Gross and histopathologic changes of porcine cholecyst assisted full thickness skin wound healing in rabbits

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Abstract

The remodeling and regenerative responses of porcine cholecyst on full thickness skin wounds were evaluated in this study. Comparison of porcine cholecyst and open wound control were made for evaluation. The *in vivo* evaluations were done on full thickness skin wound healing model in New Zealand White rabbits. The inflammatory, remodeling, and regenerative responses were evaluated 7, 14, 21 and 28 days post implantation. The H&E stained sections were evaluated for inflammatory and remodeling responses. The results of the current study indicated that porcine derived cholecyst scaffolds are one of the best suited materials for full thickness skin wound healing, owing to their biochemical, biodegradable, biocompatible and tissue remodelling responses. The use of porcine cholecyst in full thickness skin wounds shows improved epithelialization and faster remodelling devoid of infections or graft rejections in all the animals under study. Scab formation, ulcerations and other complications were also not observed in any of the scaffold assisted wounds.

Keywords: bio-scaffold; cholecyst; porcine; skin; wound

Introduction

Skin wounds ranging from abrasions, bruises, cuts, bite wounds, scratch wounds, burns etc are commonly observed in domestic animals, wild animals, birds and man. The smaller wounds heal off with no hassles, but whenever there is volumetric tissue loss, remodelling responses starts as second intention healing with epithelialization and later proceed to wound contraction resulting in scar formation, keloids and loss of sensation and function of the healed tissue. Initial focuses were to ensure minimal scarring, which later changed to compensation of lost tissue, and finally to complete regeneration of tissue and lost functions (Williams, 2008). Hence more emphasise were given to application of bioscaffolds in induced regeneration and remodelling to replace lost tissues (Badylak *et al*., 2009). Porcine cholecyst is yet another collagen based matrix, clinically proven as a gastrointestinal and entero-anastomotic buttress (Burugapalli *et al*., 2008), partial and full thickness skin wound graft (Revi *et al*., 2014), bladder repair graft (Kajbafzadeh *et al*., 2014) and abdominal hernial repair graft (Dhanush, 2014). The scaffold contains glycosaminoglycans, elastin and growth factors like VEGF and bFGF (Burugapalli *et al*., 2007; Anilkumar *et al*., 2014). This study has been designed to understand the sequential inflammatory and proliferative phases of porcine cholecyst assisted full thickness skin wound healing in rabbits against open wound healing.

Materials and methods

The experiments were carried out jointly in the Department of Pathology, College of Veterinary and Animal Sciences, Mannuthy and Division of Experimental Pathology, Sree Chitra Tirunal Institute of Medical Sciences and Technology, Trivandrum. Adult New Zealand white rabbits (n=24) of about 1.5 Kg of either sex were used. Porcine cholecyst required for the study was harvested from freshly slaughtered pigs from Department of Livestock Products Technology, College of Veterinary and Animal Sciences, Mannuthy and were processed (Anilkumar *et al*., 2014) and packed at Division of Experimental Pathology, Sree Chitra Tirunal Institute of Medical Sciences and Technology, Trivandrum. The animals were divided into two groups, viz. Group I – Porcine cholecyst derived graft (d-PC) and Group II – Open wound (control group). Sterile surgical technique was employed under general anaesthesia (Markowitz, 1964) to create a 2x2 cm full thickness skin wound dorsally on either side of the spine at thoracic region of the rabbits. Lyophilised and EO sterilized porcine cholecysts were cut to the similar size of the defect. The material was rinsed repeatedly in sterile PBS (pH 7.2) before implantation. Pieces of this were placed on the skin wound and sutured with polyamide suture material (Ethilon^{TM} 3-0, Johnson and Johnson India). The animals were monitored regularly for signs of inflammation at the surgical site. On day seven, 14, 21 and 28 post-surgery, six animals from each group (total–six) were humanely sacrificed. The gross observations were recorded prior to euthanasia. The skin at the site of implantation, along with the biomaterial were explanted and fixed in 10 per cent neutral buffered formalin (Bancroft and Gamble, 2008). Histopathology of the explants was done on the fourth day of fixation using standard techniques (Bancroft and Gamble, 2008).

