

Wound healing potential of a gel formulation of porcine cholecyst extracellular matrix in a rat full-thickness excision model

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ABSTRACT

Collagen-rich extracellular matrices (ECMs) recovered from mammalian organs and tissue have found clinical uses in several formats like thin sheets, powder, ointments, gels, *etc.*; either as a standalone tissue engineering scaffold or in combination with other biomaterials and pharmaceuticals for many regenerative medical applications. Out of these, gel formulations of 'advanced wound care products' made of animal-derived ECMs have high utility because of structural adaptability, biocompatibility, and tunable physicochemical properties. This study demonstrated the wound healing potential of a porcine cholecystic extracellular matrix gel formulation in rat full-thickness skin-excision wound model by gross and histomorphological studies.

Keywords: Extracellular matrix, Full-thickness skin excision wound, Wound healing

INTRODUCTION

Tissue engineering scaffolds (TES) fabricated out of animal-derived extracellular matrix (ECM) recovered from appropriately decellularized mammalian organs/tissues can be used as 'advanced wound care' products¹. They contain ECM constituents (e.g.: collagen, laminin, glycosaminoglycan, fibronectin, *etc.*) with variable purity. Indeed, collagen-rich ECMs have found clinical uses in several formats like thin sheets, powder, gels *etc.*, either as a standalone product or in combination with other biomaterials and pharmaceuticals². Despite several limitations in clinical utility due to shape/form/presentation, the ECM sheets are widely used as surgical grafts for wound healing applications. On the other hand, for topical wound healing applications, gel formulation is preferred over sheet forms³.

Our laboratory has developed several sheet formulations using ECM of porcine gall bladder (cholecyst) with potential clinical applications^{4,5,6,7}. Preparation of powder⁸ and gel formulations⁹ were also attempted from porcine cholecystic extracellular matrix (CECM). The original gel formulation of CECM was intended for surface modification of polymeric hernial repair graft which mitigated the nature of tissue reaction around intramuscular implants, but was not suitable for topical wound healing application because of its high viscosity and storage modulus⁹.

This study evaluated the wound healing potential of a modified CECM-hydrogel with a softer consistency in a rat full-thickness skin-excision wound model.

MATERIALS AND METHODS

Preparation of the gel

The CECM hydrogel was prepared by the original method⁸ involving solubilisation (using, pepsin-HCl), followed by neutralisation (using, 0.1 N NaOH and 10x PBS), functionalization [using, 1-ethyl 3-(3-dimethyl

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aminopropyl) carbodiimide, 2 mM and N hydroxysuccinimide acrylate, 4 mM] and gelling [using, poly(ethylene glycol) diacrylate, M_n -700, 2 mg/ml]. The acrylate modified CECM pre-gel solution and the gelling agent PEGDA were mixed in the ratio of 1:0.08 (w/w). The consistency of the original viscous CECM hydrogel was reduced by the addition of PBS solution.

Wound healing study in rats

Animal experiments were conducted on 36 sub adult (4-6 weeks of age) male Sprague Dawley rats of 150-200 mg bodyweight, with the statutory permission of the Institutional

Animal Ethics committee (SCT/IAEC-366/JULY/2020/106) under the direct supervision of a veterinarian. Essentially, rats were housed in individually ventilated cages (temperature $22 \pm 3^\circ\text{C}$; humidity 30 – 70%; 12 h day/light cycle) with *ad libitum* access to feed and water. Animals were anaesthetized using Ketamine (80-100 mg/Kg, Indian Immunologicals Limited, India) and Xylazine (10 mg/Kg, Neon Laboratories Limited, India) in combination intraperitoneally. The surgical site, on the dorsum was shaved with an electrical hair trimmer in caudo-cephalo direction and painted with Betadine Surgical Scrub 7.5% (G. S. Pharmbutor Private Limited, India) for sterilization. Loss of pedal reflex was checked to ensure that the animals were in a surgical plane of anaesthesia. In the dorsal region, about 1 cm posterior to the transverse line connecting lower angle of scapula was considered as the cranial border of wound. Full-thickness dorsal skin excision wounds of 2.5 cm x 1.5 cm dimension craniocaudally were created in each animal using a scalpel blade (scalpel size 15, BP handle 3). Underlying panniculus carnosus muscle was also removed. Immediately after surgery, rats were randomly allotted to three groups as described below. The wound areas were photographed by digital planimetry¹⁰ and was recorded as initial wound area. The first group had only open wound (OW) which served as the control without any assistance for healing. The second group received CECM-gel formulation (CG) for assisted wound healing. The third group was treated with *Gencoll* (GC), a commercially available gel formulation (Cologenes Healthcare Pvt Ltd, Salem, India) containing gentamycin, and used for assisted skin wound healing applications. The rats were observed and routine veterinary care was given daily. Rats were observed every day for any signs of infection or illness and the wounds were gently cleaned with hypertonic saline every day. The treatment with CG and GC was repeated for the first five days followed by once in every 5 days (10th day and the 15th day). Four rats were selected from each group and euthanized on three time points namely 10, 15 and 20 days, by carbon dioxide asphyxiation. Immediately after the death, digital planimetry was performed and recorded as final wound area. While recording wound area, presence of scab was considered as unhealed area.

