Abstract:
As calcium was shown to modulate cellular proliferation, maturation of keratinocytes and fibroblasts, we conducted study to observe effect of verapamil on excision wound model. Method Total 12 Albino Wistar male rats were divided in two groups and each group received either verapamil or normal saline after excisional wound. Primary outcome was percentage closure of the wound at 12th post-operative day. Results Verapamil showed significant difference in percentage reduction of wound area comparing with normal saline at day 3, 6, 9 and 12 (p 0.001 .05). The mean percentage inhibition for saline group was 68.25 5.58 and 86.64 1.63 (p 0.019 .05) for verapamil group. There was no statistically significant difference between the verapamil and saline group heart rate between pre-drug and post-drug heart rate. Conclusion Our data demonstrates that verapamil dressing has overall faster healing rate than standard saline dressing. Topical verapamil may be a suitable alternative to saline in the treatment of wound area in the developing country like India.

Keyword : Wound Healing, Verapamil, Calcium channel Blocker, Rats

INTRODUCTION:
The normal response of an organism to injury or wound is either regeneration (the complete restoration of the damaged part) or repair (the reconstruction of the injured region). When skin is injured or wounded the dermis responds primarily to repair while the epidermis responds to regeneration; the collective response of the skin to injury is termed as wound healing. Wound healing is a highly dynamic process and involves complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. (1) The immediate goal in repair is to achieve tissue integrity and homeostasis. To achieve this goal, the healing process involves three phases that overlap in time and space: inflammation, tissue formation, and tissue remodeling. (2) During the inflammatory phase, platelet aggregation is followed by infiltration of leukocytes into the wound site. In tissue formation, epithelialization and newly formed granulation tissue, consisting of endothelial cells,
macrophages and fibroblasts, begin to cover and fill the wound area to restore tissue integrity. The balance between tissue degradation and biosynthesis permits remodeling of the provisional tissue and also determines the net amount of scar tissue produced. Remodeling is characterized by cellular apoptosis and characterized by maintenance of balance between scar remodelling and scar degradation. The process is accompanied by extracellular matrix reorganization and reduction. Matrix Metallo-proteases synthesized during the proliferation stage continue to break down the extracellular matrix at a rate largely determined by physical and biochemical factors in the matrix. Synthesis, remodeling, and deposition of structural extracellular matrix molecules, are indispensable for initiating repair and progression into the healing state. Cellular responses to injury involve direct cell–cell and cell–matrix interactions, as well as the indirect crosstalk between different cell populations by soluble mediators. Indeed, complex interactions between the epidermal and dermal compartment are essential. The use of an in vivo model of wound healing is inevitable in trying to obtain information on the multifactorial nature of the wound-healing process, which may be influenced by externally introduced factors or by the presence of underlying pathology.

**Figure 1. Stages and timing period of normal cellular components of wound healing.**

In the wound healing, collagen deposition is central to wound contraction and tensile strength formation. This deposition appears to be regulated by a fine balance between synthetic and degradative pathways. Accumulating evidences from biomedical research implicate calcium ion as a key intracellular signal that modulates the expression of cellular function and gene expression in a variety of systems including the developing ones. Cell culture studies on keratinocytes and fibroblasts demonstrated the capacity of local calcium to modulate cellular proliferation, modification, maturation and the creation of epidermal lipid function through signal transduction and gene expression. Such functional capabilities implicate calcium ion in a central role in epidermal regeneration and reconstruction in wound healing. It is also possible that specific cells involved in wound healing respond differently to elevated levels of calcium. Calcium is known to affect cellular mitosis, neutrophil exocytosis and superoxide production, growth factor regulation, and it was shown that this anion plays a key role in regulating wound repair. In the wound site, calcium concentration consistently changes with the biological events of the healing process. Keratinocytes, the major epithelial cellular component, are metabolically active and involved in epithelialization and scar formation. The functions of these keratinocytes are
known to be sensitive to calcium alterations.(13,14) Experimental studies have shown that calcium channel blockers, verapamil and nifedipine to abolish terminal differentiation of cultured keratinocytes.(6,15) On the other hand, calcium concentration in the extracellular media was found to be inversely proportional to keratinocyte motility and adhesion to matrix component including collagen I and II. (7,16) Furthermore, excessive calcium concentration in the wound environment induced reduction in proliferation and chemotactic responses and is lead to delay in healing.(5) The growth factors have also been shown to act through calcium sensitive intracellular pathways on both the epidermis and dermis.(9)

**CALCIUM CHANNEL BLOCKERS IN WOUND HEALING:**

Calcium channel blockers (CCBs) have been used extensively in various cardiovascular conditions and they may have a role in non-cardiac conditions. Apart from vasodilatory effect of calcium channel blockers, there are evidences of cellular calcium metabolism regulating extra cellular matrix and collagen production.(16) Calcium channel blockers have been shown to inhibit synthesis/secretion of extracellular matrix molecules including collagen, glycosaminoglycans and fibronectin, lead to depolymerization of actin filaments, alteration of their cell shape and reduction of the fibrous tissue production, increase collagenase and transforming growth factor- activity.(17) Calcium channel blockers may suppress the neutrophil adhesion and superoxide anion (O2-) production which can hasten the wound healing process in synthesis phase.(11)

Hence the present study was taken up to assess the effect of calcium channel blockers, mainly verapamil (phenylalkamine class), on normal wound healing, using excision wound model in rats. This study was been conducted to assess the artificially created wound reduction healing comparing saline with verapamil.

