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The Effect of Topical Corticosteroid Time of Application on Fibroblast and Type III Collagen Expression in *Oryctolagus cuniculus* with Deep Dermal Burn Wound (As an Indicator for the Best Time to Start Topical Corticosteroid Application in Preventing Hypertrophic Scar)

Loelita Lumintang[1]*, I Made Suka Adnyana[1], Agus Roy Hamid[1], Hendra Sanjaya[1], Nyoman Golden[2], Putu Astawa[3], Made Darmajaya[4], I Wayan Juli Sumadi[5]

[1]Department of Surgery, Division of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia; [2]Department of Surgery, Neurosurgery Division, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia; [3]Orthopedic and Traumatology Department, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia; [4]Department of Surgery, Pediatric Surgery Division, Faculty of Medicine, Universitas Udayana, Sanglah General Hospital, Bali, Indonesia; [5]Department of Anatomical Pathology, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia

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***Correspondence:** Loelita Lumintang, Division of Plastic Reconstructive and Aesthetic Surgery, Department of Surgery, Faculty of Medicine Udayana University, Sanglah General Hospital Bali. E-mail: drloelitamlumintang@gmail.com
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Abstract

BACKGROUND: Hypertrophic scar is an abnormal scar that causes physical deteriorations, psychological problems, and esthetic issues. An excessive number of fibroblasts and collagen III expressions are histopathology indicators for the hypertrophic scar. The role of topical corticosteroids in suppressing inflammation and hypergranulation had widely demonstrated in the previous studies. However, there is no study related to the application of topical corticosteroids as prevention of hypertrophic scars from burn wound found.

AIM: Hence, this study aimed to examine the evidence of the effects of corticosteroid topical in decreasing the number of fibroblasts and type III collagen expression and the best time to start its application in preventing hypertrophic scars.

METHODS: This randomized experimental post-test only study involved 54 deep dermal burn wounds on the ventral ear of female *Oryctolagus cuniculus* that distributed into three groups based on the healing phases. Each group consisted of treatments and controls. Corticosteroid topical application on the first treatment group (inflammatory phase group), the second group (proliferation phase group), and the third group (remodeling phase group) was started on day 3, on day 10, and day 21, respectively. Specimens taken on day 35. Hematoxylin-eosin and immunohistochemically staining performed to measure the number of fibroblasts and type III collagen and to observe the epithelialization and inflammation process.

RESULTS: The number of fibroblasts significantly decreased in the second treatment group (p =0.001) and followed by the first group (p = 0.016), but no significant decrease found in the third group (p = 0.430). The type III collagen decreased significantly in the second treatment group (p = 0.000) and followed by the third group (p = 0.019), but no significant decrease found in the first group. There was no statistically different number of fibroblast and type III collagen discovered between the controls. Complete epithelialization found in all groups. Furthermore, no ongoing inflammation found in all groups.

CONCLUSIONS: Topical corticosteroids on deep dermal burn wound revealed to be effective in reducing the number of fibroblasts and type III collagen with no healing disruption. The proliferation phase found to be the best time to start the application of topical corticosteroids.

Introduction

Hypertrophic scar is an unsolved abnormal wound that is not only esthetically not pleasing but can also disrupt skin function (pruritus, contracture, deformity, and/or pain). This type of scar often triggers psychological problems, especially if it appears in a very conspicuous area. The incidence of hypertrophic scars due to deep dermal burns wound is up to 70–90%. Pathologically, it characterized by the accumulation of fibroblasts and type III collagens. Topical corticosteroids application is effective in suppressing inflammation and accelerating the stimulation of keratinocyte migration, suppressing proliferation and hyperproliferation, and improving the remodeling process (differentiation, degradation, and maturation) with its antimitotic effect [1], [2], [3], [4], [5], [6]. This topical application shows better compliance and provides better additional effects. It was cost effective, skin moistened, relatively easy to apply, and had no pain sensation. Japan Burn Wound Guideline Treatment had included corticosteroid

topical application as a treatment for burn injury and it showed good outcomes [7], [8], [9].

