Histopathological studies on the repair of the excised skin wounds of the air-breathing scalyfish *Channa striata* (Bloch)

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Regeneration and repair of excised wounds in the skin of the scalyfish *Channa striata* have been studied. The present investigation revealed that regeneration processes in fishes vary greatly from that of mammals. Following injury in the skin of *C. striata*, the free borders of the wounds retract and within an hour, the areas surrounding the wound become very dark. The normal shade is however, restored after 24 h. A mass movement of the epidermis towards the wound gap starts at about 2 h, which results in the epithelialization of the wound gap within 4 to 6 h. This is in contrast to dry mammalian skin where the granulation tissue appears first and the epithelialization takes place quite late and new epithelial cells are produced due to mitotic division of the cells of the *stratum germinativum* of the epidermis. Subsequently the thickness of the epidermis covering the wound gap in *C. striata* rapidly increases due to hyperplasia of the polygonal epithelial cells. Later, the epidermis gets equipped with numerous sac-like goblet mucous cells which lay a copious amount of slime on the newly regenerated epidermis for protecting the underlying damaged tissue components. At about 16 h, numerous ionocytes appear in the epidermis covering the wound gap, playing active roles in maintaining the normal osmotic equilibrium. The epidermis gradually becomes thinner with the development of scales in the underlying tissues and appears normal by 20 days. The denuded muscle bundles in the wound gap start disintegrating soon (30 min) after the lesions are made and by 4–5 days are replaced by the granulation tissue. With the initiation of disintegration of the denuded muscle bundles, an amorphous PAS positive substance appears in the wound gap, the quantity of which gradually increases and reaches its maximum within 6–10 h when the muscle bundles show maximum vacuolization and attenuation. The process of fibre formation in the granulation tissue begins at about 5 days; simultaneously myoblasts appear at the level of old muscle bundles and start differentiating into new muscle fibres. The appearance of melanophores and leukocytes in the regenerating tissues has been correlated with the local defence mechanism of the newly formed epidermis. The pigment cells also appear in the sub-epidermal granulation tissue. Later, the outer layer of the granulation tissue gets differentiated into *stratum laxum* with the formation of numerous fat cells, the scales, and the inner layer into *stratum compactum* which shows the appearance of compactly arranged connective tissue fibres and the fibroblasts. Differentiation of fat cells also takes place at the level of the subcutis. The wounds get completely repaired within 35 days and unlike in mammals leave no scar or scab of the wound on the surface of the skin.

THE skin of fishes is the main organ of defence against environmental hazards. Hence, its restoration in the case of a wound is of great importance for the maintenance of *milieu interior* of the fish. To achieve this, the injured skin tries to regenerate continually, even under the toxic stress of the water-borne xenobiotics[1]. Most of our knowledge on the regeneration and repair of vertebrate wounds is framed on the basis of the literature available on the process of healing in mammals and reptiles[2–4]. Various aspects of healing of fish wounds have not yet been studied on an adequately broad scale[2,5–13]. Therefore, in this paper, efforts have been made to report the various processes of healing, leading to successful repair of excised wounds in the skin of the scalyfish *Channa striata*, which is also known to respire through its skin.

Materials and methods

Fish and their maintenance

Live specimens of *C. striata* of either sex of approximately 12 cm length were collected from a single population at Varanasi and maintained for 4 weeks in the laboratory in large plastic tubs containing 50 l of tap water for acclimation in the confined condition. During their confinement, fish were regularly fed with minced

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goat liver and the water was renewed every 24 h with routing cleaning of the aquaria.

Experimental protocol

Infliction of excised wounds: The area between the anterior end of the dorsal fin and the lateral line on the left side of the fish was selected for the study. Nine scales were plucked mechanically with the help of a stout forceps and rectangular wounds of approximately 4 × 6 mm area and 2–3 mm depth, parallel to the longitudinal axis of the body, were made on the denuded skin with a sharp scalpel blade. Fishes were immediately returned to the water until sacrificed.

Histopathological analysis

Large (12 × 10 mm) skin fragments with wounds were dissected out from 3 fishes from each of the following intervals of healing and fixed in 10% neutral formalin and Boun's fluid (Oh, 1/2 h,1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 16 h, 20 h, 24 h, 30 h, 42 h, 48 h, 60 h, 72 h, 4 d, 5 d, 6 d, 7 d, 8 d, 9 d, 10 d, 12 d, 15 d, 18 d, 20 d, 25 d, 30 d, 35 d and 40 d). Paraffin sections of the tissue blocks without decalcification were protected with a thin layer of celloidin before being stained with Ehrlich's haematoxylin/eosin for routine histopathological analysis, periodic acid-Schiff (PAS) for 1,2 glycols, and alcian blue pH 2.5 for acidic moieties of the carbohydrates[14]. Glycogen was detected by digestion of the tissue sections with saliva prior to their staining with the PAS technique. Presence of nucleic acids was detected by methyl green pyronin-y technique[14].

Results

Normal (intact) skin

The skin of *C. striata* consists of three main layers: the epidermis, the dermis and the subcutis (Figure 1 *a*). The epidermis is a typical mucous membrane and is made up of epithelial cells (ECs), sacciform granular cells, goblet mucous cells and ionocytes (Figure 1 *b*)[15]. Migratory cells (mostly the lymphocytes) from the circulatory system also invade the lower layers of the epidermis. The epidermis covers the scale surfaces. The dermis is a thick layer and is made up of parallely arranged connective tissue fibres. Due to the presence of prominent scales in the outer layer of the dermis within well-developed scale-pockets, the outer layer appears loosely arranged (*stratum laxum*)[16]. The inner compact layer of the dermis is made up of parallely arranged coarse collagenous fibre bundles (*stratum compactum*). The subcutis or hypodermis is generally formed by loosely arranged fine connective tissue fibres and large-sized fat cells[16]. Both the dermis and the subcutis are highly vascular and are greatly innervated. Unlike the epidermis, the dermis does not appear to be a very active layer with various histochemical techniques applied during the present investigation.

Regeneration of wounded skin

Alteration in the skin following removal of scales prior to infliction of the wound: In normal skin, the anterior free parts (not embedded into the dermis) of the scales are covered with the epidermis, which, however, does

