar temperatures, indicating the adaptation of *N. coriiceps* to the Antarctic environment.

Keywords Wound healing · Inflammation · Skin · Antarctic fish · *Notothenia coriiceps* (Teleostei)

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Introduction

Metchnikoff (1891) demonstrated that phagocytosis and the inflammatory process occurs in almost all animals, with a few remarkable exceptions (Metchnikoff 1891; Tauber and Chernyak 1991; Silva et al. 1995, 1998b). The inflammatory process, as an essential physiological mechanism, is a component of wound repair. It is essential for the maintenance of homeostasis, being present from the beginning of ontogeny (Silva 2000, 2001).

The intensity and velocity of wound resolution in ectothermic vertebrates varies according to local temperature (Reddan and Rothstein 1965; Finn and Nielsen 1971; Grout and Morris 1987; Hardie et al. 1994). When the temperature is reduced, cellular responses tend to decrease (Finn and Nielsen 1971). Other studies have shown that Antarctic low temperatures do not inhibit phagocytosis in invertebrates (Silva and Peck 2000; Silva et al. 2001; Borges et al. 2002) or in Antarctic fish (Silva et al. 2002). The inflammatory process is not inhibited either in the Antarctic fish *Pleuragramma antarcticum* (O'Neill et al. 1987, 1988) or *Notothenia coriiceps* (= *N. neglecta*; Silva

et al. 1998a, 1999). These inflammatory studies have detected the same pattern as that observed in temperate fishes but with a reduced speed of healing. Macrophages and neutrophils predominate during acute phases but the former do not present the activation signs that characterize their ultrastructure (Silva et al. 1998a).

The ability of fish and other animals to maintain integument integrity is essential for their survival. Tissue repair always aims at closing the wound with new tissue, in order to recover the original function or to recreate a physical barrier (Majno and Joris 1996). The inflammatory process is the initial phase of tissue regeneration or wound repair and is attributable to the activity of inflammatory cells that act as scavengers, removing necrotic tissue and debris, killing potentially pathogenic microorganisms and stimulating fibroblasts and epidermal proliferation (Rowley and Ratcliffe 1988).

Studies have been carried out concerning the influence of acclimatization of fish from tropical and temperate waters on the regenerating process (Mittal and Munshi 1974; Anderson and Roberts 1975; Bullock et al. 1978; Phromsuthirak 1977; Whitear et al. 1980; Majno and Joris 1996; Quilhac and Sire 1998, 1999). However, only one study has reported wound repair under polar temperatures (Silva et al. 2004).

Wound repair is a vital mechanism for animal survival in any environment and, as there is only one description of fish wound healing at Antarctic temperatures (Silva et al. 2004), we have studied the histological processes occurring at 0°C to verify their similarity to those occurring at higher temperatures.

Materials and methods

Fish maintenance and wound induction

Specimens of *N. coriiceps* (according to Gon and Heemstra 1990; common name: Cabeçudas; n=21), with a mean weight of 792.45±220.16 g (range: 438.00–1,835.00 g), standard size of 34.61±4.73 cm (range: 25.50–47.00 cm) and total size of 38.44±5.23 cm (range: 29.00–53.00 cm) were collected in January–February in 2000 and 2001 in Admiralty Bay, King George Island, South Shetland Islands (S 62°10.168′, W 058°26.959′). The fishes were acclimatized for 1 week. Experiments were carried out in fibreglass tanks (1,000 l) filled with running sea water (1±1.0°C) in a temperature-controlled room at 0±1.0°C at the Biology Laboratories of the Brazilian Antarctic Station, "Comandante Ferraz", in which the recording of biometric data and identification of the fish took place (Silva et al. 1998a, 1999). Following anaesthesia with benzocaine (50 ppm; Silva et al. 2002), two square (2.0×2.0 cm) full-thickness excision wounds, removing scales, epidermis, dermis, hypoderm and most of the perimysium, were inflicted on both sides of the dorsal–lateral anterior region in each of the 21 fishes. Three fish were used as normal skin controls. After time spans of 0 (n=1), 1 (n=2), 2 (n=2), 7 (n=3), 15 (n=4), 23 (n=4), 30 (n=2), 45 (n=1), 60 (n=1) and 90 (n=1) days, the fish were killed by immersion in 70 ppm benzocaine. Tissue samples from the border to the centre of the wound and the area covered with the new epidermis were collected and processed for light and transmission electron microscopy (Fig. 1). One *N. coriiceps* was found with a wound scar on the posterior ventral lateral region. This fish was photographed and tissue samples from the wound centre and periphery was processed for light microscopy.

This work was carried out in accordance with the Ethical Commission of Animal Experimentation of the Biomedical Sciences Institute of the University of São Paulo (protocol number 067/03).

Light microscopy

All samples were fixed in cold McDowell's fixative solution (McDowell and Trump 1976) for 48 h and decalcified in 10.0% $Na_3C_6H_5O_72H_2O$ (sodium citrate) and 21.25% HCOOH (formic acid) for 7 days. Afterwards, the tissues were dehydrated in 95% ethanol and embedded in Historesin (Leica). Sections (2 μm) were stained by the fuchsin–toluidine blue, Romanowsky, periodic-Schiff (PAS) and Picrosirius techniques and the peroxidase (H_2O_2) test for melanin characterization (Bancroft and Stevens 1982; Junqueira et al. 1979; Silva et al. 1999). Observation, documentation and section measurement were carried out on a Zeiss (Axiomat) microscope equipped with a scale to measure the "leap-frog" diameter (see below) in tissue samples and on an Olympus (BX-60) photomicroscope.