We examined the effect of corticosteroid topical application on the fibroblast and type III collagen expression in the deep dermal burn in this study. We also compared the outcome based on the time of use to find the best timing of its application.

Materials

Model

Fifty-four samples of the female New Zealand Rabbits weighing approximately 2500–3000 g had raised and adapted in wire-bottomed cages at 22°C with a 12 h light/12 h dark cycle for 3 weeks before the experiment. The casein diet and water were available *ad libitum*. The study's ethical clearance had granted by the Ethics Committee of our institution and Udayana University Institutional of Animal Care and Use Committee.

- Medications
- Hydrocortisone 1 %.
- Instrumentation for burn

A brass rod with 10 mm in diameter, 8 cm long, and 30 g weight designed as the burn instrument. Additional loads added to produce 90 g of the device's weight. Hence, it provided equal pressure and heat distribution to the skin.

Methods

After being well-fed and adapted, three 10 mm deep dermal burn wounds were created for each of the rabbit's ears by heating the brass instrument to 90°C in a Scientific Dry Bath Incubator. The instrument placed directly on the flattened ear for 20 s. Three days after created the burn wound (day 3), consistent with current clinical practice, the burn wounds were debrided surgically with a 10 mm punch biopsy with perichondrium preservation. The right ear applied as the treatment side, while the contralateral ear applied as the control side. Then, the wounds were divided into three groups based on treatment initiation. The treatment groups on the first, second, and third groups treated with hydrocortisone 1% (2 mm thickness) on day 3, day 10, and day 21. The wound in the control groups only treated by transparent dressings. On day 35, the animals were sacrificed, and the wounds were harvested in 10% formalin and cut into the paraffin-embedded section. The staining (hematoxylin-eosin and immunohistochemistry) then observed to compare each treatment and examine the number of fibroblast and type III collagen. Epithelialization and inflammation also descriptively observed. Data were analyzed by the windows statistical software SPSS 24.0. The fibroblast data difference between groups analyzed using ANOVA statistical test. Kruskal–Wallis and post-hoc Mann–Whitney statistical test was applied to analyze the difference of type III collagen data between the groups.

Results

Effect of topical corticosteroid on number of fibroblast in rabbit's deep dermal burn wound (mean±SD)

Table 1 shows that the second group had the highest difference of fibroblast cell number after day 35 (475.78 ± 305.2 in the treatment and 1087.78 ± 322.87 in the control group) with p: 0.001. It followed by the first group (740 ± 366.1 in treatment and 1348.89 ± 566.81 in the control group) with p: 0.016. In the third group, the mean of fibroblast number on the treatment and control group was 1103.89 ± 320.31 and 1348.89 ± 404.95, respectively, with p: 0.43.

Table 1: The effect of topical corticosteroid application on number of fibroblast in rabbit's deep dermal burn wound per five visual fields with 400× between treatments and controls in each group

Group	Intervention	Fibroblast	Mean Difference	p-value
		Mean ± SD		
1	Treatment	740.0 ± 366.1	−608.9	0.016*
	Control	1348.9 ± 566.8		
2	Treatment	475.8 ± 305.2	−612	0.001*
	Control	1087 ± 322.9		
3	Treatment	1103.9 ± 320.3	139.4	0.43
	Control	964.4 ± 404.9		

*p<0.05: Statistically significant. The highest difference in the number of fibroblasts in rabbit deep's dermal burn wound.

Table 2 reveals that the second group had the highest difference of fibroblast cells on the deep dermal burns of rabbit ears after day 35. The difference between the second group's treatment with the first group's treatment was not statistically significant, but a comparison done with the third group's treatment showed statistically significant results with p = 0.001.