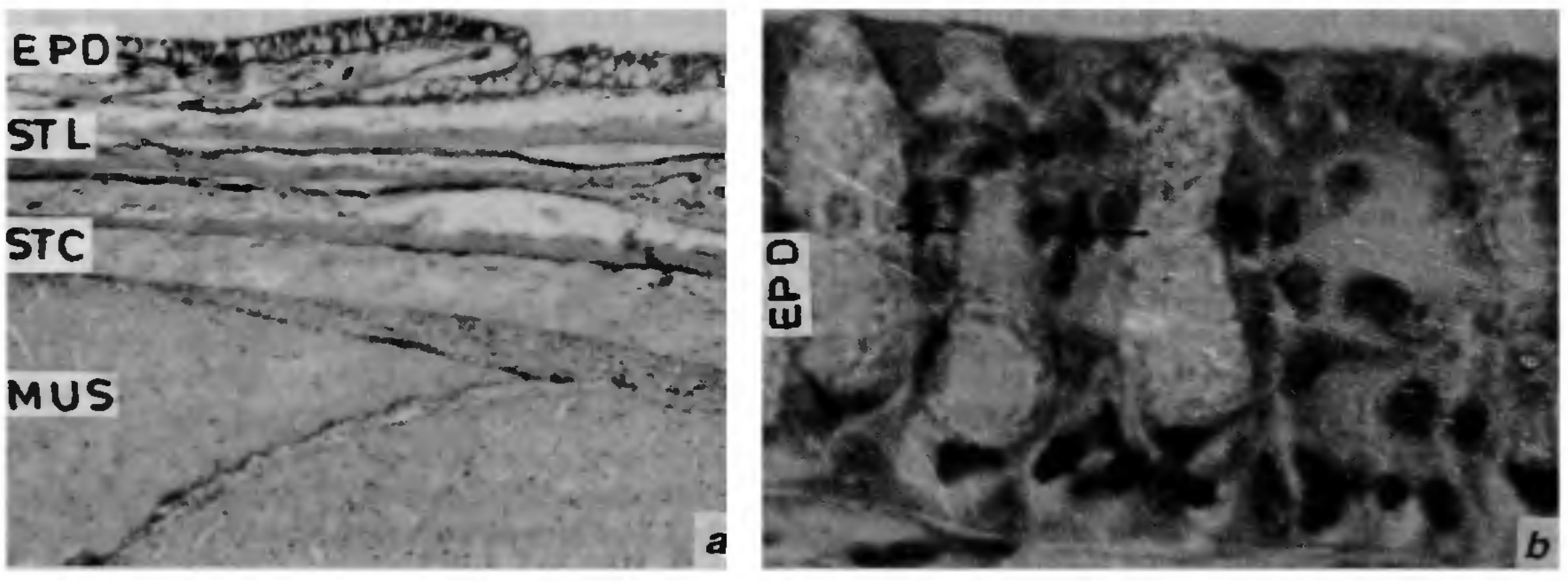
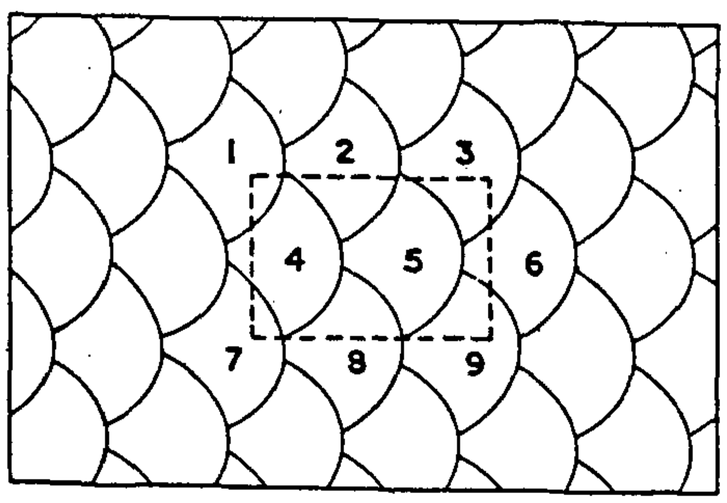
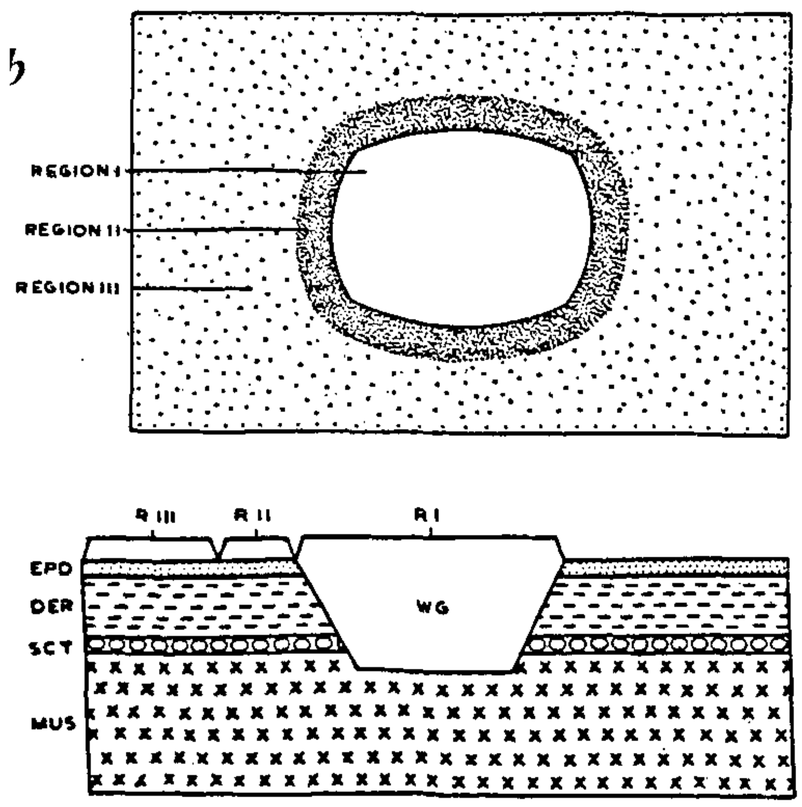
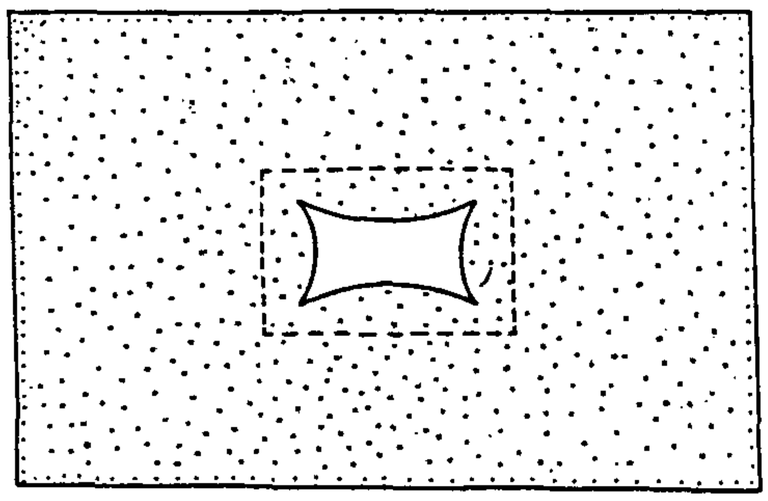


Figure 1. ***a***, Vertical section of the skin of *Channa striata* showing its structural organization (Ehrlich's haematoxylin/eosin; × 40); ***b***, Magnified view of the skin of *C. striata* showing structural organization of its epidermis (Ehrlich's haematoxylin/eosin; × 800).







ɔ. Diagrammatic representation of *a*, surface of the skin of *a* showing scales (numbered 1–9) removed before infliction ound: The barred lines represent the margins of the wound *b*, surface view of an excised wound of *C. striata* showing ent regions; retraction of the wound margins and change in ı region II surrounding the wound gap; *c*, vertical section of ed wound in the skin of *C. striata*; *d*, surface view of an wound in the skin of *C. striata* showing the development of ır wound margins resulting in the contraction of the wound. ed lines represent the original wound margins.

end up to the posterior parts of the scales that embedded deep into the dermis[15,16]. Following l of the scales and subsequent infliction of the , the posterior portion of the scales (enclosed the connective tissue pocket) lying at the anterior of the wound, loosen the epidermal lining. How- he scales lying at the posterior margin of the remain unaffected due to removal of the preced-

ing underlying scales. Thus, the epidermis still covers the anterior free portion of the scales present at the posterior margin of the wound. Hence, a topographic difference exists between the anterior and the posterior borders of the wound.

Alteration in the wounded area of the skin during healing: Following infliction of the wound, the free borders of the wound retract. For the benefit of description, the wounded area of the skin may be divided into three regions: Region I (the wounded area proper or the wound gap); region II (the small area surrounding the wound); and region III (the rest of the skin which is intact or undamaged) (Figures 2 *a–c*). Within 5–10 min of wounding, region II starts changing its colour, becoming very dark by 1 to 2 h (Figure 2 *b*). However, the normal shade is re-established only after 24 h. After about 6 days, the wound starts contracting due to proliferation of the sub-epidermal tissue at the margins of the wound boundary. The square-shaped wound acquires semilunar margins by 10 to 12 days (Figure 2 *d*). Gradually, the wound gap narrows down and by 35 days, the wound gets completely healed.