Tissue samples of the wounds were collected in 10% neutral buffered formalin along with adjacent normal area for histomorphological evaluation.

Histomorphological evaluation

Skin tissues preserved in 10% neutral buffered formalin were processed through isopropanol and xylene and four-micron thin sections were routinely stained with haematoxylin and eosin (H&E) stains. All the histomorphological evaluations were performed by a veterinary pathologist and light microscopic images

were captured using an Olympus BX51 (Olympus Corporation, Japan) mounted with a DP71 camera. The nature of wound healing was evaluated by assessing the progression through re-epithelialisation, inflammation, angiogenesis, and collagen remodelling.

Morphometry

In order to quantitatively evaluate extend of re-epithelialisation, morphometry was performed on the H&E sections using *Image-Pro*® software. For this, images were captured under low power and calibrated. The length between healthy well differentiated epithelium on either end of the neo-epithelium of the healing wound was considered as the total length of the wound. The length of the unbridged neo-epithelium was measured as the unhealed remaining wound length. Percentage of re-epithelialisation was calculated by the formula,

Percentage of re-epithelilisation =

$$\frac{\text{length of re-epithelialised wound}}{\text{length of total wound}} \times 100$$

Statistical analysis

Statistical analysis was performed using *GraphPad Prism 6*®. Students t-test was performed on the planimetry data and the percentage of re-epithelialisation.

RESULTS

Gross pathology and planimetry

Grossly, all the wounds appeared to heal under a scab without any clinical complication. Gross images of the wounds on various time points and the planimetry data are presented in Fig 1 and 2 respectively. Wound healing was similar in all rats in the first 10 days. However, by day 15, wound closure in the CG group appeared better than OW and the GC groups as evidenced by planimetry data (Fig 2) and there was significant difference between CG and GC group. However, no significant difference was statistically apparent in the morphometry data collected on day 20 between the CG and the other groups. On day 20, all the rats in the CG group had 100% healing, while all rats of OW group and GC group had scabs.

Histomorphological Evaluation

Representative histomorphological appearances of the healing wounds are presented in Fig 3, and Fig 5 to Fig 9. Morphometry data on extend of re-epithelialisation is shown in Fig 4. The nature of the healing reaction was similar in all groups, with respect to re-epithelialisation, inflammation, angiogenesis, and collagen remodelling. Additionally, multifocal granulomas and foreign body giant cell reactions were present in the CG and GC groups (Fig 7 and Fig 9).