Results

Surgical procedure and post-surgical healing were uneventful in all the cases. No animal exhibited sings of infection or rejection of the bioscaffold material. All the animals survived through the experimental duration, with normal feeding, watering and voiding. They were euthanized seven days, 14 days, 21 days and 28 days post implantation, according to the experimental design.

On day 7 PI (post implantation), the sutures were found intact and the scaffold was found slightly dried up with minimal amount of scab formation in d-PC treatment (Plate.1). There were no ulcers or seroma formation externally. The surrounding tissue appeared slightly reddish and inflamed. In open wound control group, scab formation was minimal and was not found covering the entire surface of the wound (Plate.3). The wound edges were reddish and there were no discharges or pus from any of the control groups.

On day 14 PI, scaffolds fell out from three each of d-PC, leaving the wound grossly apposed to maximum extent (Plate.2). The area of the wound appeared slightly reddish and glistening than the surrounding tissue. Three animals which had intact scaffold material showed fully dried up scaffolds integrated with the wound and had minimal scab formation. The tissue surrounding the wound area was normal in colour. The wound edges were demarcated by intact sutures in all the animals. There were no ulcerations or seroma formation in any of the animals.

Animals treated as open wound showed more reddish discolouration of the wound area (Plate.4). One of the animals in open wound control group showed loss of granulation tissue and scab, leaving the wound open at the middle portion. There were no discharges from the site.

On day 21 PI, the wounds were almost fully apposed in d-PC. The size of the wound area was smaller than actual. Wound edges were demarcated by few non-absorbable sutures (Plate.5).

The open control group showed small focal reddish discolouration at the centre of the wound area (Plate.7). The centre of the wound area was slightly raised than the surrounding tissue. There were evidences of contraction of wound.

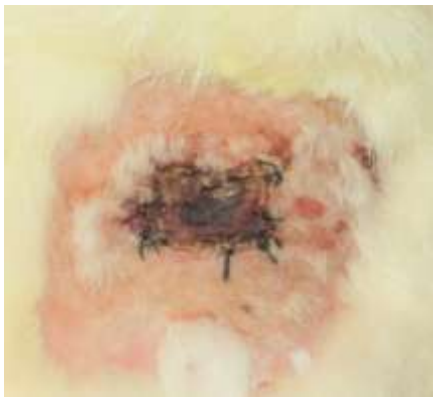

Plate.1 (d-PC: Day 7) – intact sutures, slightly dried up scaffold and minimal scab formation

Plate.2 (d-PC: day 14) – fully dried intact scaffold integrated to the wound with minimal scab formation

Plate.3 (Open wound: Day 7) – moderate scab formation, but not covering the entire wound

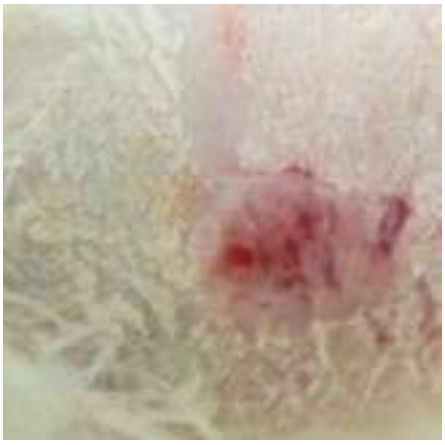

Plate.4 (OW: day 14) – scab fell off, leaving the wound open at middle portion. No discharges or ulceration present

Plate.5 (d-PC: Day 21) – wound almost fully apposed, wound edges demarcated by sutures