Regeneration and repair of the epidermis: After a period of inactivity of about 2 h, the epidermis from the posterior margin of the wound starts migrating towards the wound gap in the form of a thin sheet (2–3 cell layered) of epithelium (Figure 3 *a*) and by 4 h, it reaches the anterior margin of the wound. Due to migration of the ECs the epidermis at region II of the wound also becomes very thin. Meanwhile the epidermis at region III near the anterior side of the wound also starts migrating towards region II and reaches the anterior margin of the wound gap and by 4–6 h, the migrating ECs from all the sides come close to each other, resulting in complete bridging (epithelialization) of the wound gap (Figure 3 *b*). At this stage, the basal cells also start differentiating and acquire an almost flat or low cuboidal shape, each cell bearing a flat nucleus. Minute PAS positive granules are also observed throughout the epidermis. After about 6 h, the basal cells acquire a cuboidal shape (Figure 3 *c*), each cell bearing a centrally placed spherical nucleus having well-differentiated chromatin material. Rest of the epidermis comprises horizontally stretched ECs and a few mucous cells (MCs). The thickness of the epidermis starts increasing subsequently and by 10 h of healing, it becomes 8 to 10-cells thick (Figure 3 *d*). The basal cells at this stage get arranged closely in a row without leaving much intercellular space between them. These cells also remain filled with huge deposits of PAS positive saliva resistance granular material. A strongly PAS and weakly alcian blue positive basement membrane also differentiates as a thin sheet. Small finger-like processes from the under-

lying ground substance connect with the basement membrane for strong anchorage (Figure 3 *d*). The middle layer of the epidermis at this stage appears vacuolated and spongy due to the presence of prominent extracellular spaces between the ECs which remain connected to each other by prominent intercellular cytoplasmic bridges (Figure 3 *d*). The quantity of PAS positive granular substances increases in the ECs that mostly remain concentrated in the perinuclear areas. The density of the MCs, especially on the surface layer, also increases. The sacciform-granulated cells voiding their contents along the MCs also differentiate at this stage of regeneration (Figure 3 *e*). Simultaneously, the ionocytes[17] start appearing mostly in the outermost layer where their density increases subsequently (Figure 3 *f*). The thickness of the epidermis decreases between 16 and 30 h when the number of intercellular vacuoles reduces greatly. The quantity of the PAS positive granules in the ECs also decreases. The activity of the MCs increases greatly at this stage (Figure 3 *g*) when a thick layer of slime elaborated by them protects the newly bridged wound gap (Figure 3 *f*, *g*).

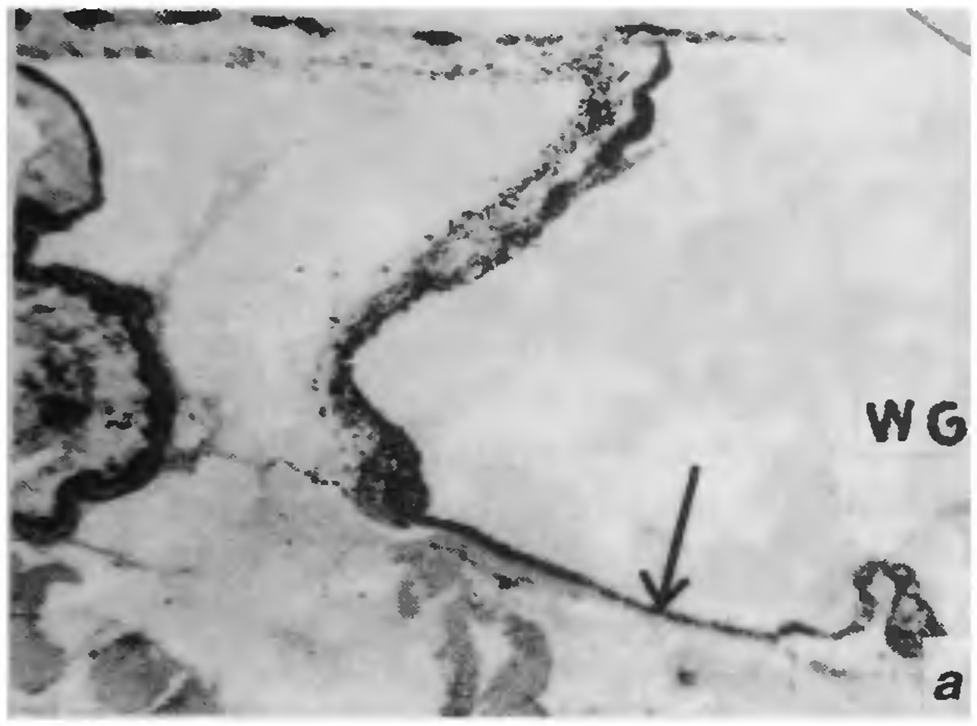
In the subsequent stages, the ECs and the MCs show hyperplasia and the epidermis becomes very thick. Developing MCs at different layers of the epidermis along with actively secreting voluminous sac-like MCs at the outermost layer are commonly observed. The epidermis also shows great vacuolization with prominent intercellular cytoplasmic bridges connecting the distantly lying ECs. Subsequently, the epidermis appears wavy giving out papilla-like projections. Later, the developing scales from the granulation tissues penetrate into these epidermal papillae which subsequently become thinner and by about 20 days, acquire normal appearance.

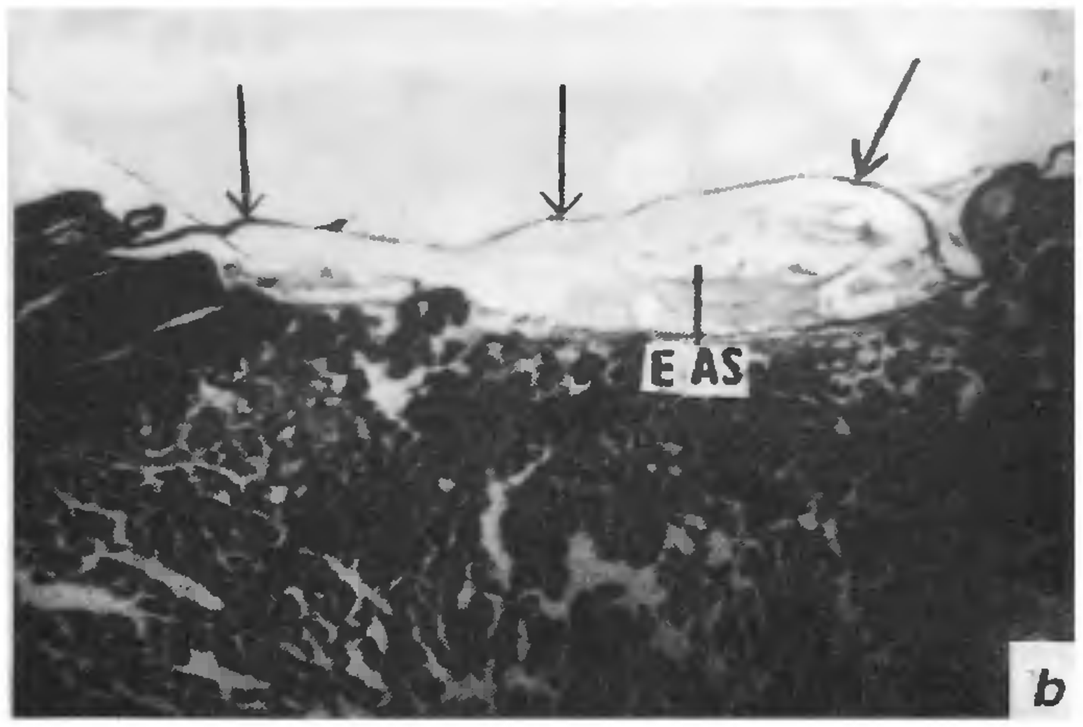
Regeneration and repair of the sub-epidermal tissue: After an initial period of inactivity of about half an hour, the most superficial layer of the denuded muscle fibre bundles start disintegrating in the form of vacuolization. Subsequently, the underlying muscle fibre bundles lying at the regions I and II get widely separated from each other and appear loosely organized. Vacuolization intensifies further and reaches its peak by 6–10 h (Figure 3 *h*). The space between the degenerating muscle fibre bundles gets simultaneously occupied by loose connective tissues and blood material (Figure 3 *h*) derived from the connective tissue septa (that binds the muscle bundles) and damaged blood capillaries present in the region. Simultaneously with the appearance of vacuolization, a thin layer of amorphous slightly eosinophilic (Figure 3 *b*) and weakly PAS positive ground substance appears in the wound gap that reaches its maximum at about 6–10 h. A large number of phagocytes which give strong PAS reaction (Figure 3 *i*) are also frequently observed along with the ground substance.