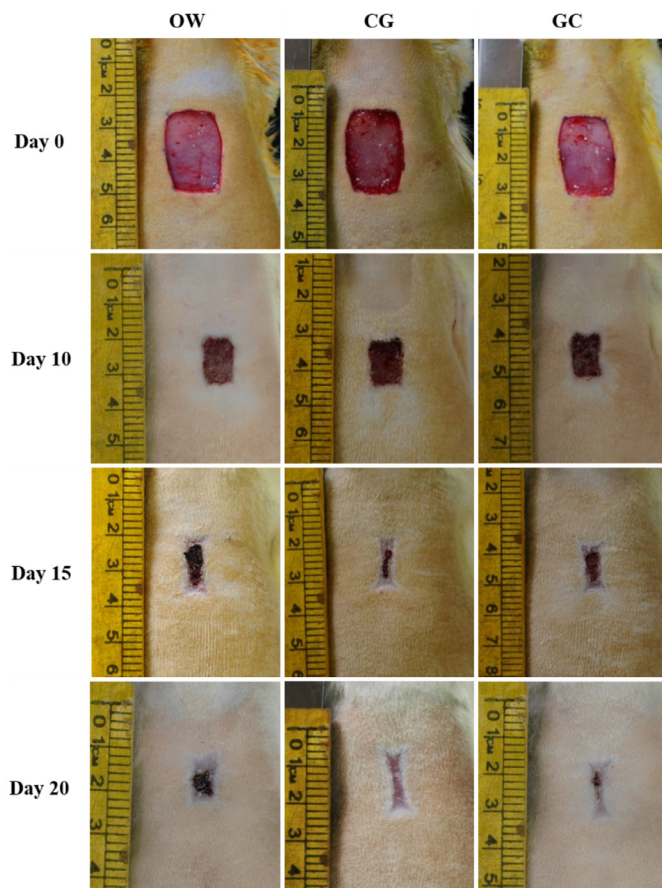


Fig 1. Gross photographs showing the morphology of wounds in rat untreated group (open wound, OW), wounds treated with the gel formulation containing porcine cholecyst extracellular matrix (CG) and *Gencoll* (GC, a commercially available gel formulation containing Gentamycin) on day 0, day 10, day 15, and day 20 after surgery. Note the complete closure of the wound and absence of scab on day 20 in the CG group.

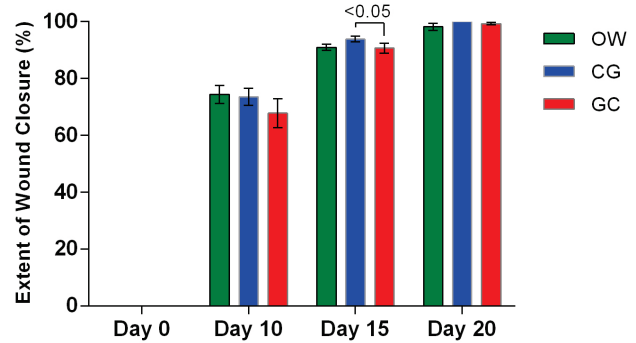


Fig 2. Bar diagram drawn from the planimetry values depicting the extend of wound closure in rats.

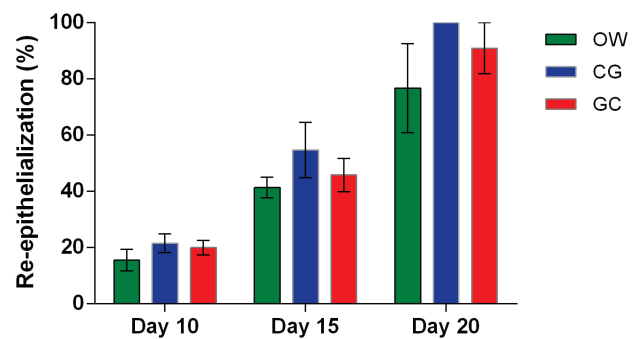
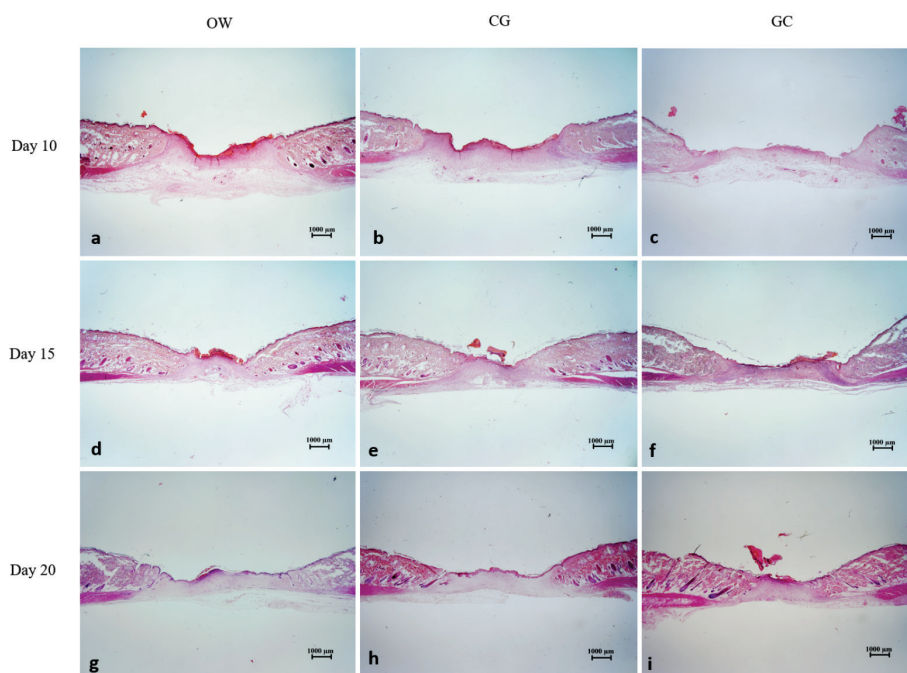


Fig 4. Bar diagram showing percentage of re-epithelialisation in rats with open wound (OW and those treated with cholecyst extracellular matrix gel (CG) and the *Gencoll* (GC). No significant difference between groups was seen, although the re-epithelialisation was complete in all animals of the CG group.



