Effect of andrographolide on the pathological events during the surgical open wound healing process

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Abstract. Wound healing is an important physiological process and several natural and synthetic drugs are being tested to speed up the process to avoid infection and other undesirable pathological events. Although andrographolide has been reported to be an excellent bioactive compound that can influence multiple pathways in the cells, its wound healing property has not been widely appreciated. In this study, we wanted to exhibit the improvement in the pathological events concerning wound healing process by injecting andro in a surgical wound model in rats. The right paw of female SD rats was operated and after 5 days, pathological events in the operated right paw was observed by staining the sections using hematoxylin & eosin dyes to elucidate the dermal and epidermal changes, while Mason’s trichrome staining was performed on the sections to show the granulation layer formation and provide supporting evidence for angiogenesis and ECM deposition. Pathological score was given to the sections according to the extent of the changes observed and the results suggested that epidermal changes were scored almost similar in both saline and andro injected rats (2-3), while angiogenesis and inflammation were moderately improved in favor of wound healing in the andro injected rats (2.5 & 3.7 respectively) compared to saline-injected rats (3 & 4 respectively). The results clearly suggested that, though the improvement due to andro injection was moderate, these observations might create an awareness regarding the wound healing property of andro. Further studies are required to optimize the dosage of andro to influence the molecular pathways in order to show a better wound healing activity.

1. Introduction

Medicinal plant-based treatments of various diseases are being carried out for several decades and exploration of natural bioactive compounds from such medicinally valuable plant parts has gained the interests of biomedical researchers and medical practitioners [1, 2]. Andrographis paniculata is one of the popularly known medicinal herbs with numerous bioactive compounds that were reported for its ability to treat many infectious and metabolic diseases [3, 4]. Analgesic, anti-inflammatory, anti-apoptosis, free radical scavenging property, etc are some of the commonly reported biological activities of A. paniculata [5]. The plant is widely known as “King of bitter” and this is mainly because of its high terpenoid content [3]. Among several terpenoids, Andrographolide (Andro) was the major constituent with more than 1% of the whole plant material. Andro and structurally similar terpenoids were explored...
for many biological activities and were also chemically modified to enhance their aforementioned activities [6].

Though several researchers were still progressing towards identification of compound or group of compounds for accelerated wound healing process, still there is a lacuna in compounds in targeting several biochemical processes that can improve the physiological stages of wound healing like angiogenesis, reduced inflammation, ECM formation, etc [7, 8]. Andro and their analogs, though possess an appreciable wound healing property, were not explored enough, despite of their ability to inhibit inflammation [9, 10], aids in angiogenesis [11], and recruit mesenchymal stem cells [12, 13].

In the current study, we wanted to report the effect of andro on specific pathological events of the wound healing process of a surgical wound model in hind paw of rat. We studied the tissue architecture of the operated paw tissues with respect to specific changes at the epidermal and dermal region by staining with Hematoxylin and Eosin staining protocol for observing the crust formation, epithelialization, and recruitment of inflammatory cells. On the other hand, events like angiogenesis and granulation were illustrated through Mason’s trichrome staining.

2. Materials and methods

2.1. Animals
Sprague-Dawley (SD) rats (weight 250 g, about 8 weeks old) were used for this study. Animals that had undergone surgery were kept in individual cages in properly ventilated, humidity and temperature-controlled rooms with a 12h light/dark cycle. They received food pellets and water ad libitum. The Ethical Committee for Animal Research of National Chung Hsing University approved all experiments.

2.2. Surgery
A longitudinal incision of 1 cm length using surgical blade No. 11, was made on the medial plantar surface of the right hind paw. The incision began 0.5 cm distal from the end of the heel to the first set of footpads. The skin, fascia, and plantaris muscle were all elevated using forceps. The epidermis and dermis were separated by scissors in the area directly underneath the wound and then the skin was closed with 5-0 nylon sutures. After surgery, the animals were allowed to recover from the anesthesia in their cages.

2.3. Treatment groups
Experimental groups were segregated as follows: (i) Pain control (Saline) groups: the rats were operated on and treated with 150 µL saline locally in paw after surgery. (ii) Andro injected (injection) group: the rats were operated on and treated with Andro (Sigma-Aldrich - 364645-500MG; 98%) (150 µg), which was administered on the paw locally. Six rats were used for each treatment group and pathological tests were performed on the sections obtained after sacrificing the rats on 5th-day post-operation.

2.4. Collection of tissue samples
The rats were deeply anesthetized with sevoflurane and sacrificed by bloodletting. The paw skin was collected from both the operated and non-operated feet of the same rat. The samples were fixed by 4% paraformaldehyde in 1x PBS overnight, followed by several washes of PBS. Paw tissue was subjected to a treatment of graded alcohol to dehydrate, followed by embedding in a wax block. These were stained with hematoxylin & eosin (HE) and mason’s trichrome stain (MT) for pathological study and grading.