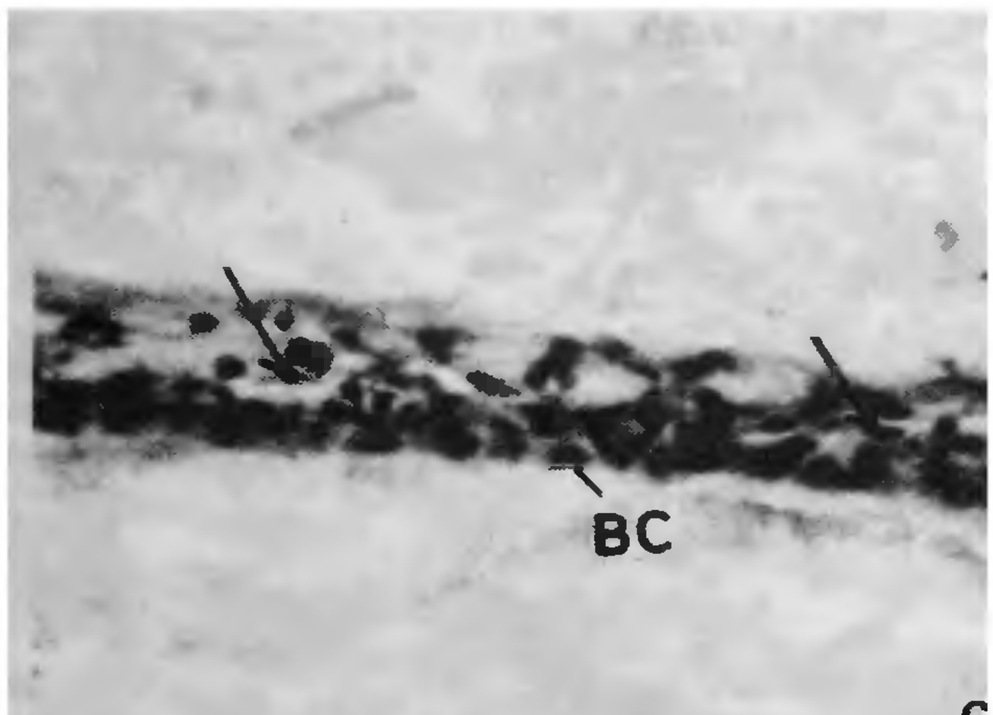
Most of the denuded muscle fibre bundles get almost completely disintegrated by day 3. This space is occupied by phagocyte and different types of blood and connective tissue cells (Figure 3 *j*). The amount of ground substance also decreases markedly within three days and disappears completely within 4–5 days of healing. Fine blood capillaries start invading the sub-epidermal tissue of the wound gap region after 24 h of healing. At region II of the wound, a row of serially arranged cuboidal or low columnar scale-forming cells are formed just below the existing scales after 3 days. The sides of these scales later extend towards the wound gap (Figure 3 *k*). Each scale-forming cell, which appears identical to the osteoblasts, is provided with spherical or oval lightly stained nucleus having a prominent nucleolus and an intensely basophilic, weakly PAS positive cytoplasm (Figure 3 *k*). They also show positive reaction for RNA. By day 4 of healing, a granulation tissue which is marked by the presence of a large number of loosely and irregularly arranged fibroblasts, fine blood capillaries, wandering blood cells and connective tissue cells, develops and occupies the entire sub-epidermal area of the wound gap. Within next 24 h, the granulation tissue becomes compact due to orientation of parallely arranged numerous fibroblasts, especially in the outer layers of the wound gap. Simultaneously, at the lateral and inner margins of the granulation tissue, small myoblasts (Figure 3 *l*, *m*) start appearing more or less at the level of the old muscle fibre bundles. A large number of vertically oriented blood capillaries (Figure 3 *n*) which are more numerous in the outer layers of the wound gap and small-sized pigment cells are also noticed in the granulation tissue.

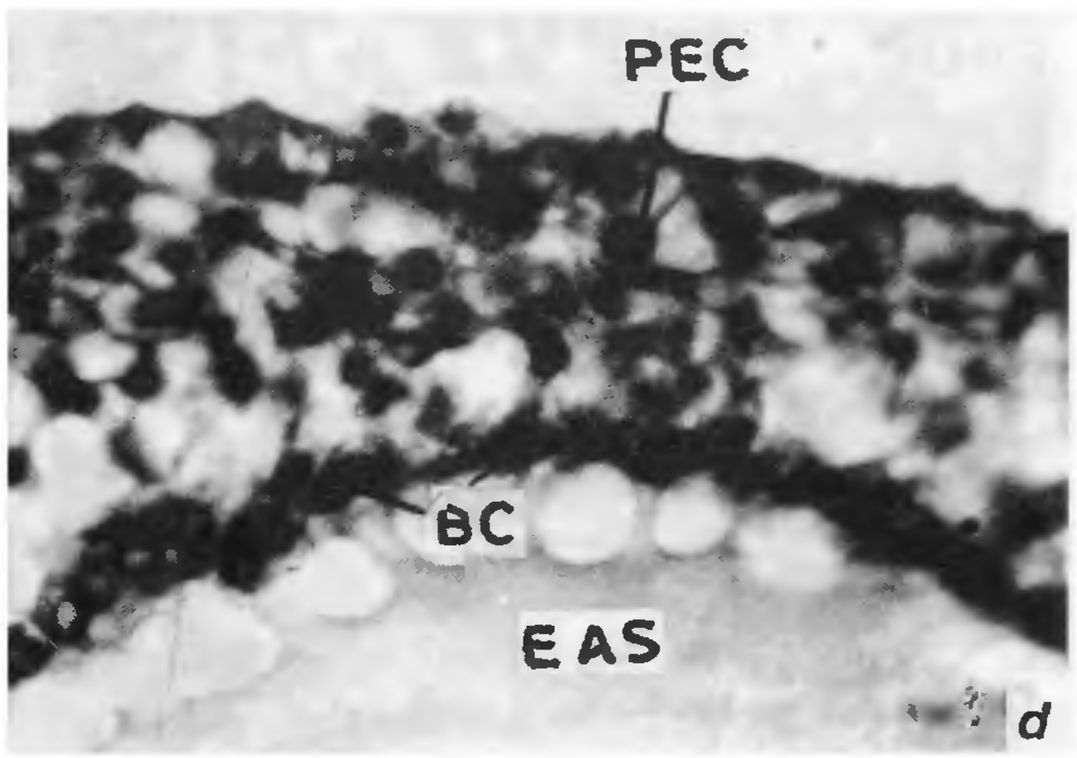
Extensions of scale margins from the anterior and posterior sides of the wound appear to migrate further in the wound gap region. Minute developing scales are also observed in the outer layers of the granulation tissue (Figure 3 *o*, *p*). At these outer layers, the granulation tissue was found to be composed of loosely arranged connective tissue cells, infiltrated with a network of blood capillaries. Fat cells also start forming at these areas and between day 7 and day 10, well-developed fat cells appear at the anterior and posterior margins of the wound gap (Figure 3 *q*). The inner layers of the granulation tissue (mostly at the level of *stratum compactum*) become compactly arranged (Figure 3 *n*) where the number of the blood capillaries decreases markedly. In the deeper layers, myoblasts show gradual development and enlargement.