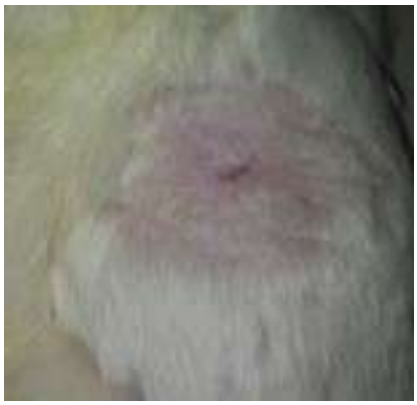

Plate.6 (d-PC: day 28) – wound completely healed, wound area marked by an elongated diamond shaped raised patch

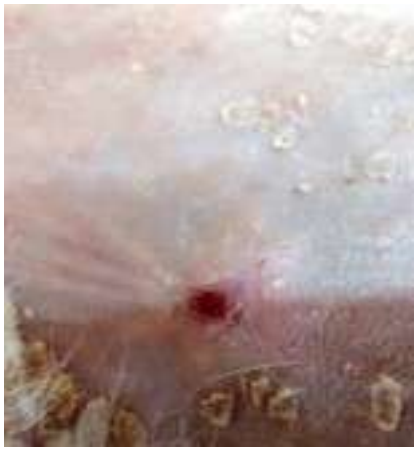

Plate.7 (OW: Day 21) – evidences of wound contraction observed. Centre of the wound is slightly raised

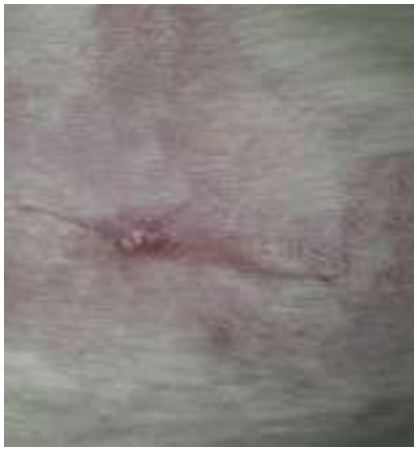

Plate.8 (OW: day 28) – wound completely healed, wound area marked by an elongated diamond shaped raised patch

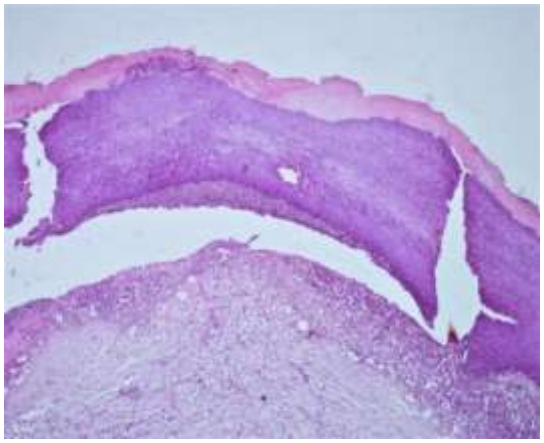

Plate.9. d-PC: Day 7 – Mononocluear cell infiltration (star) around the newly formed keratinocytes (H&E X 100)