Figure 2. This diagram summarizes our present concepts of the factors responsible for wound healing. The keratinocytes differentiate in the epidermis in response to elevated calcium concentrations. The fibroblasts produce collagen whereas collagenase is produced by both macrophages and fibroblasts. Tumor necrosis factor (TNF) and calcium modulate collagenase production. This collagenase breaks down the collagen into smaller fragments. The growth factors, depicted to the right of the diagram, act through calcium-sensitive intracellular pathways on both the epidermis and the dermis. Epidermal growth factor (EGF) and transforming growth factor (TGF) enhance epithelialization, whereas TGF- and platelet-derived growth factor (PDGF) appear to act on the dermis. (Source: Sank A, et al. Surgery. 1989 Dec;106(6):1141–7)
MATERIALS AND METHODS
The study was carried out with prior approval Institutional Review Board, CMC, Vellore and the Institutional Animal Ethics Committee, CMC, Vellore and all animal handling was done according to CPCEA guidelines. Healthy inbred Albino Wistar male rats weighing between 200 and 220 g were procured from the College Animal House, CMC, Vellore. They were individually housed and maintained on normal food and water ad libitum. Animals were periodically weighed before and after experiments. All the animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study. Rats are divided in two groups with 6 rats in each group. After giving ketamine (100 mg/kg i.p.), full skin thickness wound of around 400 mm^2 area was created on the dorsum of the animal under sterile conditions by skin excision with scalpel. After achieving hemostasis, the wounds were covered with an occlusive dressing. Group 1 received 0.5 ml of normal saline (0.9% NaCl) as topical treatment daily and group 2 received 0.5 ml of verapamil hydrochloride (2.5 mg/ml) commenced on the day of wounding and continued for 12 post-operative day. Wound boundaries for each wound were traced by a fine-tipped permanent marker onto a transparency sheet. This procedure was performed daily starting from day 0, the day of wounding and continued for 12th days post wounding on every third day wound tracing been taken in the transparent sheet. After the procedure, the animals were kept in separate cage in the post-operative period. Figure 3. Wound Tracing of verapamil and normal saline groups

STATISTICAL ANALYSIS:
The sample size for the study was calculated to be 6 in each group. Every three days, wounds were traced on to transparencies. These results were digitally traced and saved on to a computer using Pixel software. The percentage inhibition of wound area was calculated by following formula

\[ \text{Percentage Inhibition of Wound Area} = \left( \frac{\text{Wound Area Day}_0 - \text{Wound Area Day}_{12}}{\text{Wound Area Day}_0} \right) \times 100 \]

This gives the percentage of area of wound which has taken up and healed, starting day 0 till final assessment day 12. For each parameter, mean values ± SEM were calculated. Student’s t-test is used to compare both group in terms of the reduction of wound area from the base line to 3, 6, 9 and 12th post of day. A p-value of < 0.05 was considered statistically significant. All analyses were done using SPSS11.0.

RESULT
Figure 4 and 5 shows the percentage area at day 3, 6, 9 and 12 in relation to the original wound area at day 0 for individual animal for both saline and verapamil group. **Figure 4: The bar chart showing the Percentage of wound area for normal saline group from day 0 to 12th post of day for each animal.**

**Figure 5: The bar chart showing the Percentage of wound area for verapamil group from day 0 to 12th post-operative day for each animal.**

**Figure 6: The bar chart for comparison of mean percentage wound area at day 3, 6, 9 and 12 between normal saline and verapamil group.** **indicates p-value <0.05)**