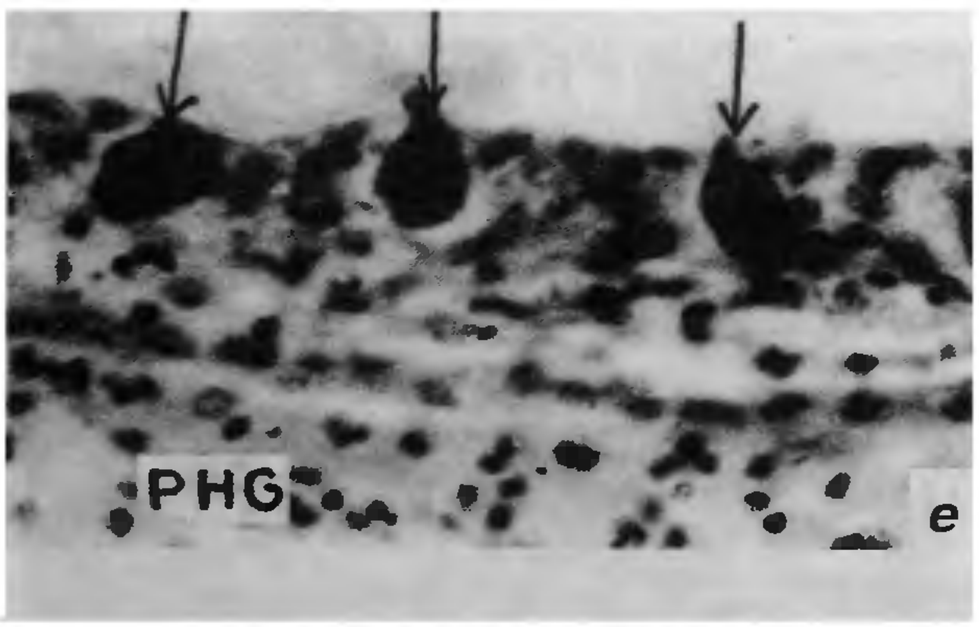
Regeneration of the subepidermal tissue further continues and between day 12 and day 20, the granulation tissue gets further differentiated into *stratum laxum, stratum compactum* and the underlying muscular layer. *Stratum laxum* may be differentiated from the underlying compactly-oriented *stratum compactum* by the presence of