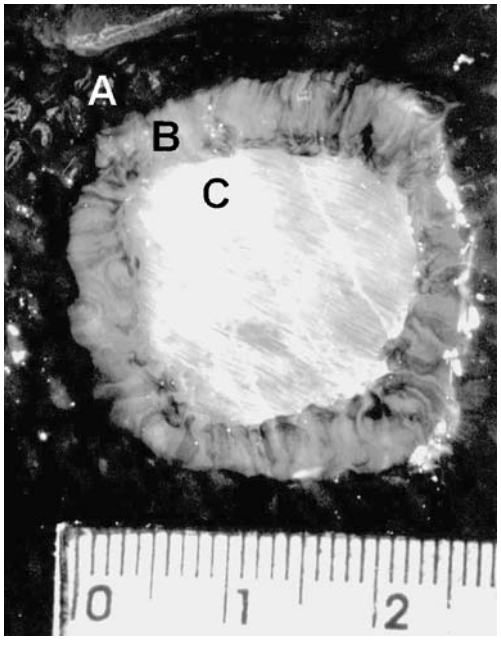

Fig. 1 Wound area. *Region A* Wound periphery composed of normal skin with scales. *Region B* Regenerating epidermis. The region next to the normal skin is the border. The area in contact with the wound centre is the tip. *Region C* Wound centre in which the muscle with the remaining perimysium is in contact with the external environment. *Scale* (*bottom*) is in centimetres

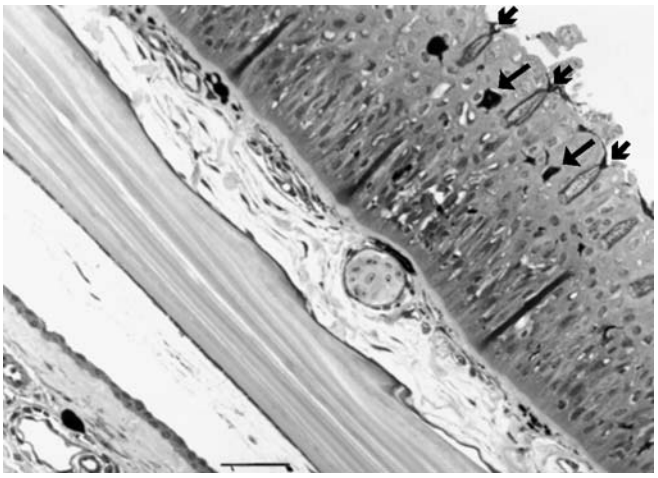

Fig. 2 Normal skin preparation of the dorsal–lateral anterior region of *Notothenia coriiceps*. The epidermis is scaled and stratified, with mucous cells (*short arrows*) and melanocytes (*long arrows*). The lamina propria has a layer of melanocytes bordering the basal lamina, with many fibroblasts and vessels below it. Under the lamina propria, the calcified scales are lined by simple squamous epidermis, lying on top of both loose and dense connective tissue stratum. The presence of collagen is predominantly at the scale and dense connective tissue. Toluidine–fuchsin. *Bar* 50 μm

Transmission electron microscopy

Samples at each time interval were collected for ultrastructural analysis. They were fixed at 0°C in 2.5% (w/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.2), postfixed in 1.0% OsO_4 (Hayat 1984) and embedded in Spurr resin (Sigma). Ultra-thin sections of 70 nm were gathered onto copper grids and stained with 2% uranyl acetate in distilled water for 1 h, washed in distilled water and then stained again in 0.5% lead citrate in distilled water. Ultrastructure was examined in a Jeol 100 CX-II electron microscope at the Institute of Biomedical Sciences at the University of São Paulo (Silva et al. 1999).

Results

Normal skin and site of injury

Normal skin has a stratified epidermis (n=10, including region A in lesioned fish; see Fig. 1) composed of eight to 11 epidermal cell layers with a diameter of: 135.1–201.7 μm (168.4±33.3 μm). Among the superficial epidermal cell layer, some unicellular serous–mucous cells were characterized by the PAS stain and some by toluidine-stained granules. Cells with basal nuclei and clear granules (immature mucous cells) were observed in the middle layers. Several star-shaped melanocytes and melanocyte projections were present along the epidermal layers. The adjacent loose connective tissue (lamina propria) had a discontinuous layer of melanocytes bordering the basal lamina, below which many fibroblasts and vessels were present. Underneath the lamina propria, calcified scales were lined by the simple scaled epidermis (Fig. 1) surrounded by both loose and dense connective tissue. The scale and dermis had a collagen-rich matrix (Fig. 2).

Immediately after surgery, a visible haemorrhagic reaction was characterized by the presence of many blood clots, mainly on the wound borders (but also in the central region). Some muscle cells were necrotic.