Table 2: The highest difference in the number of fibroblasts in rabbit's deep dermal burn wound per five visual fields with 400× between treatments in each group

Treatment group	Treatment group	p-value	Mean difference
1	2	0.104	264.2
	3	0.209	(−)363.9
2	1	0.104	(−)264.2
	3	0.001*	(−)628.1
3	1	0.029	363.9
	2	0.001*	628.1

*p<0.05: Statistically significant. Effect of topical corticosteroid on number of type III collagen in rabbit's deep dermal burn wound (median±iq).

Table 3 shows that the number of type III collagen with parametric data on deep dermal burns of rabbit ears after day 35 had the highest number of median differences, 15(10) and 27(0) in control and treatment, respectively, with p: 0.000. It followed by the third group with 24(5) and 27(3) in the treatment

and control groups, respectively, with p: 0.019. While in the first group, the median of the treatment and control groups was 21(11) and 27(5), respectively, with p: 0.82.

Table 3: The effect of topical corticosteroid application on number of type III collagen in rabbit's deep dermal burn wound per five visual fields with 400× between treatments and controls in each group

Group	Intervention	Type III collagen	p-value
		Median and interquartile range	
1	Treatment	21 (11)	0.82
	Control	27 (5)	
2	Treatment	15 (10)	0.000*
	Control	27 (0)	
3	Treatment	24 (5)	0.019*
	Control	27 (3)	

*p<0.05: Statistically significant. Comparison of topical corticosteroid application on number of type III collagen in rabbit's deep dermal burn wound between treatments of each group.

Table 4 shows a statistically significant difference of the number of type III collagen comparison between the treatment groups in each group.

Table 4: Comparison of topical corticosteroid application on number of type III collagen in rabbit deep dermal burn wound per five visual fields with 400× between treatments in each group

Group's treatment	p-value
1	0.004*
2	
3	

Descriptive evaluation of epithelialization and existing PMN.

Figure 1 reveals a complete epithelialization that occurred in 52 samples without existing inflammatory cells. There were two samples in the control group which not completely epithelialized and had existing PMN infiltration (Figure 2.).

Discussion

This was an experimental study with a randomized posttest-only control group design that aimed to examine the effect of topical corticosteroid application (hydrocortisone 1%) on deep dermal burns in rabbits to reduce the number of fibroblasts and collagen type III as a marker of hypertrophic scars. This study

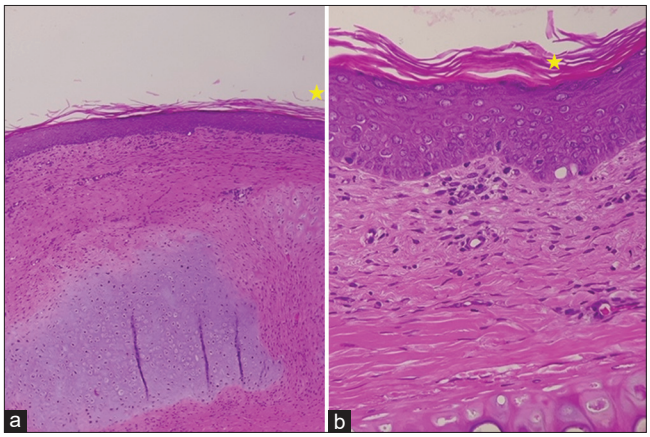

Figure 1: Complete epithelialization (a: 100×; b: 400×)

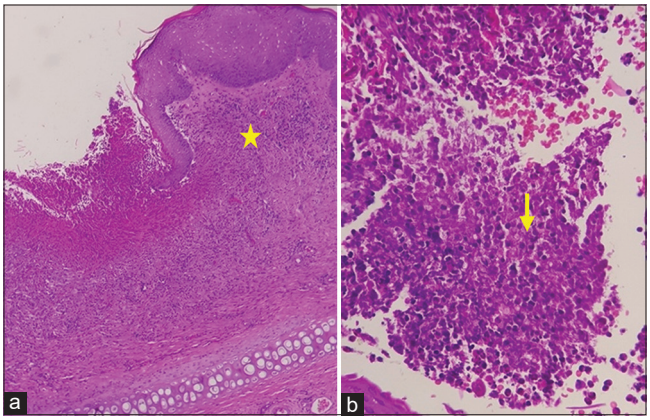

Figure 2: Incomplete epithelialization (★) with PMN infiltration (⬇) (a: 100×; b: 400×)