2.5. Pathological grading
The grading was performed according to the criteria set in earlier reports [14-16]. Briefly, the crust in the epidermis and degree of inflammation of the dermis was evaluated according to a one-to-five grading system, in which 1 = minimal (< 1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); 5 = severe/high (76-100%). In this system, the higher the grade worse is the inflammatory reaction. The extent of angiogenesis, granulation, and re-epithelialization was evaluated according to
the criteria reported previously. The degree of lesions was graded from one to four. The higher the index, the better was the recovery taken.

3. Results

3.1. Gross observation

The right paw tissues were obtained after 4 days of surgery by carefully dissecting out of the saline and andro injected animals after perfusion and visually observed for any gross changes that are relevant to the healing process and compared (Figure 1). The tissues did not show any significant infection and the right paw showed swelling and edema right after the surgery while after 5 days, they were reduced significantly and looked similar in both the saline and andro injected paw tissues. Based on gross anatomy we could observe that the wound healing process has been initiated in both the animals and stages of changes the tissues are at, pertaining to wound healing, can be illustrated microscopically by specific staining protocols.

![Figure 1. Gross anatomy of the incisional surgical wound in the right foot collected on day 4 after surgery. Saline injected (A & B); Andro injected (C & D).](image)

3.2. Histopathological observations

3.2.1. Hematoxylin and Eosin staining. The left paw in which the surgery was not performed, showed normal tissue architecture with an intact epithelium layer, and the dermis layer was also observed to be normal with well-appreciated blood vessels rich in collagen and elastin fibers. Nerve endings were prominently located with minimal macrophages and fibroblast. These findings indicate the undisturbed tissue architecture and absence of inflammation (Figure 2A). However, the right paw in which surgery was carried out showed significantly increased inflammatory cells like macrophages and mononuclear cells infiltration (Figure 2B & 2C).
Figure 2. H & E staining of foot tissue sections of saline and andro injected animals showing changes in the epidermal region. Control without surgery (A); Saline injected after surgery (B); Andro injected after surgery (C). The images clearly show re-epithelialization (Black arrow) in andro and saline-injected animals. The normal epithelium is indicated with a green arrow in the un-operated paw.

Epidermal changes: Major change in the epidermal layer of the operated paw tissues is that the focal crust formation was found to be moderate and is at a grade of 2-3 in both saline and andro injected animals, which is not statistically significant (Figure 2 B & 2C). Apart from crust formation, re-epithelialization was also appreciated in the operated right paw by the pathologists and the animals from both the groups showed grade 2-3, while the average re-epithelialization grade was found to be 2.5 grade in both the saline and andro-injected groups.

Dermal changes: Inflammation and angiogenesis are the major pathological changes observed at the dermal region and according to Figure 3B-C, we could find no statistically significant change in both the saline and andro injected groups. The grading of the dermal changes also indicated moderate to severe inflammation and newly formed angiogenesis in both the groups, but the andro injected group showed slightly lower inflammatory changes compared to the saline-injected animals which shows the already well-established anti-inflammatory property of andro. However, the difference was statistically insignificant and it is obvious that we cannot infer with the currently available data that andro showed better inhibition of inflammation but it was promising to observe that within the short study period andro could render moderate anti-inflammatory property. On the other hand, angiogenesis followed a similar pattern as an inflammation where andro showed slightly lesser angiogenesis than saline treatment, but according to this result, in andro injected rats, wound healing was slightly slower than saline-injected group. But according to the grading system, the angiogenesis pattern was similar in both groups.
Figure 3. H & E staining of foot tissue sections of saline and andro injected animals showing changes in dermis region. Control without surgery (A); Saline injected after surgery (B); Andro injected after surgery (C). Figure 3A depicts minimal infiltration of inflammatory cells (Blue arrow) & intact blood vessels (Black arrow) in un-operated paw. Figure 3B & C clearly show the infiltrated inflammatory cells (Green arrow) in saline (Grade: 4) and andro (Grade: 4) injected animals. Newly formed blood vessels (Red arrow) in saline (Grade: 3) and andro groups (Grade: 3).

Figure 4. H & E (A, B & C) and Mason’s trichrome (D, E & F) staining of foot tissue sections from saline and andro injected animals showing granulation in the dermis region. Control without surgery (A & D); Saline injected after surgery (B & E); Andro injected after surgery (C & F). Granulation layer (Black arrow) was clearly shown in saline (Figure 4B & E; Grade: 2) and andro (Figure 4C & F; Grade: 2) treated groups respectively.
3.2.2. Mason’s trichrome staining. MT staining was mainly observed for granulation in the dermal region and regenerating dermis with newer blood vessels (Figure 4A-C). Compared to the un-operated paw, where distinctly separated dermis with cross-section of blood vessels were appreciated, the operated paw of both saline and andro injected group showed moderate granulation with very few newly formed blood vessels were observed (Figure 4B & 4C). This shows that the angiogenesis and granulation layer formation was were graded statistically insignificant.