However, the nature of the healing reactions progressively varied between groups. On day 10, prominent neo-epithelium characterized by hypertrophied epidermal cells was evident that appeared to migrate from the healthy wound edges towards the centre of the wound bed (Fig 5a to c). These relatively undifferentiated cells having occasional mitotic figures (Fig 5h and 5i), were situated a few cells behind the leading edges in both

Fig 3. Representative light microscopic images of hematoxylin and eosin-stained sections showing the nature of re epithelialization at low magnification. Re-epithelialisation was complete in CG group by day 20.

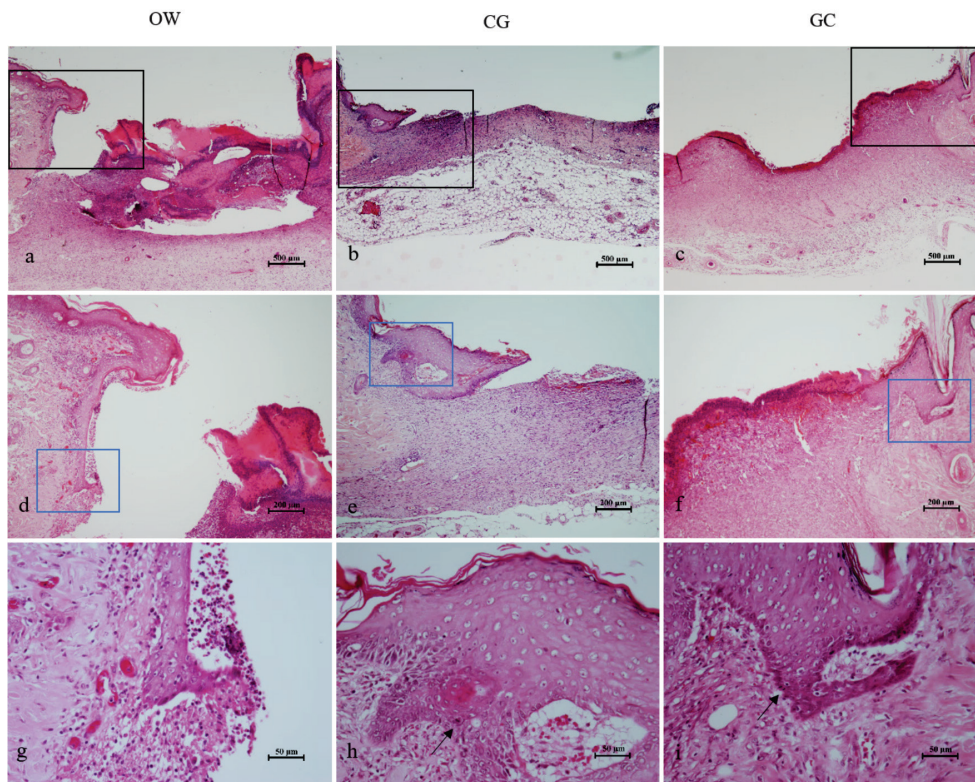
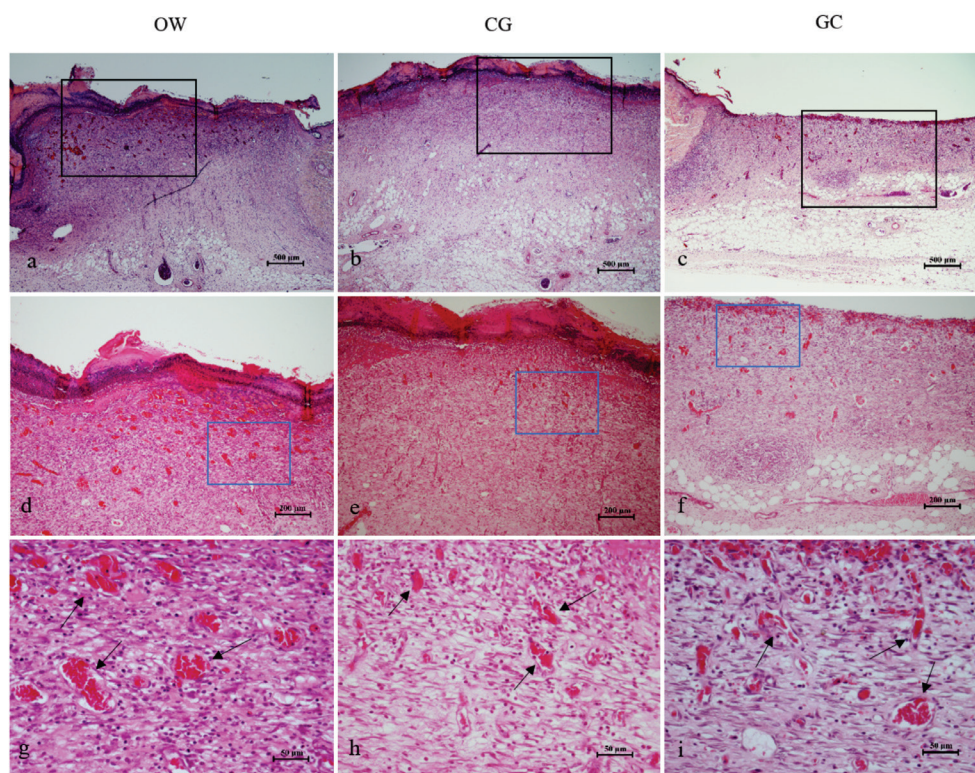


