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Influence of Ethanol Extract of *Vinca rosea* on Wound Healing in Diabetic Rats

Shivananda Nayak
Department of Pre Clinical Sciences, Biochemistry Unit, Faculty of Medical Sciences
The University of the West Indies, St. Augustine, Trinidad

Abstract: *Vinca rosea* (*Catharanthus roseus* L.) is native to the Caribbean Basin and has historically been used to treat a wide assortment of diseases. European herbalists used the plant for conditions as varied as headache to a folk remedy for diabetes. The objective of the study is to evaluate the diabetic wound healing activity of *Vinca rosea* using the excision wound model in a streptozotocin induced diabetic rats. The animals were weight matched and placed into five groups (n=6 per group). Animals in groups 1 and 2 were normal control (Vaseline) and normal experimental (extract treated) respectively; those in groups 3 and 4 were the diabetic control and diabetic experimental batches. Diabetic animals in a reference group 5 were treated with topical mupirocin ointment. All animals were experimentally wounded on the posterior surface. The ethanol extract of *Vinca rosea* (100 mg kg^{-1} body weight) was applied to animals of group 2 and 4 for ten days. Wounds were measured on days 1, 5 and 11. The granulation tissue formed on the wound was excised on the 11^{th} day and used for the histology and biochemical work up. The wound size in animals of the *Vinca rosea* treated group were significantly reduced (P<0.001) when compared with the diabetic control and mupirocin treated animals. Significant increases in the weight of the granulation tissue (P<0.001) and the hydroxyproline content (P<0.001) were also observed in extract treated animals. Our present study showed that the ethanol extract of *Vinca rosea* promotes significant wound healing and closure in diabetic rats compared with mupirocin and further evaluation of this activity in humans is suggested.

Key words: Hydroxyproline, wound area, excision wound model

INTRODUCTION

Natural products are a source of synthetic and traditional herbal medicine and are still the primary health care system[1]. The presence of various life sustaining constituents in plants made scientists to investigate these plants for their uses in treating certain infective diseases and management of chronic wounds.

Normal wound healing response begins the moment the tissue is injured. The healing cascade begins immediately following injury when the platelets come into contact with exposed collagen. As platelet aggregation proceeds, clotting factors are released resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing[2]. The inflammatory cells also arrive along with the platelets at the site of injury and they provide key signals are known as cytokines or growth factors[3]. The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury. Collagen is the most abundant protein in the animal kingdom, accounting for 30% of the total protein in the human body[4]. In normal tissues collagen provides strength, integrity and structure. When tissues are disrupted following injury, collagen is needed to repair the defect and restore anatomic structure and function.

Diabetic wounds are slow, non-healing wounds that can last for weeks despite adequate and appropriate care. Such wounds are difficult and frustrating to manage[5]. Diabetic wound healing is an enigmatic and debilitating complication and poses a serious challenge in clinical practice. The exact pathogenesis of the poor wound healing with the diabetic wound is not clearly understood, but evidence from studies involving both human and animal models reveal several abnormalities in the various phases of wound healing process[6,7].

Catharanthus roseus L. (apocyanaceae) also known as *Vinca Rosea*, is native to the Caribbean Basin and has historically been used to treat a wide assortment of diseases. European herbalists used the plant for conditions as varied as headache to a folk remedy for diabetes. It has more than 400 known alkaloids, some of which are approved as antineoplastic agents to treat leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilms' tumor and other cancers. Its vasodilating and memory-enhancing properties have been shown to alleviate vascular dementia and Alzheimer's disease[8,9]. The two classes of active compounds in *Vinca* are alkaloids and tannins. The major alkaloid is vincamine

Corresponding Author: Dr. Shivananda Nayak, Dept of Pre-Clinical Sciences, Biochemistry Unit, Faculty of Medical Sceinces, The University of the West Indies, Trinidad and Tobago, Tel: 001-868-6621873-4641, Fax: 001- 868-6621873

and its closely related semi-synthetic derivative widely used as a medicinal agent, known as ethyl-apovincaminate or vinpocetine, has vasodilating, blood thinning, hypoglycemic and memory-enhancing actions[10, 11]. The extracts of *Vinca* have demonstrated significant anticancer activity against numerous cell types[12].