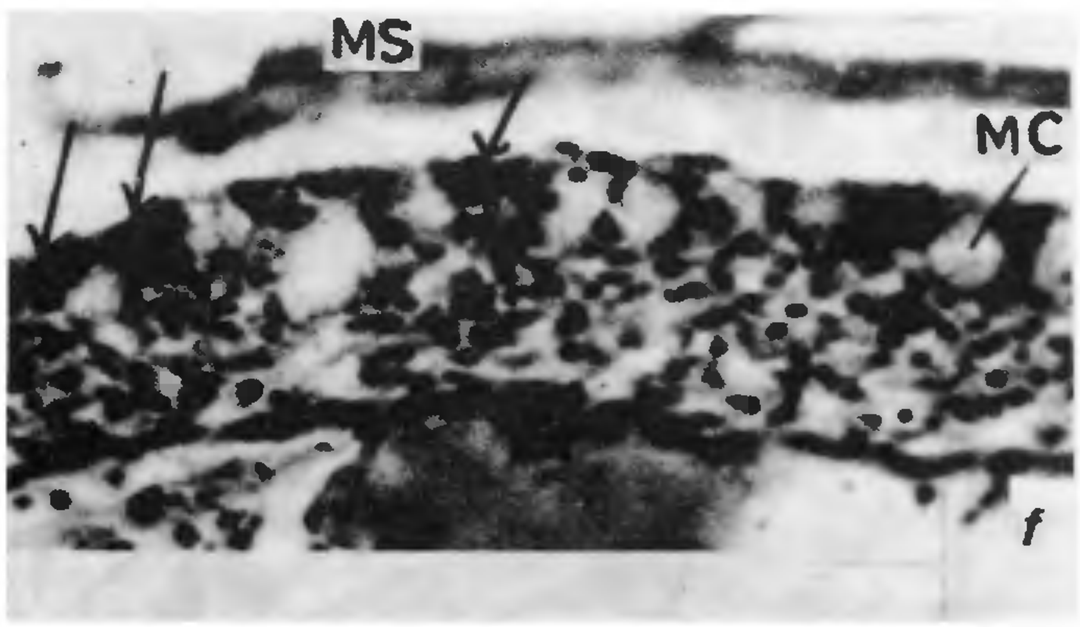


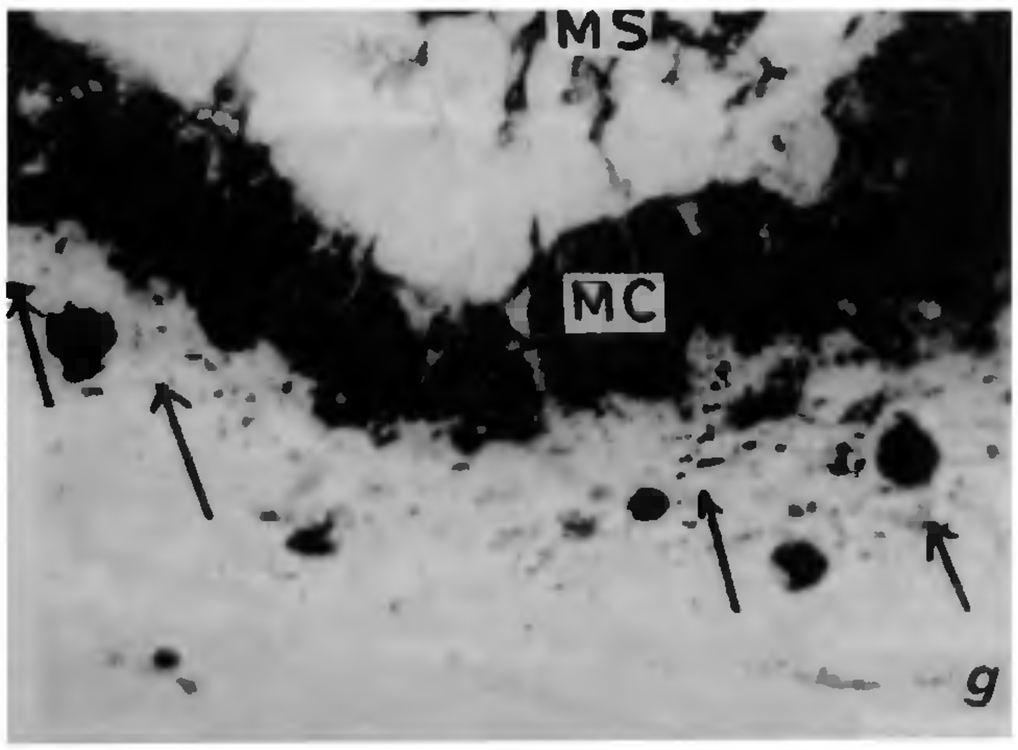












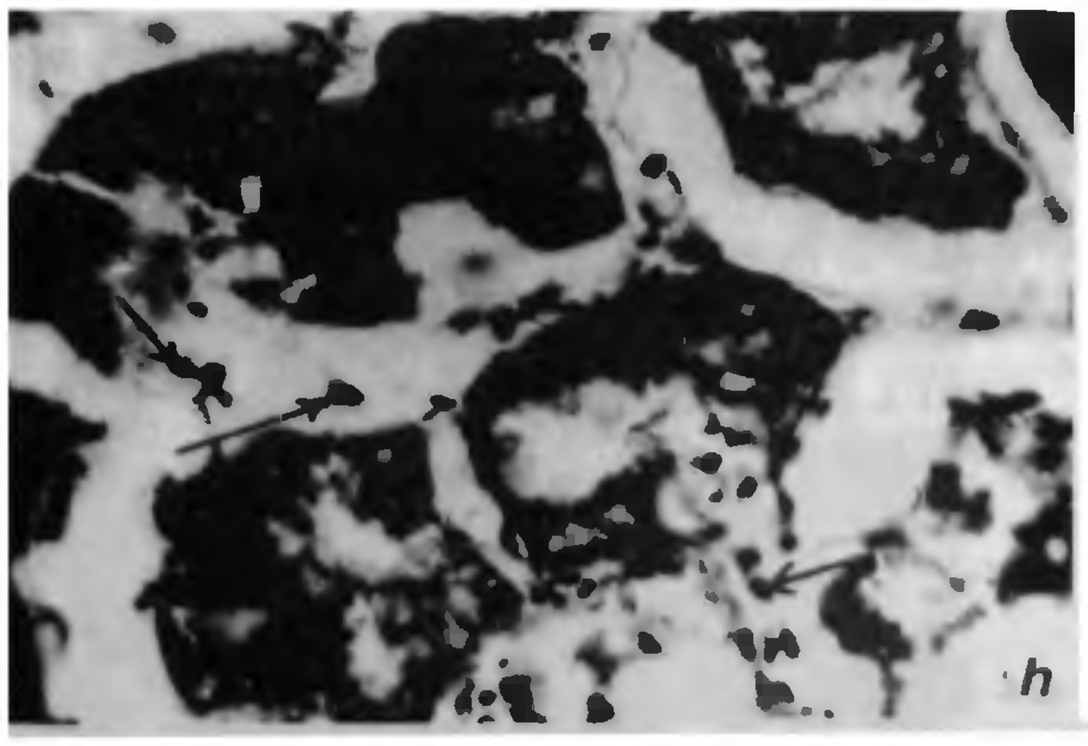


Figure 3. *Continued.*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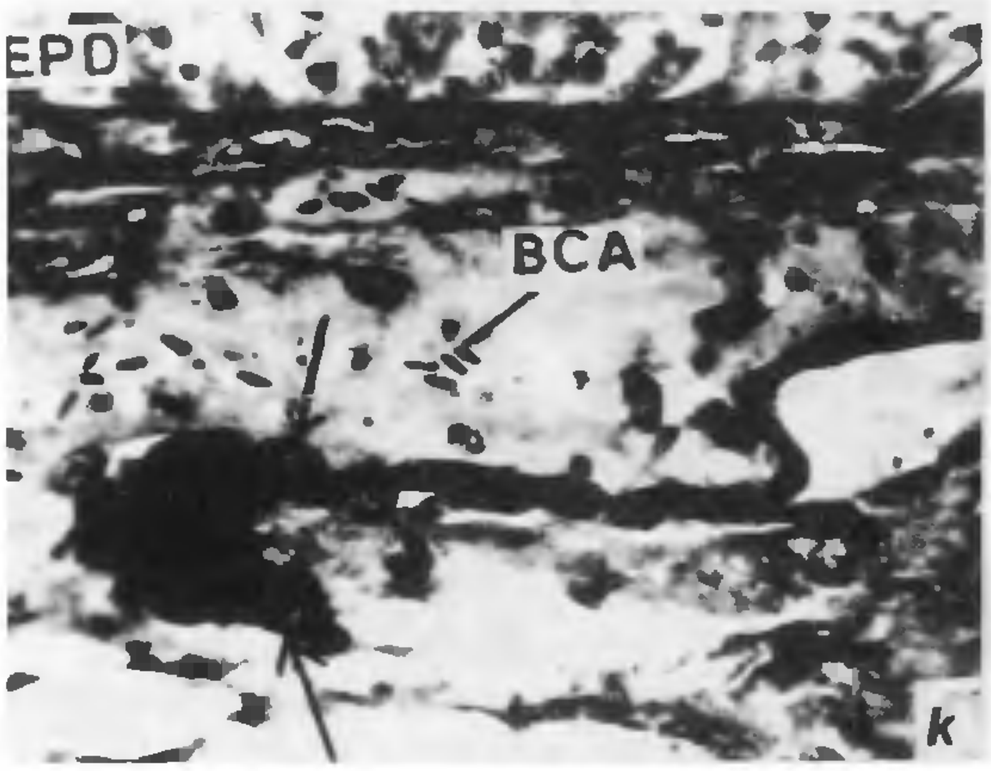
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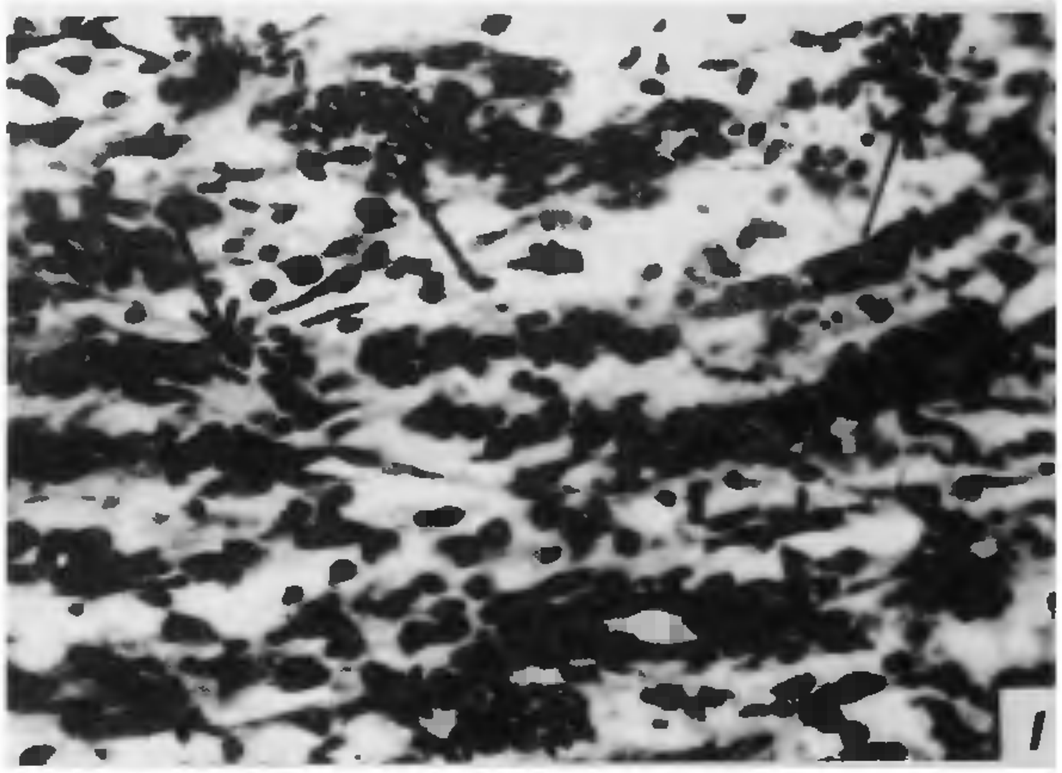
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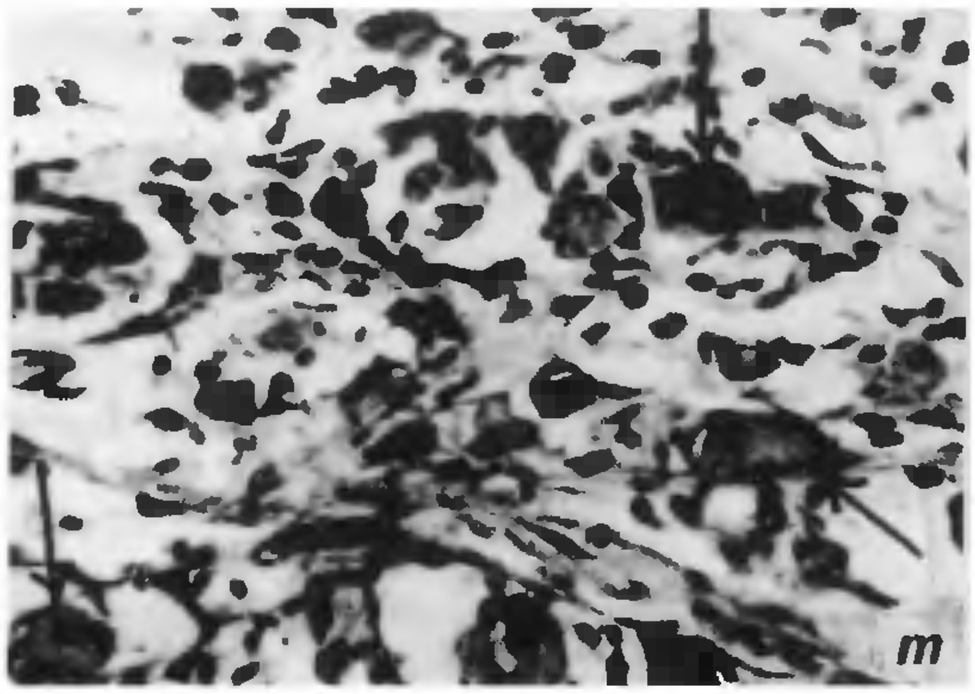
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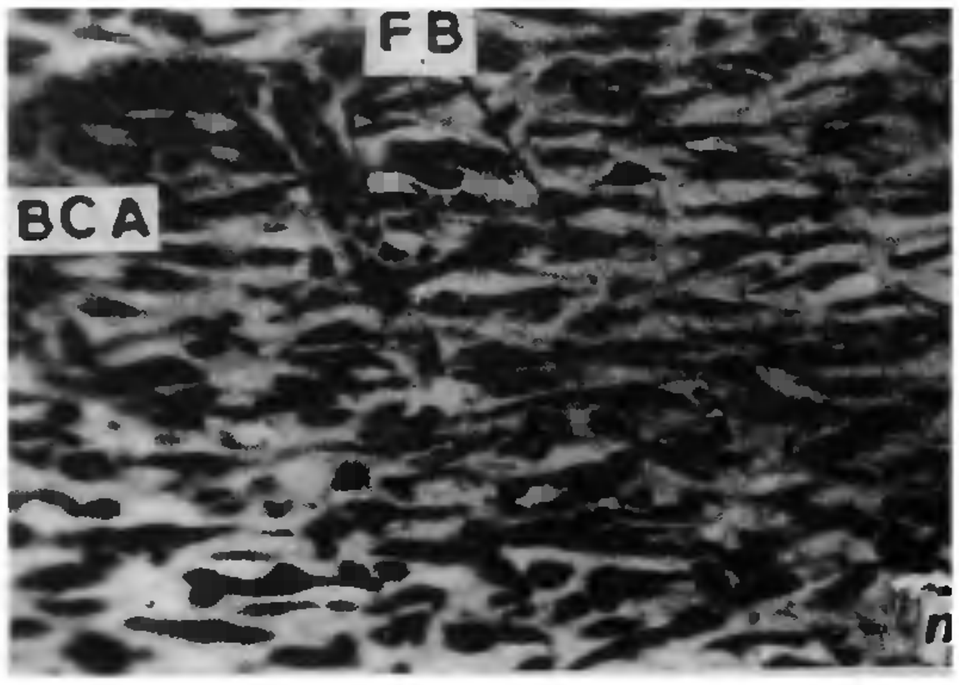
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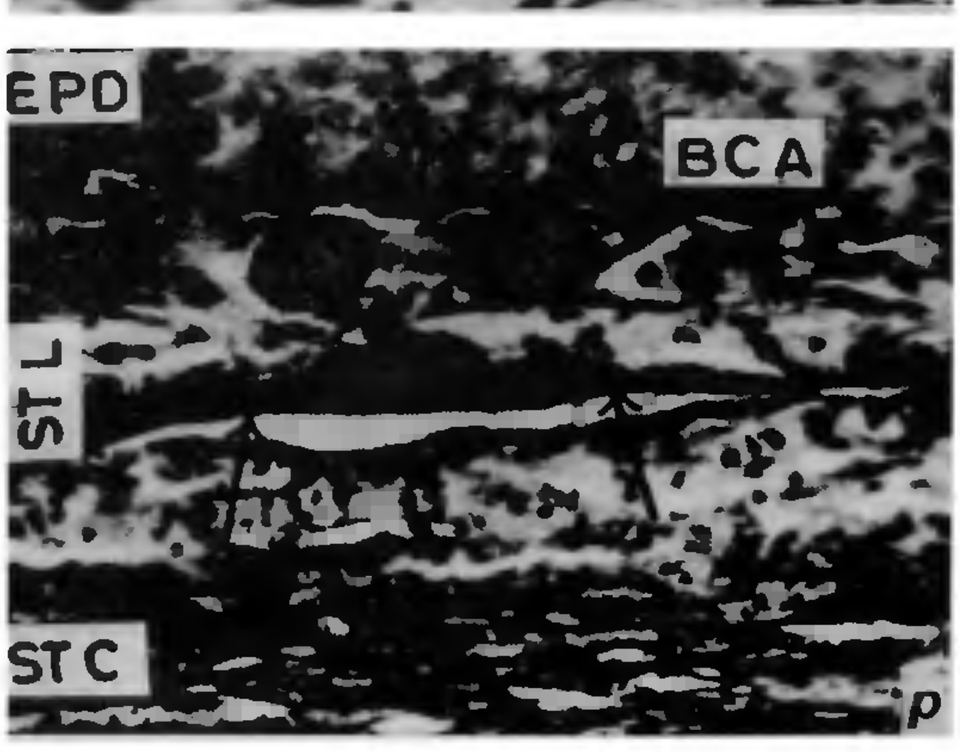
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prominent deposits of fat cells and blood capillaries in these regenerating loosely-oriented connective tissues where developing scales invariably remain embedded. Small fat cells also develop at the inner layer of the *stratum compactum*, thus marking the initiation of differentiation of the subcutaneous area (Figure 4).