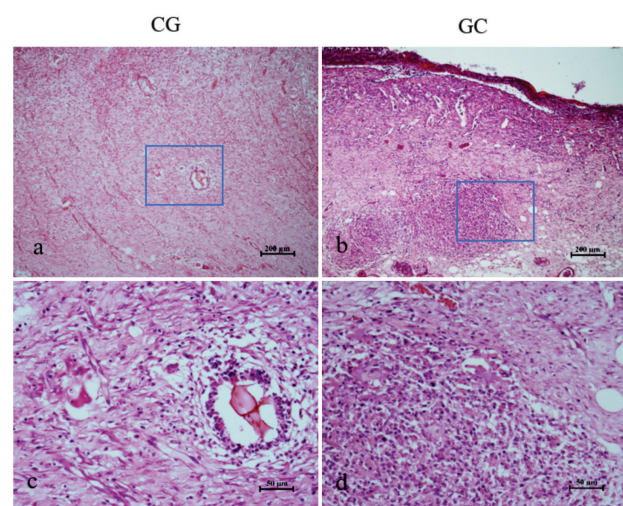
Fig.5. Light microscopic images of hematoxylin and eosin-stained sections showing extend of re-epithelialization on day 10. Occasional mitotic figures (arrow) were present, a few cells behind the tip of the leading edges in the CG (h) and the GC (i) groups, compared to the OW group (g). Mild to moderate inflammation, mostly mononuclear cells was seen in all sections. The marked rectangle represents the area enlarged in the corresponding photographs of the immediate lower row.



CG and GC groups. Scabs were present in many of the wounds. In the dermis, inflammatory cells (mostly mononuclear), sprouting thin-walled capillaries, and plump fibroblasts were present; as expected in a mild to moderate granulation tissue (Fig6). In addition, granulomas were present in CG and GC treated rats (Fig 7).

Between day 15 and day 20 day, the extent of re-epithelialisation was marked by a reduction of wound length and the epidermal height reduced, as epidermal cells matured and differentiated (Fig 9). Re-epithelialization was complete in all rats of the CG group by day 20, as evident from the quantitative morphometry data. Besides, functional differentiation evaluated by the degree of hyalinisation, keratinisation, as well as rete peg formation were also prominent in the CG group (Fig 9h). In the dermis, by day 15 and 20, granulation tissue were markedly reduced, and thin-walled capillaries were replaced by mature muscular blood vessels. The presence of plump fibroblasts was still evident in CG group (Fig 9k) even at day 20, contrasting sharply with OW and GC groups featuring mature fibrocytes and compact collagen (Fig 9g and 9i).

Fig.6. Light microscopic images of hematoxylin and eosin-stained sections showing granulation tissue on day 10 characterized by the presence of capillaries filled with RBCs in the lumen (g, h and i; arrow), fibroblasts and mononuclear inflammatory cells.



Histomorphological features of different groups at various time points are provided in tabular form (Table 1).