Initial events of wound closure at days 1–7

At day 1, the wound borders presented blood clots with an exudate and large amounts of mucus. A few layers of debris were present at the muscle surface. The epidermal border diameter was 96.7–111.3 μm (Fig. 3). On the second day, a discrete increase in the space between the connective tissue collagen fibres could be seen at the wound borders. A projection of the epidermis on the wound borders pointed towards the centre. The epidermis exhibited intercellular oedema and some melanocytes. The epidermal

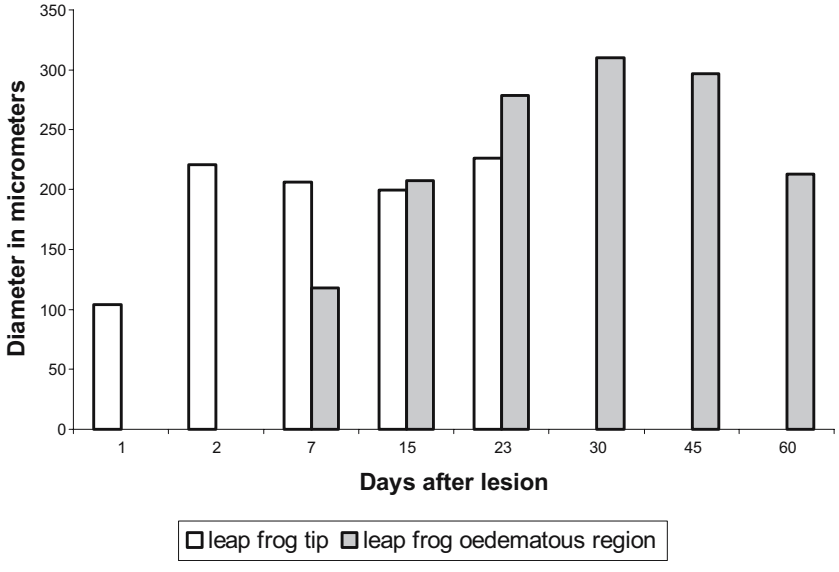

Fig. 3 Regenerating epidermis diameter after an experimental 4.0-cm^2 lesion in the dorsum lateral anterior region of the integument of *N. coriiceps*. The "leap-frog" tip and the variation in the diameter of the oedamatous region is shown in micrometers at various times. After 23 days, the lesion closed to form a diaphragm and, after this period, region C (Fig. 1) disappeared. Thus, the data of the "leap-frog" diameter after this time was coincident with the size of the total lesion

border diameter was 153.3–287.5 μm. The unattached epidermal tip of the "tongue" resembled the normal epidermal surface. This tip is also referred to as the "leap-frog" tip below.

After 7 days, the central region of the wound displayed layers of dead cells with erythrocytes. Muscle cells presenting different degrees of degeneration and adipose tissue necrosis were evident. Inflammatory infiltrate was widespread, mainly being composed of macrophages lying along congested vessels within loose and dense connective tissues near the wound area. Over the necrotic region, a tongue-like projection of regenerating epidermis was composed of two distinct areas. The extremity or tip of this projection (Fig. 4) was composed of a mass of globular cells, with round nuclei and homogeneous cytoplasm. Among these cells, neutrophils were identified, as were star-shaped and rounded melanocytes and macrophages. Mucous cells, characterized as serous–mucous cells by PAS, were observed on the epidermal surface of the projection. No typical basal layer of epidermal cells was seen, although this "tongue" seemed to be detached from the necrotic tissue underneath it (apparently floating on top of it) with an epidermis diameter of 103.4–308.6 μm (206.0±102.6 μm; Fig. 3).

The region from the wound borders to the projection (Fig. 5) was more organized than the earlier stage of wound repair. A basal-lined layer was seen below an enlarged superficial layer of the intercellular space. The diameter of this epidermis was 92.4–143.6 μm (118.0± 25.6 μm; Fig. 3). By transmission electron microscopy, the cytoplasmic membranes of these epidermal cells appeared to be attached to each other by thin projections with tight junctions. Neutrophils (characterized by their content of round granules of 1–1.5 μm diameter, which were stained salmon-pink in toluidine–fuchsin preparations; Fig. 4) were also present among epidermal cells. Macrophages were identified by their intra-cytoplasmic phagocytosed material. Melanocytes with cytoplasmic projections could be identified by their typical melanin granules.

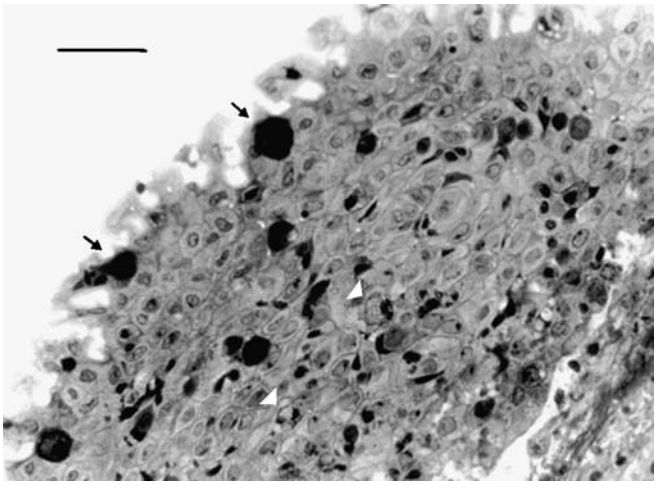

Fig. 4 Wound section showing the regenerating epidermis after 7 days of skin excision (4.0 cm^2) in *N. coriiceps*. Epidermis projection (tongue) containing mucous cells (*arrows*) in the epidermis, surrounded by epidermal cells. Infiltrating neutrophils (*arrowheads*) are present. Toluidine–fuchsin. *Bar* 100 μm