Between day 21 and day 35 of regeneration, the various layers of the sub-epidermal tissue including well-developed scales get fully differentiated (Figure 5) and it becomes very difficult to distinguish the wounded area from the rest of the skin excepting the *stratum compactum* which appears comparatively thinner. No scar or scab as observed in higher vertebrates is left behind on the wounded region. However, development and elongation of the scales continue for some period of time.

Discussion

The process of healing of superficial wounds in fishes differs greatly from those of mammals and other higher vertebrates. In contrast to mammals, epithelialization in fishes is a rapid process with movement of the epidermis starting from the wound edge within 1–4 h of infliction of the wound. This is achieved perhaps to prevent the heavy influx of water through the damaged skin. Even the large wound gap of the excised wound in *C. striata* is rapidly bridged by a thin layer of epidermis which provides an immediate protective barrier to maintain the *milieu interior* of the fish. While epithelialization in mammals takes place by division of cells of the *stratum germinativum* several hours after infliction of the wound, bridging of the wound gap in fishes takes place only by mass movement of the epithelial cells, causing decreased thickness of the epidermal lining around the wound gap (region II). Mitotic division of the epithelial cells at the wound edge during or before the epithelialization could not be observed because completion of several cell cycles within 2–4 h (time required for epithelialization) for the supply of new cells to bridge the wound gap is never possible. Mittal and Munshi[10] correlated the quicker rate of epithelialization with the late formation of granulation tissue. In mammals where the wounds are dry, epithelialization starts after a latent period of 24 h. The wound gaps in mammals are plugged by coagulated blood material.

The epidermis covering the wound gaps of fishes (including *C. striata*), which is devoid of scales and other dermal structures, rapidly becomes much thicker than the epidermis of the unwounded areas. This unusual increase in thickness of the epidermis in these otherwise unprotected areas may perhaps be an immediate adaptation for protecting the denuded areas of the fish from external hazards and maintaining the *milieu interior*. Further, a thick epidermis is also a common feature of the skin of non-scaly fishes[10,18,19]. While studying the toxic impact of heavy metal salts (mercury[20,21] and zinc[22,23]) and ambient ammonium salt[24–27], similar hyperplasia of the epidermis of xenobiotics-induced fish skin has frequently been observed. The polygonal epithelial cells at these newly-regenerated sites remain separated from each other by lanes of intercellular spaces. However, these cells remain attached to their neighbours by thin filamentous intercellular cytoplasmic bridges (Figure 3 *d*).

Maintenance of *milieu interior* is also performed by the large number of eosinophilic ionocytes which appear in large numbers in the newly bridged epidermis and prevent any possible disturbance in the ionic equilibrium of the fish caused by influx of water through the damage of the boundary tissue. With the gradual appearance of