Morphometry

The extent of re-epithelialisation was more in the CG group than OW and GC groups. This was perceptible at day 15 (Fig 4), where CG group had 54.76% re-epithelialisation when compared to GC (45.88%) and OW (41.39%) groups. By day 20, re-epithelialisation was complete in all four animals of the CG group, three animals of the GC and two animals of the OW group.

Fig 7. Photomicrographs of hematoxylin and eosin-stained sections showing granuloma in CG and GC group on day 10. Multifocal granulomas comprising of mononuclear inflammatory cells were also clearly visible in the boxed areas of first row which are magnified in the second row.

Table 1. Table showing summary of important histopathological lesions

Time points	Histomorphological parameters	Open Wound	Cholecystic Extracellular Matrix gel	GenColl®
Day 10	Nature of re-epithelialisation	Prominent neo-epithelium characterized by hypertrophied epidermal cells was evident that appeared to migrate from the healthy wound edges towards the centre of the wound bed.	Prominent neo-epithelium characterized by hypertrophied epidermal cells was evident that appeared to migrate from the healthy wound edges towards the centre of the wound bed.	Prominent neo-epithelium characterized by hypertrophied epidermal cells was evident that appeared to migrate from the healthy wound edges towards the centre of the wound bed.
	Mitotic figures	Mitotic figures were not evident.	Occasional mitotic figures were found, a few cells behind the leading edges of proliferating epithelium.	Occasional mitotic figures were found, a few cells behind the leading edges of proliferating epithelium.
	Inflammation and granulation tissue formation	Presence of granulation tissue and mild to moderate inflammation (mostly mononuclear cells) was also observed.	Presence of granulation tissue and mild to moderate inflammation (mostly mononuclear cells) was also observed.	Presence of granulation tissue and mild to moderate inflammation (mostly mononuclear cells) was also observed.
	Presence of granulomas	Absent	Present	Present
Day 15	Granulation tissue	Persistence of unorganised granulation tissue.	Granulation tissue were markedly reduced, and thin-walled capillaries were replaced by mature muscular blood vessels.	Persistence of unorganised granulation tissue.
Day 20	Completion of re-epithelialisation	Incomplete re-epithelialisation	Complete re-epithelialisation	Incomplete re-epithelialisation
	Extend of functional differentiation of epithelial layers	Incomplete differentiation	Functional differentiation of epithelium was marked by degree of hyalinisation, keratinisation, as well as rete peg formation.	Incomplete differentiation
	Nature of fibroblast/fibrocyte	Mature fibrocytes were abundant in dermis	Presence of plump fibroblasts was still evident in the dermis, rather than fibrocytes	Mature fibrocytes were abundant in dermis
	Collagen deposition	Compact mature collagen were present in the dermis.	less compact collagen deposition in dermis	Compact mature collagen were present in the dermis.
	Presence of foreign body giant cells	Not evident	Occasionally, foreign body giant cells were present	Occasionally, foreign body giant cells were present