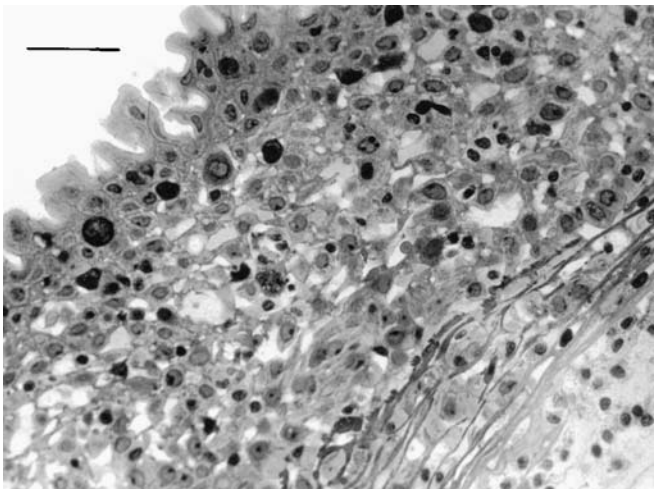

Fig. 5 Same preparation as in Fig. 4 but at the distal region of the migrating epidermis. Intercellular oedema can be seen in the epidermis. The migrating epidermis lies over layers of necrotic superposed cells. Toluidine–fuchsin. *Bar* 100 μm

At the wound border, the basal layer of the epidermis was highly organized with globular cells lying over the necrotic tissue. The superficial layer of the epidermis was paved with surface projections. Under this regenerating epidermis, muscle necrosis was characterized by the desegregation of myofibrils and an inflammatory infiltrate composed mainly of macrophages. Intense inflammatory infiltrate was observed in the resting perimysium, as was haemorrhage. Mainly macrophages and a few neutrophils were found after specimens had been stained by the Rosenfeld method.

Regenerating epidermis at day 15

The regenerated epidermis increased in length with a more organized basal layer, except for the region near the "tongue" tip, where the bond with underlying necrotic tissue was weak (Fig. 6). Intense inflammatory infiltrate remained on the muscle layer and was mainly composed of thrombocytes, macrophages and small numbers of neutrophils.

In the epidermis, we observed melanocyte projections with many granules, immature mucous cells in the middle layer and mature mucous cells at the top layer. In the middle layer of the "tongue" , the epidermis possessed small intracellular vacuoles and enlarged intercellular spaces. The mucous cells showed small electron-dense spots inside the granules (throughout the cytoplasm) and a clear homogeneous surface with peripheral nuclei. The epidermis diameter here was 183.4–231 μm (207.2±23.8 μm, n=10 lesions; Fig. 3).

The middle epidermal layer of the region between the migrating "tongue" and the wound border showed an enlarged intercellular oedematous pattern by light microsco-

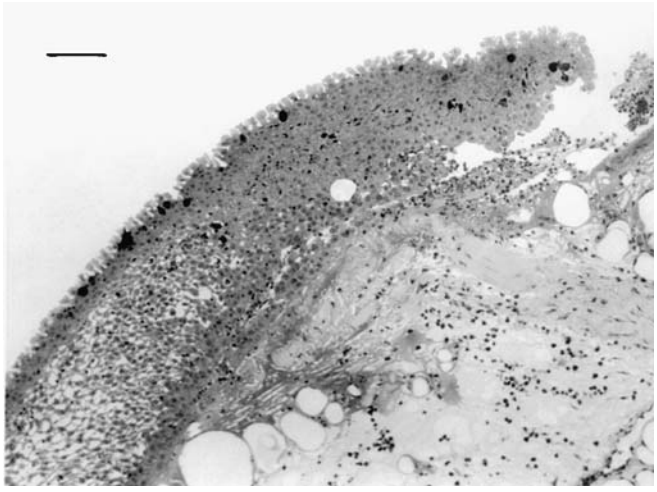

Fig. 6 Migrating epidermis at the wound border after 15 days of induced skin lesion (4.0 cm^2) in *N. coriiceps*. Oedematous and tip regions. Toluidine–fuchsin. *Bar* 100 μm

py. Transmission electron microscopy revealed that this region had projections connecting the epidermal cells by tight junctions. A large region of vacuoles filled with amorphous material (Fig. 7) was also observed. Typical neutrophils infiltrated the intercellular spaces and star-shaped melanocytes and melanocyte cytoplasmic projections were evident (Fig. 8). The melanocytes, were characterized by the presence of homogeneous electron-dense granules and nuclei with both dense and loose chromatin.

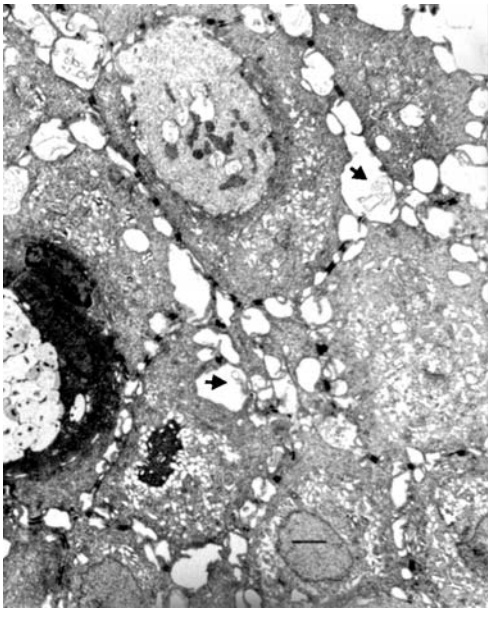

Fig. 7 Micrograph of the migrating epidermis section, at the wound border, after 15 days recovery showing the transition between the oedematous epidermis and the more compact epidermis in *N. coriiceps*. Intercellular oedema is seen between epidermal cells (clearly characterized by tight junctions). Note the vacuoles filled with amorphous substance (*arrows*). *Left* Part of a mucous cell. *Bar* 4 μm