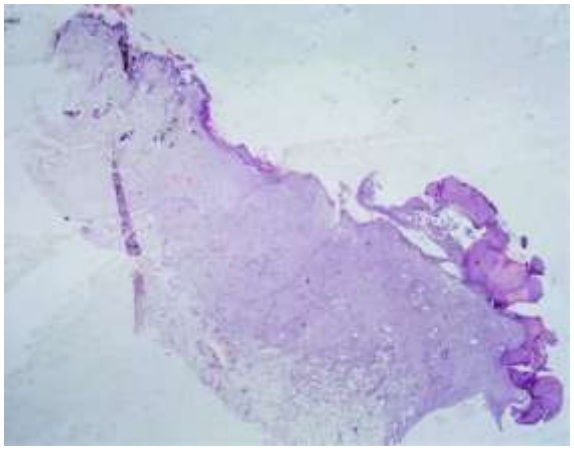

Plate.10 OW Day 14 showing areas of incomplete epithelialization

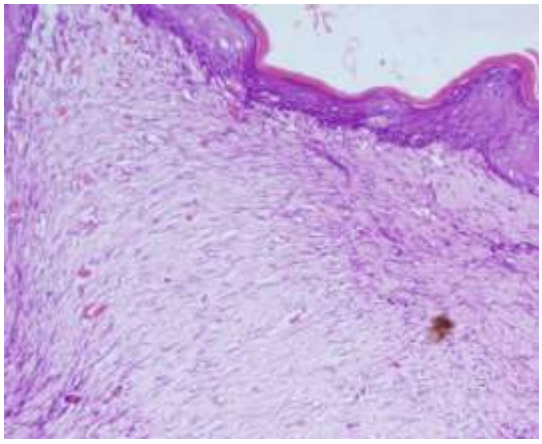

Plate.11. d-PC: Day 21 – Neoangiogenesis within the loose wavy collagenous matrix (H&E X 200)

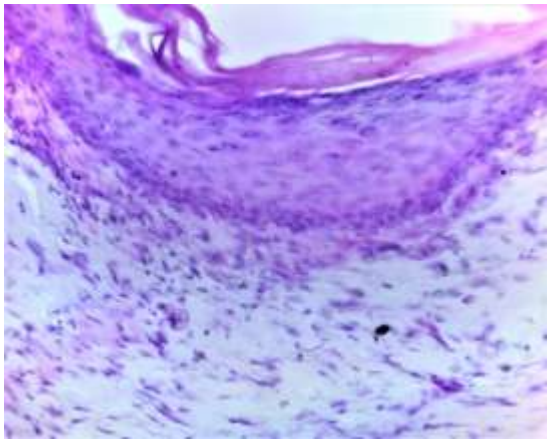

Plate.12. d-PC: Day 28 – Well-formed epidermal layer, wavy collagenous matrix with mature spindle shaped fibroblasts (H&E X 400)

On day 28 PI, the wounds were almost indistinguishable grossly except for small raised areas with a central red line in few of the animals. The wounds were fully apposed leaving an elongated diamond shaped red patch at the centre in all the treatment groups (Plate.6). The wound areas were identified after clipping of hairs only. Few of the animals had one to two sutures still over the skin. The wound area and the surrounding tissues showed normal colour in all the groups. Hair growth was also seen towards the wound area in all the groups. The control group showed evidences of contraction grossly but were not significant (Plate.8).

Histopathology using H&E staining revealed neo-epithelialization and granulation tissue formation in both groups on day seven post implantation. Newly formed keratinocytes occupied just beneath the neo-epithelial layer (Plate.9). Inflammatory cells, predominantly lymphocytes were found in large numbers just around the newly formed keratinocytes. Granulation with wavy collagenous matrix deposition and moderate degree of neovascularization were also observed in the dermis. Open nucleated actively dividing fibroblasts were seen, indicating collagenous deposition. Mild degree of fatty infiltration was seen in the dermis.

On day 14 PI, re-epithelialization was complete in d-PC, whereas the control group showed areas of wound without epithelialization (Plate.10). Keratinocytes were organized and formed a pattern of arrangement and rete pegs and ridges were also found. The newly formed epidermis was thicker than the regular epidermal layer. Keratohyaline deposition was observed outside neo-epidermis. Mild degree of cellular infiltration was evident between neo-epidermis and dermis. The scaffold was found integrated with the keratin layer and mononuclear cell infiltration of the scaffold material was also evident. Fibroblasts became more elongated and less number of open nucleated fibroblasts were seen. The collagen fibres were organized in a circular pattern, forming the centre of the circle at the middle of the wound. Newly formed blood vessels were seen scattered within the collagenous matrix.