also revealed the effect differences on different times of application. Study population randomly classified into three study groups. Each group had three control wounds on the left ears and three treatment wounds on the right ears. Treatment wounds administered topical corticosteroid ointment (hydrocortisone 1%), while the control ears covered with a transparent dressing only. Corticosteroid application on the wounds conducted 3 times: On day 3 (first treatment group), day 10 (second treatment group), and day 21 (third treatment group). Then, the wound specimens taken on day 35.

The time of application selected according to the peak of each deep dermal wound healing process (inflammation, proliferation, and remodeling) without interfering the healing process itself. In the previous studies, animal trials have shown that corticosteroids administration at least 3 days after a wound happened did not inhibit the wound healing process [10], [11]. Friedrich, 2017, stated that 3 days (day 3) after a deep dermal burn wound formation, the primary necrotic phase had completed and there were significantly upregulated levels of tumor necrosis factor (TNF)-α at this point [12]. TNF-α gene expression is an inflammatory cytokine associated with burn progression and HMGB1 release reduction [11]. Therefore, on day 3, the inflammation process was about to be completed [12]. On day 28, the same study showed that abundance of new collagens deposited with fine mature collagen (collagen type I). The proliferation phase had been completed, and the remodeling process was also starting to happen at that time. Then, on day 35, hypertrophic scars with complete epithelialization started to be discovered.

The wound healing process in the human body is different from animal wound healing due to the differences in structure, physiology, and immunology of the skin [13]. Rodents, with loose skin and an underlying panniculus carnosus, heal predominantly by contraction, which makes hypertrophic scar challenging to study, and studies are often limited to a 10–14 days time point at which time there is no measurable scar, so it is often poor models for testing effects on scarring as an endpoint [14]. Pigs may serve as a better model

for the study of healing and scar formation after burn, but the significant limitation of the pig is the enormously thickened and stiffer skin with the contraction differs from human skin, because of that, the time-to-endpoint can be on the order of months [15]. Rabbits have similar main inflammation cytokine with humans and the closest similarity to human after pig. According to the previous study, hypertrophic scar happened due to deep dermal burns wound also successfully conducted on day 35 at ear of rabbits [12].

Hypertrophic scar is an abnormal scar that results from deep dermal burn wounds. The etiology of hypertrophic scar formation is not fully understood. The factors causing hypertrophic scars associated with burns include the degree and persistence of increased inflammation, burn progression, the depth of the wound, and total burn area. These things cause healing process disruption [16]. It characterized pathologically by the accumulation of both fibroblasts and type III collagen.

Corticosteroids are known as an effective therapy for various types of skin disorders. Several lines of evidence suggest that glucocorticoids alter collagen metabolism in the skin and decrease the proliferation of fibroblast-collagen, also increase the collagenase effect. Topical corticosteroid (hydrocortisone 1%) elected as the treatment utilized in this study because it was a non-surgical therapeutic modality currently available and has been frequently used for the management of hypertrophic scar caused by hypergranulation in almost all cases of wounds, including deep dermal burn wound, [7], [8], [9], [17]. The occurrence of hypergranulation is consistent with the incidence of abnormal scarring, including hypertrophy [18], [19], [20].

The mean value applied to measure the number of fibroblasts because the data were homogeneous and normally distributed. A significant number of fibroblast reductions considered as the effect of topical corticosteroid application. Application of topical corticosteroids in deep dermal burns that started on day 10 (second group) recognized as the most effective time in reducing the number of fibroblasts (612, p = 0.001). The second most effective time of application found in the first group (p = 0.016). However, there was no significant difference in fibroblast number in the first and second groups found. The reduction in the number of fibroblasts in this study is in line with the literature on hypertrophy treatment. The ability of corticosteroids to reduce excess fibrous tissue proved by Moio *et al.*, 2014 [21]. It also found to be able in suppressing tissue growth [18], [19], [20], [22].