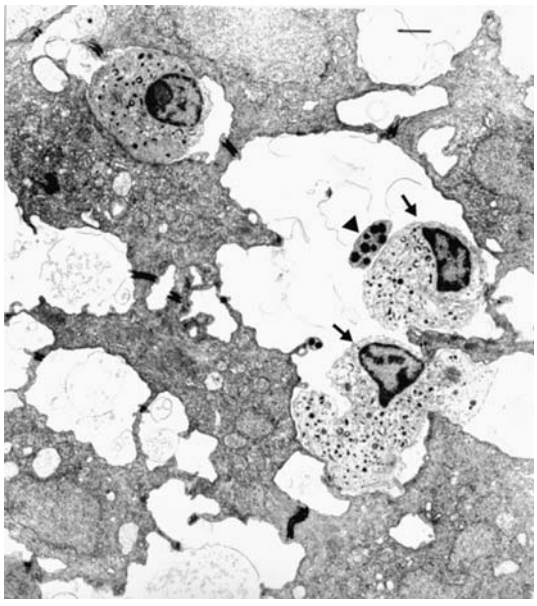

Fig. 8 Micrograph of the epidermal layer middle region between the migrating tip and the wound border in *N. coriiceps*, after 15 days recovery, showing the oedematous area. The oedema is enlarged and neutrophils (*arrows*) have infiltrated between epidermal cells, together with some melanocyte projections (*arrowhead*). *Bar* 4 μm

Near the wound borders, a monolayer of melanocytes was present underneath the epidermis (with discrete intercellular spaces). Deeper within the skin, we observed a dense connective tissue layer, followed by loose connective tissue above the scales. Inflammatory infiltrate was found in both the above-mentioned layers and was composed mainly of macrophages (Fig. 9). Some macrophages had

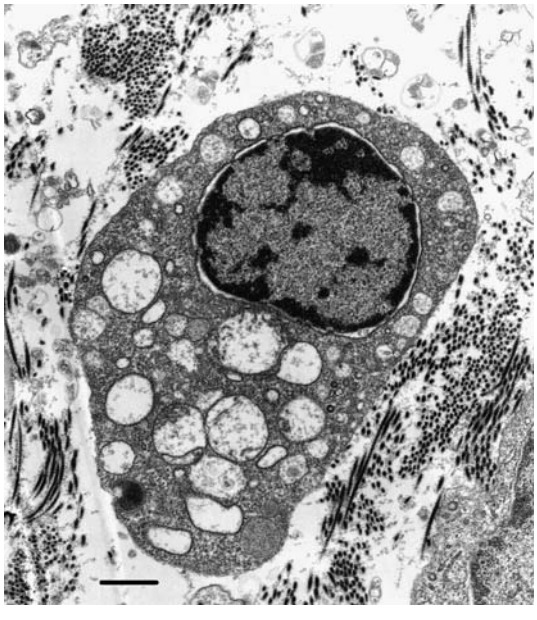

Fig. 9 Micrograph of the connective tissue under the migrating epidermis at the wound border, after 15 days of experimental injury in *N. coriiceps*. Activated macrophage in the connective tissue, with collagen fibres partially disassembled by oedema. *Bar* 1 μm

melanin granules in their cytoplasm. Intense oedema was characterized by the dispersion of loose connective tissue components.

The epidermis near the wound border was more compact with a diameter of 199.2±7.3 μm (range: 191.9–206.5 μm, *n*=10) and contained macrophages, neutrophils, melanocytes and melanocyte projections between epidermal cells (Fig. 10). A multilayered tissue composed of necrotic cells and debris was present under the basal epidermis.

Fusion of regenerating borders and increased cellular participation at 23–45 days

The "tongue" had grown longer and thicker, closing the wound, by this stage. The regenerating borders had fused; macroscopically, the general aspect of the wound resembled a "sphincter" (Silva et al. 2004) with radial black lines. The number of star-shaped melanocytes had also increased in some regions. The diameter of the intercellular oedematous epidermis was 278.4±34.6 μm (range: 243.8–313.0 μm, *n*=10 lesions), whereas the tip diameter was 226.0±13.6 μm (range: 212.4–239.6 μm, *n*=10; Fig. 3) after 30 days. The oedema at the connective tissue under the wound was reduced. The epidermis that covered the wound (278.4±34.6 μm of diameter, *n*=10 lesions) had a basal layer composed in rounded cells near to intercellular oedematous epidermal cells. The closed wound also showed certain specific features. The epidermal cells contained numerous vacuoles and debris, as described for macrophages (Langerhans cells), whereas infiltrated neutrophils were rare. The epidermal superficial layers did not present intercellular oedema and were covered by an outer paved cell layer. In the intermediate layers of the epidermis, mitotic figures could be frequently observed. The epidermis tip diameter was 226.0±13.0 μm (range: 213.0–239.0 μm, *n*=10 lesions). The connective tissue among the muscle layers was heavily infiltrated by macrophages filled with phagocytosed material, together with a few plasma cells. The myotomes frequently contained macrophages packed with phagocytosed material. Few areas of haemorrhagic tissue could still be seen.

After 45 days, melanocyte number had increased predominantly in the middle layers of the wound. The inflammatory infiltrate was composed mainly of macrophages and a few neutrophils. The remaining epidermis diameter, after the fusion of the regenerating epidermis borders, was 227.2–366.4 μm (range: 296.8±69.6 μm, *n*=10).