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>N</th>
<th>Mean ± SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Saline</td>
<td>6</td>
<td>74.6 ± 3.69</td>
<td>0.001</td>
</tr>
<tr>
<td>3</td>
<td>Verapamil</td>
<td>6</td>
<td>56.1 ± 2.63</td>
<td>0.001</td>
</tr>
<tr>
<td>6</td>
<td>Saline</td>
<td>6</td>
<td>56.1 ± 5.96</td>
<td>0.001</td>
</tr>
<tr>
<td>6</td>
<td>Verapamil</td>
<td>6</td>
<td>36.6 ± 1.96</td>
<td>0.001</td>
</tr>
<tr>
<td>9</td>
<td>Saline</td>
<td>6</td>
<td>43.7 ± 3.61</td>
<td>0.001</td>
</tr>
<tr>
<td>9</td>
<td>Verapamil</td>
<td>6</td>
<td>26.6 ± 1.82</td>
<td>0.001</td>
</tr>
<tr>
<td>12</td>
<td>Saline</td>
<td>6</td>
<td>31.8 ± 5.54</td>
<td>0.001</td>
</tr>
<tr>
<td>12</td>
<td>Verapamil</td>
<td>6</td>
<td>13.8 ± 1.63</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Table 1: Mean percentage wound area at day 3, 6, 9 and 12 between normal saline and verapamil group.** Figure 6 & Table 1 shows the wound area in percentage of initial wound comparing 3rd day of saline with 3rd day verapamil, 6th day saline compared with 6th of verapamil, 9th day of saline compared with 9th day of verapamil and 12th day of saline compared with 12th day of verapamil. The above analysis show that verapamil is highly significant in percentage reduction of wound area comparing with normal saline at day 3, 6, 9 and 12 (p= 0.001; <.05). Lower the absolute value of percentage wound area to that of initial wound area, better is the wound healing.

**Figure 7: Percentage inhibition of wound area comparing verapamil with normal saline at day 12**

GROUP N MEAN ± SEM
Saline 6 68.2 ± 5.58 Verapamil 6 86.63 ± 1.63

**Table 2: shows Percentage inhibition in wound area comparing verapamil with normal saline**

Figure 6 & Table 2 show in each and every rat, there is gradual decline in the wound area starting from day 0- till the final assessment day 12, in both saline and verapamil groups. Higher the percentage values, better is the wound healing. The mean percentage inhibition for saline group was 68.25 ±5.58 and that for the verapamil group was 86.64 ± 1.63 which is statistically significant (p= 0.019; <.05).
This shows that verapamil dressing in the wound area has overall better and faster healing rate in comparison to standard normal saline dressing in terms of percentage inhibition in wound area.

No changes in heart rate were noticed after verapamil application to the wound which suggest minimal/no systemic absorption.

**DISCUSSION:**
Present results establish wound healing promoting activity of verapamil comparing to that of normal saline. Verapamil is highly significant in percentage reduction of wound area comparing with normal saline at day 3, 6, 9 and 12 (p= 0.001; <.05) (Table 1). The mean percentage inhibition for saline group was 68.25 ± 5.58 and that for the verapamil group was 86.64 ± 1.63 which is statistically significant (p= 0.019; <.05) (Figure 7, Table 2). It showed better and faster healing of wound with verapamil. Wound healing generally requires support at three levels. First is by improving general resistance and support mechanisms. Second is by stimulating the repair and regenerative mechanisms to prolong cell life, cell migration and cell binding and improve tensile strength or elasticity of the skin. Third, therapeutic and nutritional activities including anti-inflammatory, anti-septic and antimicrobial, protein and collagen synthesis and increased stability of biomembranes. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers. Free radicals and other reactive oxygen species (ROS) are considered to play an important role in wound healing. Verapamil has been shown to inhibit superoxide production by neutrophil and their adhesion which can help to hasten the anti-inflammatory stage. Some CAs (verapamil, diltiazem, and nicardipine) may possess analgesic properties that are useful in the treatment of post-herpetic neuralgia when administered alone or in combination with lidocaine by iontophoresis. Intralesional verapamil injection for the treatment of keloid based on experimental findings show that calcium antagonists may significantly affect fibroblast function. Calcium channel blockers inhibit collagen and its mRNA production, inhibit collagen gene expression, increase matrix metalloprotease activity, inhibit transcription of tissue inhibitor of matrix metalloprotease and thus affect cell proliferation, extracellular matrix protein synthesis and secretion, as well as collagen degradation. Topical application of verapamil to chronic wounds in experimental animals resulted in improved wound contraction and healing. Our data demonstrate that the topical verapamil may be capable of promoting wound-healing activity. It also supports the role of calcium in regulating wound healing. Calcium channel blockers increased enhanced wound contraction rate. Calcium channel blockers also are known to cause vasodilatation, which increases blood supply to wounded region and hence can promote wound healing. Present data suggest that verapamil can be used to hasten the recovery of wound in patient. Economically it is also cheaper. It can decrease wound associated morbidity significantly. This will make verapamil an attractive candidate to hasten the wound healing process. Further studies using different animal models, different topical verapamil concentration, and durations of treatment are necessary to elucidate any possible effect and any other molecular mechanism are involved in wound healing.
SUMMARY AND CONCLUSION

It was found in the present study that verapamil, significantly improved all clinical parameters of the wound healing that were investigated. The improvement was observed after 3 day from the baseline till 12th post operative day (p value < 0.05). Verapamil dressing in the wound area has achieved overall better and faster healing rate in compared to standard normal saline dressing in the wound area. Our data demonstrate that the topical verapamil may be capable of promoting wound-healing activity. Hence, topical verapamil may be a suitable alternative to saline in the treatment of wound area in the developing country like India. However, it needs further evaluation in clinical settings before its consideration for the treatment of wound healing.

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