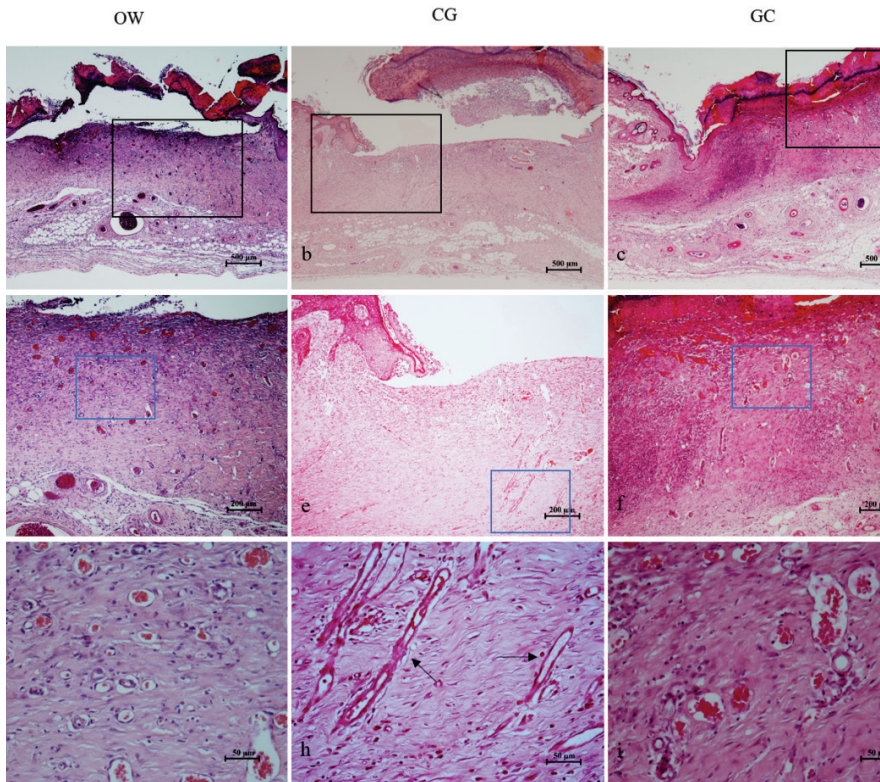


Fig. 8. Light microscopic images of hematoxylin and eosin-stained sections showing angiogenesis and collagen remodeling on day 15. There was regression of vascular channels and accumulation of fibroblasts in dermis of CG group (b,e and h). Note that in the CG group, there was matured blood vessels (arrow) seen perpendicular to the wound surface, probably primary branches of the main blood vessels beneath the panniculus carnosus muscle (e and h). However, in the OW and GC groups the neo-blood vessels are terminal branches in tufts as commonly seen in unorganized granulation tissue indicating a delayed healing reaction.

DISCUSSION

Healing of skin wounds happen through regeneration and/or repair depending on the extent/depth of the injury. Based on the depth of wounds, excision skin wounds can be classified into superficial, partial-thickness, or full-thickness¹². Skin wounds involving only epithelium heal by regeneration and restoration of the pristine architecture; wounds involving dermis heal by a repair process involving variable degrees of inflammation, angiogenesis, collagen deposition and remodelling¹¹. In this study, healing response of a full-thickness skin excision wound in a rat model assisted by the application

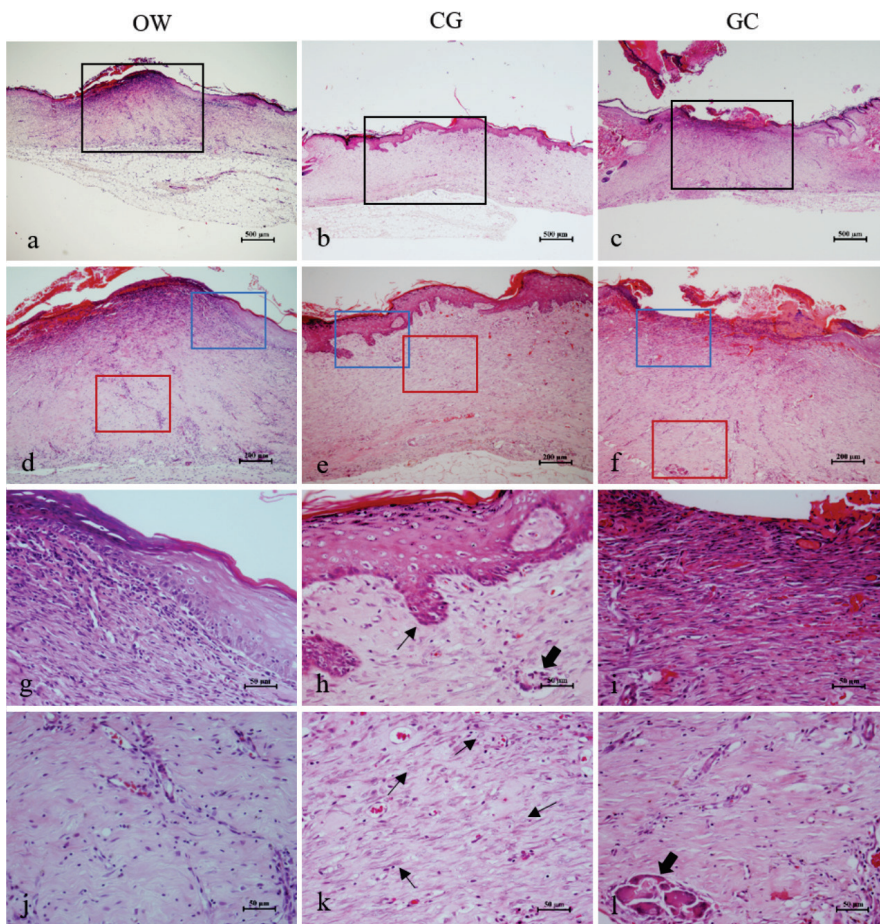


Fig. 9. Light microscopic images of hematoxylin and eosin-stained sections showing extent of wound closure and maturation of collagen by day 20. Complete re-epithelialisation was observed in the CG group (Fig b, e, h) with differentiation of epithelial layers visualised as hyalinisation and keratinisation of epidermis and restoration of rete pegs (Fig h, thin arrow). Relatively, number of plump fibroblasts (Fig k) was abundant in CG group compared to the OW and the GC groups. In the OW and the GC groups, re-epithelialisation was not complete. Moreover, the inflammation, granulation tissue, angiogenesis and excess fibrosis (g and i) were persistent in the OW and GC groups. Occasionally, foreign body giant cells were present in both the CG and the GC groups (Fig h and i; thick arrow). Blue and red boxed areas of the second row are magnified in the third and fourth rows respectively.