Oedema and persistence of inflammatory infiltrate at 60–90 days

By 60–90 days, the star-shaped melanocytes had increased in number and the epidermal cells had acquired a globule-like shape. The epidermis diameter had reduced to 179.7–245.9 μm (range: 212.8±33.1 μm, *n*=10 lesions). A small number of neutrophils remained in the inflammatory infiltrate but these were outnumbered by activated macrophages with many vacuoles, lysosomes and debris. We found rounded melanocytes with small projections in the epidermis. A new basal lamina had been formed between the connective tissue and epidermis, although some lacunae were present. The connective tissue was filled with collagen and some melanocytes had lined up below this layer (Fig. 11). After 90 days, the basal lamina was more continuous and a few small melanocytes were present in the connective tissue. A few granulocytes (mainly neutrophils) were observed in the epidermis, together with an increasing number of serous–mucous cells with well-

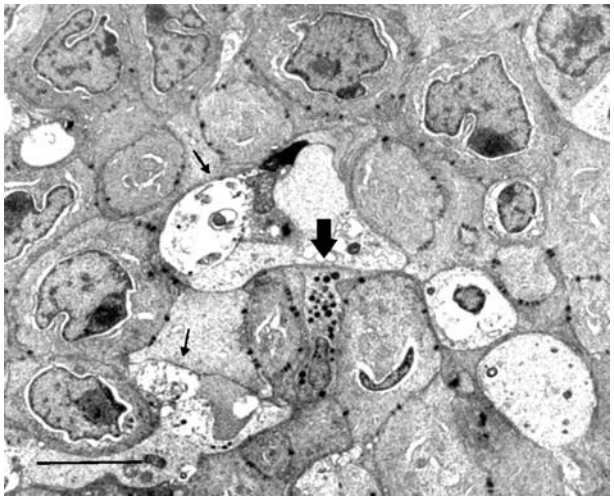

Fig. 10 Micrograph of the migrating epidermis at the wound, after 15 days of recovery following injury in *N. coriiceps*. Some neutrophils (*arrows*) are still present at this time, together with melanocytes (*large arrow*) between the epidermal cells, which have many tight junctions. *Bar* 2 μm

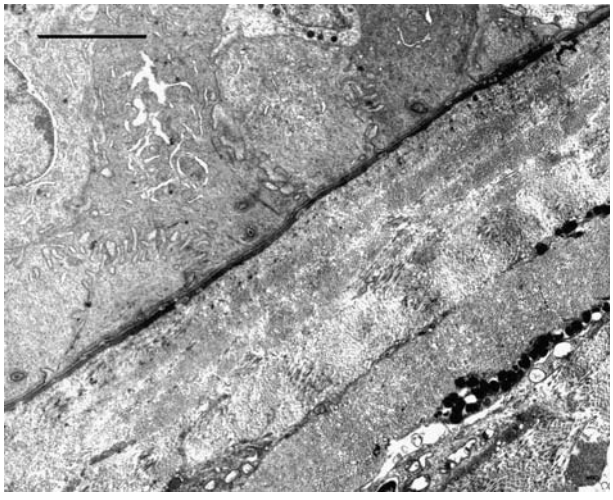

Fig. 11 Micrograph of the wound border covering the epidermis after 60 days of wound repair in *N. coriiceps*. Below the regenerated epidermis, a new basal lamina has been formed, albeit imperfectly, and melanocytes can be seen in the connective tissue, as can many collagen fibres. *Bar* 2 μm

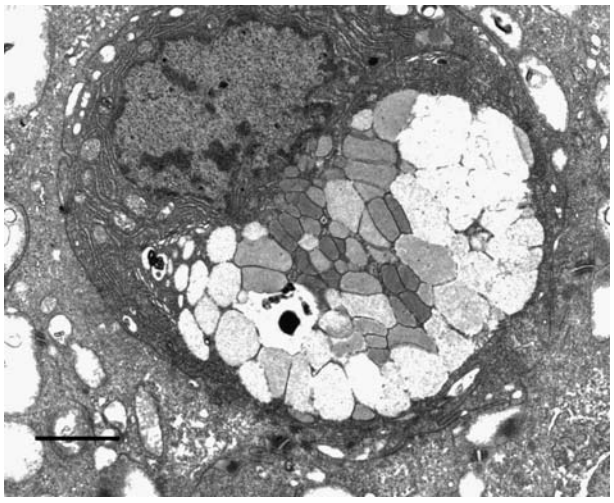

Fig. 12 Micrograph of the wound border in regenerating epidermis 90 days after injury in *N. coriiceps* showing a mucous cell with many granules at different maturation stages between the epithelial cells. *Bar* 2 μm

developed rough endoplasmic reticulum and diverse electron-dense secretory granules (Fig. 12). The inflammatory infiltrate was still present, was intensely stained and was composed mainly of foam macrophages (Fig. 13). The necrotic tissue had been replaced by tissue exhibiting fibrosis and neovascularization. Many macrophages with phagocytosed particles remained. Star-shaped melanocytes still lay among the epidermal cells but only a few of them were found under the lamina propria. After Picrosirius staining, a small quantity of stained collagen and the absence of loose connective tissue were observed, when compared with normal skin.