3.2.3. Pathological score. Paw tissues of both saline and andro injected animals showed there was no significant difference in the pathological score and only moderate changes were observed which were slightly favourable for andrographolide with regards to wound healing property. Figure 5A shows that the average grade for epidermal crust formation was 2.2 in the case of saline and 2.7 for the andro group, which shows that the wound healing process has been initiated in both the groups and it was obviously better in the andro group. But in the case of re-epithelialization the average grades for both groups were 2.5. In the case of changes in the dermal region (Figure 5B), the major finding was reducing trend in (statistically insignificant) inflammation in the andro group (3.7) compared to the saline group (4). Angiogenesis was also moderately reduced in the case of andro injected animals (2.7) compared to saline-injected animals (3). However, granulation was graded in both the groups similarly, and no major difference was reported.

![Pathological score of control, saline-injected and andro injected rats.](image)

4. Discussion
Andrographide is one of the highly acclaimed terpenoid compounds isolated from Andrographis paniculata, because of its diverse biological activities [17, 18]. It is a naturally obtained phytocompound and its analogs obtained naturally and through chemical modifications were also explored for its specific bioactivities [19]. Among the reported biological activities of andro, though anti-inflammatory [10] and anti-microbial properties [3], stem cell trafficking ability were already established at in vivo level [12], the wound healing property of andro is still underrated and this might be due to its dose-specific angiogenic property [20]. In the current study, we wanted to report evidence for pathological events during the wound healing process effected by andro in a surgical wound model in rats. The right paw of the rats was operated and pathological observation was done on the dissected paw from the sacrificed animal by staining the tissue with haematoxylin and eosin method for observation of changes in dermal and epidermal regions such as inflammation, blood vessel formation, etc. Mason’s trichrome staining was performed to establish granulation layer formation in the dermal region.

Haematoxylin and eosin staining clearly indicate major changes in the epidermal and dermal regions. Current observations were in favour of the andro injected group with improved wound healing processes compared to the saline-injected group. Re-epithelialization, also known as epidermal healing is an important stage of a wound repair mechanism because, epidermal closure is the endpoint of wound repair and the newly formed epidermis acts as a barrier for chemical, physical or microbial...
manifestations [21]. Andro injection has not significantly improved the formation of a new epithelial layer, and the results between saline and andro injected groups were not statistically significant. The reason behind this irregularity is that the observations with the andro-injected animals were not consistent as some of the replicates showed significantly prominent wound healing events while some of them did not support this evidence. Another major change observed in the epithelial region is crust formation due to fibroblast proliferation [22], which also showed increasing trend in case of andro injected animals (Grade-2.7) compared to the saline group (Grade-2.2) but the result obtained were statistically insignificant.

Dermal region observed in sections with hematoxylin & eosin and trichrome staining showed changes like inflammation, angiogenesis, and granulation layer formation in the healing wound. Severe infection at the wound site will increase the inflammatory process and infiltration of various immune cells such as neutrophils, macrophages, etc [23]. In the case of animals injected with andro, the degree of the inflammatory process was slightly reduced compared to saline-injected animals. The difference between the groups was not significant and a similar pattern was observed in the case of angiogenesis and granulation. New blood vessel formation and increased collagen deposition around the newly formed blood vessels were appreciated in andro injected animals while granulation was not different between the groups. This is an expected observation as our intention was that the use of analgesics should not influence the wound healing process. Andro was well-known for its anti-inflammatory property [17] which was observed as mild changes in our results, while the angiogenic property of andro was not widely appreciated by researchers because it was a long-standing debate that the angiogenic/anti-angiogenic property of andro is strictly concentration-dependent [20]. Some research groups have indicated the inhibition of angiogenesis by andro in cancer management [24], while another set of experts reported the ability of andro in angiogenesis with significant stem cell recruitment through upregulation of SDF-1α [25]. Hence, the moderate effect of andro on angiogenesis was understandable and further exploration is required on optimization of the dosage at which the appreciable angiogenic property of andro can be reported.

5. Conclusion
In conclusion, the pathological events pertaining to wound healing were observed in the study and at the epidermal region, crust formation and re-epithelialization were prominently illustrated in saline and andro injected animals. Similarly, dermal architecture was also observed to have similar changes between the two groups, but the extent of angiogenesis and inflammation showed slightly better improvement in the andro injected animals' tissue. Results show that andro has moderately increased blood vessel formation than the saline-injected animals. On the other hand, mild and less significant reduction in inflammation due to andro injection, which also was in favour of the wound healing process which was slightly higher in the saline group. In summary, though the results are statistically insignificant, this provides a promising start for further exploration of andro with regards to wound healing property. We can further explore the ability andro at different concentrations to optimize a dosage at which various molecular events are influenced to accelerate the wound healing process in different model systems.

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