of a gel formulation of cholecyst extracellular matrix. The wound involved removal of the epidermis (epithelium), entire dermis down to subcutaneous tissue and the panniculus carnosus muscle. The removal of the panniculus carnosus muscle avoided healing by contraction¹². The observations on the OW group facilitated the healing response of unassisted healing, whereas the GC group gave an option for comparison with nature of assisted healing with a predicate device.

Grossly, the nature of wound closure was similar in all groups up to 10 days. Faster wound closure in CG group was visibly observed by 15 days. By day 20, wound closure appeared to have completed amongst four rats in CG, while wounds still had scabs in GC and OW groups. However, by day 20 re-epithelialisation was complete in all four rats in CG, three and two rats GC and OW groups respectively. This could probably be due to the presence of scab observed during planimetry and absolute re-epithelialisation observed at histomorphometry. The morphometry data corroborated the histomorphological observation. Lack of significant difference in morphometric data of re-epithelialisation on day 20 could be because, the wounds of the CG might have healed much before day 20 and the wound in the OW and GC groups were only nearing completion of the healing process. This notion is supported by previous observations by similar experiments using different preparations of the CECM formulation^{10,13}. These reports suggest complete re-epithelialisation of rat excision wound by day 16 and day 18 respectively compared to OW which healed only by 21 or 24 days. Presumably, more interim assessments of the healing process between 16 to 19 days would have been more precise and cogent. However, extend of re-epithelialisation gives a strong indication of the better healing potential of the CG, even in comparison to the established commercial product GC.

Histomorphological evaluation showed infiltrating inflammatory cells in all samples collected on day 10. Although there was a reduction in acute inflammation, infiltration of mononuclear cells (granuloma) and foreign body giant cell reaction persisted even on day 15 and day 20. The sprouting blood vessels were abundant in day 10 but regressed by day 15 especially in the CG group. This could probably be an indication of the faster healing potential of the CG compared to GC and unassisted healing in OW groups.

In this study, granulomas with foreign body giant cells, considered as histological markers of chronic inflammation in response to foreign materials¹⁴, were observed in the wounds treated with CG and GC formulations. Considering that 20 days of duration is too short a period for complete degradation of CECM formulations⁴ the presence of foreign body reaction was not surprising. Further, gel components which did not

degrade might have also stimulated the granulomas and the foreign body giant cell reactions. Remarkably, the foreign body giant cell reaction were not present in the OW group.

Collagen remodelling also had some qualitative difference between groups. Although plump fibroblasts and mature fibrocytes were present throughout the study points in all groups. It is known that the CECM can stimulate fibroblasts that synthesise Type III degradable collagen and less of Type I mature collagen⁵, that contribute scar formation. In the present study the CG group had a lot of plump fibroblast still actively secreting collagen even at day 20 and the formation of thick bundles of collagen was relatively less. However, a detailed study for differentiating Type I collagen and Type III collagen was not attempted in this experiment. A quantitative evaluation for differentiating the proportion of the Type I collagen and the Type III collagen might have given a better indication of the scarring potential of CG.

The persistence of inflammation and granulation tissue at variable degrees also indicated the differential healing potential of the gel-assisted healing. A detailed histomorphological and morphometric evaluations deploying immunohistochemistry and molecular pathology tools are required for deciphering the nature of healing with respect to inflammation, angiogenesis and collagen remodeling¹⁵. Nevertheless, the overall preliminary results suggested that assisted healing with either of the gels healed the wounds faster compared to OW. The CG-treated rats probably had a better healing response than those treated with the predicate device, as evidenced by the extent of gross observations on wound closure and morphometry data on re-epithelialisation. Therefore, it is plausible to think that the CG formulation has better wound healing potential than GC, but further corroborative evidence is required to establish the notion.

In conclusion, CG promoted better healing than the GC or the OW groups. However, the present CG formulation may need further modifications before advocating its use as an effective wound healing gel for faster healing with minimal scarring.

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