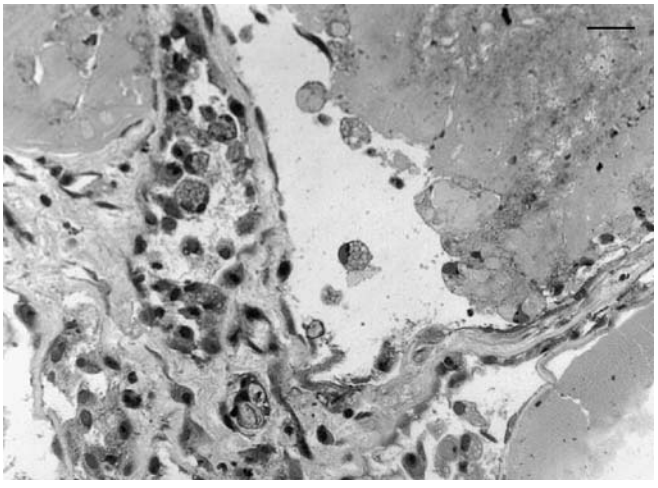

Fig. 13 Light-microscopic preparation of the connective tissue underlying the wound border of the regenerating epidermis after 90 days of wound repair in *N. coriiceps*. The perimysium is filled with many activated macrophages and tissue debris being phagocytosed by foam macrophages. Toluidine–fuchsin. *Bar* 20 μm

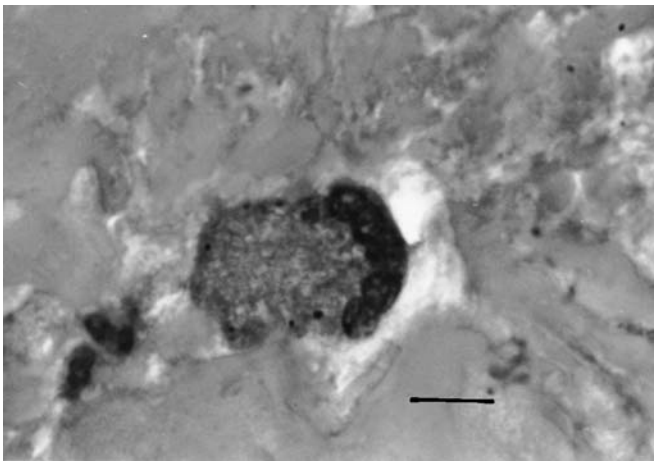

Fig. 14 Connective tissue of the epidermis in a naturally produced wound in *N. coriiceps*. Note the giant multinuclear cell in the oedematous connective tissue. Toluidine–fuchsin. *Bar* 10 μm

Naturally occurring wound

In the naturally occurring wound, the epidermis was moderately vacuolated with a visible basal lamina. Between the epidermal cells, many globular melanocytes (about 27 μm of diameter), sometimes in groups in two or three, were found to be surrounded by two layers of flat cells. Other melanocytes, with a more dispersed pattern of granules, remained on the surface. No mucous cells were seen in the epidermis. Some haemorrhagic regions could be discerned and the connective tissue was intensely infiltrated with macrophages, lymphocytes and plasma cells. A few erythrocytes were dispersed in the muscle and connective tissues. Intense inflammatory infiltrate was found in the muscle connective tissue and was mainly composed of macrophages and lymphocytes. Some of the degenerated muscle cells contained many activated macrophages and giant cells (Fig. 14). The Picrosirius technique revealed the presence of a collagen-rich matrix in the fibrous tissue formed under the epidermis. This tissue was composed mainly of fibrocytes and fibroblasts, among the collagen fibres, and did not have melanocytes.

Discussion

This histological study was designed to determine the events occurring during of integument wound repair in an Antarctic fish at 0°C. The existing literature has only one study of the wound repair kinetics of *N. coriiceps* (Silva et al. 2004), whereas a few other reports deal with the influence of acclimatization at different temperatures on the processes of healing and regeneration of tropical and temperate fish. Bullock et al. (1978), using *Pleuronectes platessa* maintained under various temperatures (5, 10 and 15°C), have observed that a 5×1 mm lesion is completely closed by epidermal migration and that the thickness of the newly formed layer is thinner at lower temperatures; they

point out that, at low temperatures (such as 5°C), the main mechanism of wound repair in fishes involves cellular migration, seen as the sliding of the epidermal layer over the wound. Mittal and Munshi (1974) have also shown that the initial wound closure in fish results from epidermal sliding and not by the multiplication of epidermal cells.

Similarly, wound repair in *N. coriiceps* occurs by epidermis sliding, favoured by the intercellular oedema at the regenerating epidermis border. The regenerating epidermis is thicker than control epidermis after 15 days, despite the low temperature (0°C). This strategy allows the same number of cells to occupy a larger area without multiplication and enables the cells to slide towards the wound centre. However, when compared with previous studies at higher temperatures, epidermal sliding is not rapid after the first few hours following wounding, with the wound being completely closed only after 23–30 days. The same pattern has been observed in an ultrastructural analysis of wound healing in *Notophthalmus viridescens* showing epidermal cells proximal to the wound site within an evident intercellular oedema and a reduction in desmosome numbers of the migrating cells (Repesh and Oberpriller 1980).

Wound repair depends on several other factors, such as the lesion size, contamination and the blood and nerve supply (Majno and Joris 1996). Despite the long period required for wound closure in *N. coriiceps*, no signs of contamination by bacteria or fungi have been seen to retard the process. This finding suggests the highly effective mucous protective action of the mucus-covered skin or the low pathogenicity of the microorganisms in Antarctic seawater.

The stratum for cell migration is composed of a matrix, membrane debris and fibrils. The literature indicates a "leap-frog" type of cellular migration, despite the occurrence of cell adhesion to the substrate (Quilhac and Sire 1999). *N. coriiceps* are able to deal with the external environment by means of a multilayer cell debris barrier associated with the mucous secretion, thus isolating the muscles from the hyperosmotic environment.