Figure 3. Vertical sections of the wounded skin of *C. striata* showing changes occurring following infliction of the wound. ***a***, Posterior wound margin showing movement of the epithelium (marked by arrow) towards the wound gap (after 2 h of healing; Ehrlich's haematoxylin/eosin (H/E); × 40); ***b***, Complete epithelialization (marked by arrows) of the wound. Note the presence of an amorphous acellular substance in the wound gap (after 6 h; H/E; × 18); ***c***, Magnified view of the newly regenerated epidermis covering the wound gap. Note the presence of cuboidal basal cells and 3–4 layers of horizontally stretched polygonal epithelial cells (marked by arrows; after 6 h; H/E; × 375); ***d***, Newly regenerated epidermis over the wound gap showing prominent intercellular spaces and bridges between the epithelial cells. Note the finger-like processes from the underlying amorphous substance attached with the basal cells (after 10 h; H/E; × 400); ***e***, Regeneration of sacciform granulated cells (marked by arrows) in the newly bridged epidermis. Note the presence of numerous wandering blood cells, mostly the phagocytes infiltrating the wound gap (after 20 h; H/E; × 320); ***f***, Numerous ionocytes (marked by arrows) and mucous cells in the outer layers of the newly regenerate epidermis over the wound gap (after 16 h, H/E; × 340); ***g***, Numerous actively secreting mucous cells and PAS positive granules (marked by arrows) (stained magenta) in the newly regenerated epidermis after 6 d (PAS, × 180); ***h***, Denuded muscle bundles showing vacuolization and disintegration. Note the blood cells and connective tissue cells (marked by arrows) in the space between the disintegrating muscle bundles (after 6 h; H/E; × 180); ***i***, Huge deposition of an amorphous PAS positive material infiltrated with numerous phagocytes below the newly regenerated epithelium (marked by arrow; after 6 h; PAS × 100); ***j***, Invasion of numerous phagocytes (marked by arrows) in the degenerating muscle bundles (after 2 d; H/E; × 320); ***k***, Serial aggregation of scale-forming cells (marked by arrows) causing enlargement of the existing scale resulting in its penetration in the wound gap from the margin (after 3 d; H/E; × 300); ***l***, Beginning of formation of granulation tissue with irregularly arranged fibroblasts, myoblasts, phagocytes and numerous fine blood capillaries (marked by arrows; after 4 d; H/E; × 180); ***m***, Regeneration of myoblasts (marked by arrows) at the level of old muscle bundles (after 5 d; H/E; × 180); ***n***, Differentiation and fibre formation due to parallel arrangement of fibroblasts in the granulation tissue (after 6 d; H/E; × 180); ***o***, Differentiation of *stratum laxum* and *stratum compactum* from the granulation tissue. Note the development of scales (marked by arrows) in *stratum laxum*, and papilla-like projections of the epidermis (after 7 d; H/E; × 40); ***p***, Magnified view of development of scales (marked by arrows) in *stratum laxum* (after day 7; H/E; × 180); ***q***, Development of numerous fat cells (marked by arrows) in the granulation tissue at the level of *stratum laxum* (after day 10; H/E; × 120).

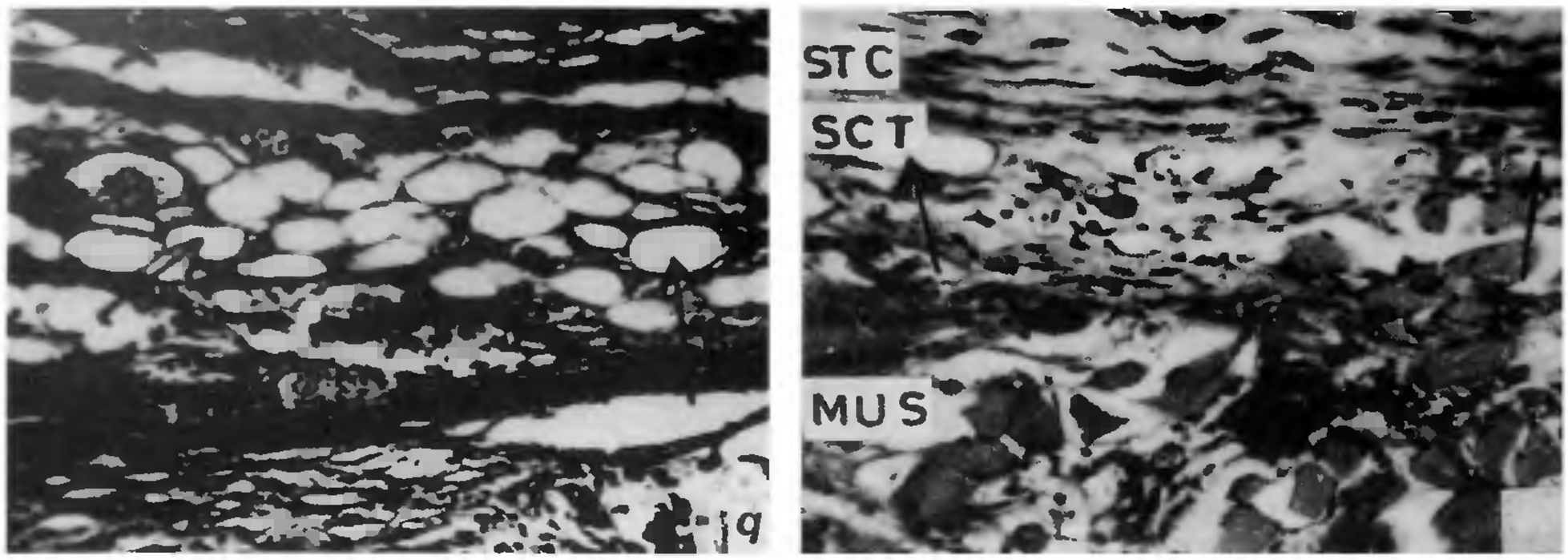

Figure 4. Development of subcutis following development of fat cells (marked by arrows) between the *stratum compactum* and muscle bundles (after day 20; H/E; × 180).

Figure 5. Completely regenerated skin with well-established epidermis, dermis and subcutis and the underlying muscle bundles identical to the intact skin of control fish. Note well-developed scales enclosed within connective tissue pockets in the *stratum laxum* (after day 35; H/E; × 90). BC, basal cell; BCA, blood capillary; CTP, connective tissue pocket; DMB, denuded muscle bundle; EAS, eosinophilic amorphous substance; EPD, epidermis; FB, fibroblast, MC, mucous cells; MS, mucous secretion; MUS, muscle; PEC, polygonal epithelial cells; PHG, phagocyte; RI, region 1 around the wound gap; R II, region 2 around the wound gap; R III, region 3 around the wound gap; S, scale; STC, *stratum compactum*, STL, *stratum laxum*; WG, wound gap.

the scales at the wounded region in the later stages of healing, the thickness of the epidermis decreases gradually. This is because the regenerating scales and other sub-epidermal tissues subsequently take over the protective role. Differentiation of other cellular elements of the epidermis (including the unicellular glands) starts quite late. However, Rajan and Banerjee[20] did not observe any differentiation of glandular elements in the regenerating epidermis following exposure to lethal concentration of mercuric chloride.

The denuded muscle bundles in the early stages of healing show massive degeneration and severe inflammatory reaction causing appearance of macrophages, neutrophil granulocytes, lymphocytes and eosinophilic granulocytes (apart from fibroblasts and myoblasts)[2]. Apart from supplying nutrients required for regeneration and repair of the damaged tissue, the network of fine blood capillaries perhaps also helps in local respiration of the regenerating tissue components. The pigment cells which frequently appear in the granulation tissue might also help in local defence mechanism by eliminating the debris of the degenerating cells and the infectants. The phagocytic action of the melanophores is well illustrated. The granulation tissue develops at the wounded area which is mainly formed by densely packed free fibroblasts, a network of fine blood capillaries along with a large quantity of undifferentiated extracellular substances. The rich vascularization of the granulation tissue supplies nutrients for active regeneration of the subepidermal tissues. It also provides protection to the underlying tissues against penetration of infectants into the denuded tissues of the deeper layers by facilitating the active migration of leukocytes in the superficial layers. The various sub-epidermal layers differentiate from the granulation tissue only. The granulation tissue shows strong basophilia due to the presence

of large quantities of RNA, indicating active protein synthesis for laying the connective tissue and muscular layers.

Although new scales also regenerate from the granulation tissue, extensions from the existing scales in the wound margins also migrate towards the wound gap, thus penetrating into the granulation tissue. This results in haphazard arrangement of the scales (irregular but increased size) at the regenerated sites. Regeneration of the scales takes place by serial arrangement of the strongly basophilic scale-forming cells (scleroblasts) below the invading portion of the existing scales. According to Goss[28], these scale-forming cells remain in the scale pockets even after removal of the scales and following injury to the scale pockets these cells get stimulated to differentiate[29,30]. However, a large number of scattered scale-forming cells are also observed in the granulation tissue of the regenerating skin even after complete elimination of the scale pockets by inflicting excised wounds on the body of *C. striata*. The strong basophilia and positive reaction for RNA also suggest active metabolic stage of these cells leading to the synthesis and accumulation of the scale-forming materials (e.g. proteoglycans). These cells appear quite identical to those observed at the sites of regenerating fractured bones.

Unlike mammals, no scar or scab is left behind on the skin surface (Figure 5) after completion of healing.

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