Contrarily, on the third treatment group (day 21–day 35), the number of fibroblasts was higher than the control grout but was not statistically significant. The different absorption rates in different epidermal thicknesses explained this finding. In the remodeling phase, the raw surface area had been healed and filled with immature epithelialization. The absorption of topical corticosteroids would become lower in comparison with the application on the inflammatory and proliferative phases, which still have raw surface area. Therefore, injection suggested is the only way of administering intralesional corticosteroids [23], [24]. It can also demonstrate that in the remodeling phase, the fibroblasts no longer increase in number, but transform into myofibroblasts. Immature fibroblasts, especially dermal fibroblasts, have larger receptors that are more sensitive to therapeutic modulation. Those reasons explain the absence effect of topical corticosteroids on the remodeling phase [23], [24], [25], [26], [27].

Elevated fibroblast production raised collagen synthesis. This process can lead to collagen build-up if it does not follow by its degradation. Type III collagen also could not be converted into type I that causes hypertrophic scars in the remodeling phase [25], [26], [27].

The median value applied as the collagen type III measurements because the data were not normally distributed. Collagen type III in the controls from each group had similar values, about 27 with a slightly different interquartile range. The median value on the first group, the second group, and the third group was 5, 0, and 3, respectively. The median score of treatment on Group 1, Group 2, and Group 3 was 21 (11), 15 (10), and 24 (5), respectively. There was a significant difference between treatment and control of the second and third groups, where the second group showed the lowest type III collagen value. Significant differences also found in treatments of each group comparisons (p = 0.004).

This result can be explained by the proliferation of productor cells (fibroblasts) decreasing significantly at best in the proliferation phase so that their production (collagen type III) also significantly decreases. This result is in line with Uva *et al*. and Coondoo *et al*. that found the role of corticosteroids (anti-mitotic effect) in the dermis inhibits cell proliferation and collagen synthesis [28], [29]. The highest reduction of the amount of type III collagen occurred in the proliferation phase group, followed by the reduction in the remodeling phase group. The effect of topical corticosteroids not only able affects collagen synthesis but also the degradation of type III collagen. In the remodeling phase, there was no decrease in the number of fibroblasts, so the decrease in the amount of type III collagen was also not as high as the one that started in the proliferation phase where the number of fibroblasts, as a productor of type III collagen synthesis, also decreased. Hence, it is clear that corticosteroids play a role in increasing the degradation of type III collagen [25], [26], [27]. This finding is in line with other studies. It found that corticosteroids strongly interfere collagen synthesis and degradation, corticosteroids drastically against type III collagen by reducing tropocollagen levels from collagen type III [30]. Corticosteroids also have a significant impact on type III fibrils of collagen. In 1982, glucocorticoids reported able not only to decrease the

synthesis of collagen both type I and type III collagen, but it also found changed the ratio of procollagen type III to procollagen type I in rat skin fibroblasts [31]. This finding explains that the change of type III procollagen to type I procollagen is not due to different synthesis regulatory processes, This finding explained that the change of type III procollagen to type I procollagen happened due to the result of extracellular processes and selective extracellular degradation of it.

The administration of corticosteroid was not significantly affected the amount of type III collagen in the inflammatory phase. The application of corticosteroids in the inflammation phase suppressed the PMN immune cells that result in cell function deterioration, which results in a longer inflammatory phase. It proven by the higher number of fibroblasts in the inflammatory group treatment than in the proliferation group [33], [34], [35]. Therefore, the amount of collagen type III in this burn group was higher. But, the statistical analysis identified no significant difference in the number of collagen type III compared to the control group. This finding was in line with a previous study by Todoroki *et al*. that stated that the use of topical steroids should be limited to the 1st day after injury to 1st or 2nd degree burns because these drugs delay wound healing and suppress epithelialization [36]. In 2014, Taheri proposed topical corticosteroid application after epithelialization to limit persistent inflammation in the dermis, which would be responsible for excess fibroblasts and collagen, leading to hypertrophy [36], [37]. There have been three RCTs (including a double-blind trial) showing that topical corticosteroids have no anti-inflammatory effect on burns [38], [39], [40].