The lack of mitotic figures at the regenerating epidermis of *N. coriiceps* for up to 30 days agrees with other studies showing that the closing mechanism of injured skin occurs primarily by epidermis sliding (see above) and secondarily by cellular differentiation and mitotic burst (Mittal and Munshi 1974; Bullock et al. 1978; Quilhac and Sire 1999). We have been unable to use percentage of cell nuclear antigen (PCNA) protocols, commonly used in mammals, for the analysis of cell proliferation. Therefore, the closure mechanisms under our experimental conditions cannot be more precisely described.

Adequate blood perfusion within the healing tissue is crucial but the influence of low temperatures rather than blood supply may be responsible for differences in regeneration and fibrosis. The inflammatory response of *N. coriiceps* is slower than that in other temperate fishes, although faster than expected for any temperate fish exposed to Antarctic temperatures (Silva et al. 1998a, 1999, 2002). We do not support the hypothesis of a delay caused by deficient blood perfusion in *N. coriiceps*, since inflammatory infiltrate is intense after the first day. Nevertheless, the efficiency of this infiltrate needs further elucidation, since macrophages of *N. coriiceps* show a low phagocytic index in vitro (Silva et al. 2002). The participation of regenerating epidermal cells of *N. coriiceps* in the phagocytosis of debris may also be an important cleansing mechanism. Phagocytosis has also been observed in *Gasterosteus aculeatus* (Phromsuthirak 1977) and *Notophthalmus viridescens* (Repesh and Oberpriller 1980) and may help the inflammatory cells to cleanse the region.

These findings regarding the inflammatory process in Antarctic temperatures are in agreement with other data previously described by our group (Silva et al. 1998b, 1999, 2001). Neutrophils are predominant at first in the inflammatory infiltrate and are replaced by mononuclear macrophages at later time points. This description confirms with other studies on wound repair (Phromsuthirak 1977).

Fish scales usually regenerate quickly after the completion of wound repair in skin, as demonstrated in *Hemichromis bimaculatus* whose scleroblasts migrate from the wound border (Sire and Géraudie 1983, 1984; Sire 1989). However, in *N. coriiceps*, neither scale formation nor scleroblast migration or organisation was observed after the maximum experimental period (90 days). This suggests that a different process for wound repair occurs in *N. coriiceps* compared with other studied temperate and tropical teleosts. The wound retraction borders would be than of primary importance to protect soft regenerated tissue for longer periods. The absence of scales in the naturally occurring wound found in one *N. coriiceps* and in the experimentally induced wounds after 90 days reinforces this hypothesis. Studies involving longer observation periods are necessary to clarify this point.

In *N. coriiceps*, epidermis sliding begins between 24 to 48 h after lesion and folding occurs at the "tongue" tip. The folding has been confirmed by the presence of mucous cells at both sides of the epidermis surface and by the thicker projection diameter compared with regenerating epidermis. This region does not attach to the wound as the epidermis lies behind it. The thicker projection diameter of the epidermis generally increases up to 30 days and, although it subsequently decreases, it remains larger than the original epidermis after 60 days.

The presence of star-shaped melanocytes in the epidermis and has been described in other fish, e.g. *Pollachius virens* (Bereiter-Hahn et al. 1986). The importance of the number of melanocytes in the epidermis and dermis should be noted as this is responsible for the dark colour of the normal skin of *N. coriiceps*. After the induced wound, the black colour found after 60 days is exclusively produced by the epidermal melanocytes, since they are seen in the dermis only after 90 days and only in small numbers.

The intense replacement of melanocytes brought about by the sliding of the epidermis from the borders, first in the epidermis and later (after 90 days) in the dermis, is unusual and promotes remarkable changes in the original fish skin colour at the injury site after 60 days (see also Silva et al. 2004). This may provide protection against predators (a bright wound in a dark animal could be at-

tractive) and ultraviolet radiation, which is especially strong in Antarctica.

The morphology of the naturally found wound suggested that this was an old injury because, when compared with 90-day induced wounds, the presence of giant cells in the connective tissue under the epidermis indicated a chronic process. No giant cells were observed in any of the induced wounds. Precocious formation of multinuclear cells (6 h) occurs in *N. coriiceps* after glass coverslip implantation in the abdominal cavity, indicating that the attached macrophages are activated by the intense infestation of parasites present in the viscera and abdominal cavity (Silva et al. 2002). Nevertheless, even after 30 days of implantation of a cotton suture thread in the muscle of *N. coriiceps*, no giant cells have been discerned (Silva et al. 1998a), indicating that a different kinetic processes take place in the abdominal cavity and in the muscle. The fibrous connective tissue of the naturally occurring wound of *N. coriiceps* would probably have hindered the regenerating process, because of the presence of a scar composed of fibroblasts and collagen fibres. This also confirms the significant magnitude of this injury, since the haemorrhage and the intense inflammatory infiltrate were still present (macrophages, few lymphocytes and plasma cells); however, neutrophils were absent.

These data confirm the ability of *N. coriiceps* to deal with large injuries and to survive even if scales are not regenerated. The dark wound colour is attributable to the increase of the number and size melanocytes in the epidermis. However, the different pattern of the melanocytes (they were rounded or absent in the connective tissue) requires further elucidation. We describe, for the first time, by light and transmission electron microscopy, the regenerating integument of an Antarctic fish under polar temperatures. Our study indicates the efficiency of wound healing at Antarctic temperatures (0°C), despite the response differences in other euteleosts. *N. coriiceps* is able to deal successfully with relatively large wounds (4 cm^2) with no subsequent infection, reflecting its adaptive history through evolution in an Antarctic environment.

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