On day 21 PI, complete re-epithelialization was observed in both groups. There was no inflammatory reaction anywhere in the healing matrix. Granulation tissue became more organized, with wavy collagenous matrix and mature spindle shaped fibroblasts. Basal cells were tightly packed and keratinocyte proliferation was minimal. The collagen fibres were seen parallely arranged in most areas except at the centre of the wound, where wavy unorganized pattern of fibres were seen. The epidermal layer thickness was smaller than day 14 PI. Neovascularization was evident throughout the collagenous matrix (Plate.11).

Mature epidermal layer with differentiated rete pegs and ridges were seen in all the animals on day 28 PI. Keratinocytes and basal cell proliferation was minimal. The thickness of the neo-epidermis was comparable to normal skin. Fibroblasts were elongated and spindle shaped (Plate.12). The area of remodeled matrix was minimal compared to previous observations. The area of wound showed more of dense wavy collagenous deposition at the dermis. The collagen fibers formed a regular parallelly arranged wavy matrix, as similar to normal skin. Evidences of regeneration of skin structures like hair follicles were seen towards the wound healing areas in all the animals.

Discussion

The graft assisted healing technique, as a whole, appeared more visually acceptable and the gross observations itself were vital evidences of improved healing compared to open wound. Graft assisted wounds were devoid of inflammation, seroma formation and ulcerations, suggesting better acceptance of the scaffold *in vivo*. Revi *et al.* (2014) also reported similar hassle free gross healing observations. Open wounds were also non-infective, but ulcerations were seen in a few animals as early as 7 days post implantation and scab formation were also evident. The scaffolds were seen partially dried by day 7 PI and fully dried by day 14 PI in all the animals, and few of the scaffolds shed off from the wounds, exposing a smooth glistening wound surface. The wounds which were treated with porcine cholecyst were found fully apposed by about 14 days, suggesting an improved healing response compared to open wounds. Cholecyst has proven to have a higher water retention ability marked by a lower water vapour transmission rate (Anilkumar *et al.*, 2014). Moisture in the wound bed was proven to improve the healing rate (Bryan, 2004). Thus, it can be inferred that improved rate of wound healing in cholecyst assisted wounds are related to the water retention properties of the scaffold also. Though all the wounds healed by 28 days post implantation, wound contractions and distorted wound edges were observed when the wounds were left to heal as open wound. This suggests that in a large sized wound (above critical wound size), graft assisted healing is having a better prospect.

The earliest histopathological observations were taken at day seven PI, which revealed mononuclear cell infiltration and actively dividing fibroblasts present in abundance. This represented the proliferative stage prior to remodeling. Mononuclear cell infiltration was more in scaffold assisted wounds comparing to open wounds. There were large numbers of these inflammatory cells, predominantly lymphocytes, towards the scaffold wound interface, and active fibroblasts at the dermis, revealing the scaffold enhances the proliferative stage of wound healing. Mononuclear cell infiltration reduced considerably by day 14 PI in all the treatments, and by day 28 PI, there were no inflammatory reaction in any of the animals. Early infiltration of fibroblasts and inflammatory cells helps in faster epithelialization and keratinocyte migration. Though it is observed that

materials that induce inflammation tends to fasten healing of partial and full thickness skin wounds (Chvapil *et al.*, 1991) it is also observed that more inflammatory infiltration leads to rejection of graft according to studies conducted by Thampi (2011), where gluataraldehyde treated bovine pericardium was found to elicit a higher inflammatory response and lead to graft rejection in rat subcutaneous biocompatibility model and Filho *et al.* (2017), where the intense inflammatory reaction elicited by canine amniotic membrane prevented graft integration leading to rejection in rabbit full thickness skin wound healing model. In contradiction to these findings, in this study, even though on day 7 there was heavy infiltration of inflammatory cells, graft assisted wounds tend to heal faster than open wound, as observed by a complete re-epithelialization of wounds by day 14 PI. This suggested that porcine cholecyst was better biocompatible and tends to heal the wound by enhanced inflammatory response.

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