However, these study findings were contrary to results from several studies and expert opinions. Yoshino *et al*. stated that topical steroids in burns could apply with the recommended level: C1, as written in a guideline in 2016 for burn management [8]. Topical corticosteroid administration in burns also had widely implemented in Japan. These findings recorded, but it is still an expert opinion about the effectiveness of topical corticosteroids in burn wounds.

Corticosteroids are often associated with wound healing disorders and infections. In this study, almost all wounds in experiment animals had covered microscopically with complete epithelialization on day 35. We identified only one inflamed sample from control that had not fully epithelized. Furthermore, there was a sample that was completely epithelialized but still inflamed. These samples were the control group's sample. These findings are consistent with several studies that found acute use did not cause systemic effects (3–4 weeks) and had no significant influences on the incidence of dehiscence and wound healing [41], [42], [43]. Topical use of low potent could apply for chronic intermittent [44]. A systemic or local injection has no significant effect on wound healing disorders [45]. Side effects reported so far are side effects of systemic and prolonged administration of corticosteroids [45], [46]. Chowdri found that in intraoperative administration of corticosteroids, a complete symptom improvement was achieved in all patients within 5 weeks after surgery. The objective response in terms of no recurrence noted in 91.9% of patients with keloids and 95.24% of patients with hypertrophic scars with a mean follow-up of 30.5 months. Local or systemic complications also concluded as insignificant [47].

Based on the results, topical corticosteroid application starting from day 10 was the best timing selected in reducing the number of fibroblasts and type III collagen to prevent hypertrophic wounds on deep dermal burns.Our findings also had indicated that the topical administration of topical corticosteroids (hydrocortisone 1%) initiated on the proliferation phase provided better hypertrophic scars prevention on deep dermal burns, compared to its administration initiated in the inflammatory (day 3) and remodeling phase (day 21). Topical corticosteroids have shown the ability to assist wound closure and prevent hypertrophic scars in deep dermal burns by reducing the number of fibroblasts in line with the decrease of collagen type III synthesis and increasing the degradation of type III collagen. These findings supported by the study of Guo and DiPietro [48] and Hofman *et al*. [20] that found that the application of low-dose topical corticosteroids for the treatment of chronic wounds and hypergranulation wounds has been found to accelerate wound healing, accelerate epithelialization, reduce pain and wound exudate, and suppress hypergranulation tissue formation, especially if it prevents the occurrence of infection [20], [48]. The use of low potent corticosteroid (hydrocortisone 1%) too early in deep dermal burns potentially causes prolonged inflammation. It also found that deviant or excessive inflammation was associated with the incidence of hypertrophic scarring [49], [50], [51].

Conclusions

Application of topical corticosteroids in deep dermal burn wound effectively reduced the number of fibroblasts and type III collagens. Application in the proliferation phase showed the most effective decrease of the number of fibroblasts and type III collagens. Further studies necessary to be conducted to examine: (1) The mechanism of topical corticosteroids in limiting the inflammatory and abnormal fibrotic response without impeding the wound healing process (the corticosteroid potential, the dose, and duration), (2) the extent of deep dermal burns that can be treated without causing negative effects (local or systemic), (3) possibility to matrix modulation components of corticosteroids, (4) the role of corticosteroids in ECM (extracellular matrix)

apoptosis without causing scar atrophy, (5) detailed characterization of fibroblasts in scar tissue, and (6) combination of anti-inflammatory with antibiotics and antifungals. These findings could complement the unstudied variables, such as local and systemic infection markers, myofibroblasts, α-sm-actin expression, PCNA expression, hyaluronic acid, apoptosis, and p53 levels.

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