Current methods used to treat chronic diabetic wounds include debridement, irrigation, antibiotics, tissue grafts, proteolytic enzymes and corticosteroids, which possess major drawbacks and unwanted side effects. Extracts from the dried or wet leaves of plants are applied as a paste on wounds in some rural communities. The fresh juice from the leaves of *Vinca rosea* made into a tea has been used by Ayurvedic physicians in India and other countries for external use to treat skin problems, dermatitis, eczema and acne. There is no previous report on diabetic wound healing activities of *Catharathus roseus* in literature to the best of our knowledge and in this paper, we report for the first time, the efficacy of *Vinca rosea* leaf extract in the treatment and management of diabetic wounds.

MATERIALS AND METHODS

Plant material and extraction: The *Vinca rosea* leaves were collected locally in March 2006 and identified by Mrs. Yasmin S, plant taxonomist and curator, National herbarium of Trinidad and Tobago, The University of The West Indies, St. Augustine, Trinidad. The voucher specimen was also deposited at the above mentioned herbarium (specimen number: 36458).

The *Vinca rosea* leaves (100g) were cleaned with water following which the leaves were ground into solution using an electric blender with 200 ml of ethanol. The solution was kept at room temperature for 2 hours in a closed glass container. Then the contents were filtered and the clear solution (50 ml) was used for these studies. The extract was subjected to preliminary phytochemical tests.

Animals: Healthy in bred female Sprague Dawley rats weighing 180-200g were procured from the School of Veterinary science, Trinidad. They were individually housed and maintained on normal food and water ad libitum. Animals were periodically weighed before and after experiments. The rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anesthesia (10 mg kg^{-1} body weight of an animal). Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study. An acute toxicity study was conducted for the extracts by the stair-case method[13]. The LD_{50} of ethanol leaf extract was found to be 1000 mg kg^{-1}, b.w. One tenth of the dose was selected for the evaluation of wound-healing activity i.e., 100 mg kg, $^{-1}$ b.w. The study was carried out with prior ethical approval (Animal Ethics Committee, Faculty of Medical Sciences, The University of The West Indies, EC= 04/05). After infliction of wounds as described in the succeeding paragraph, 6 animals each were randomly distributed into five groups according to similar weights (n=6 per group)

Group 1= Normal control –treated with Vaseline
Group 2= Normal experimental- treated with extract
Group 3= Diabetic control- treated with vaseline
Group 4= Diabetic experimental–treated with plant extract topically.
Group 5= Standard-treated with mupirocin ointment

Induction of diabetes: Animals of group 3, 4 and 5 were weighed and their fasting blood glucose was determined before inducing diabetes. Then the animals were induced for diabetes by administering a single dose of streptozotocin (STZ, 50 mg kg^{-1}) in cold 0.1M citrate buffer, pH 4.5 (freshly prepared) via the tail vein. Control animals were administered 0.1M citrate buffer. Fasting blood glucose was measured three days later to ensure the induction of diabetes. Animals with a blood glucose level of ≥150 mg dL^{-1} were considered diabetic. Blood glucose measurements were done using blood drawn from the tail vein. A glucometer was calibrated using the hexokinase method with standards and quality controls.

Excision wound creation: All the rats were anesthetized with 1 ml of intravenous ketamine hydrochloride (10 mg kg^{-1} body weight) and shaved on both sides of the back with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. The full thickness of 2.5 cm length and 0.2 cm depth of the excision wound was created along the markings using toothed forceps, a surgical blade and pointed scissors.

The group 2 and 4 animals were treated topically with the extract (100 mg kg^{-1} body weight) which was applied as a single layer thickness to the wound for 10 days. On the 11^{th} post-operative day, the granulation tissue which was formed on the wounds was excised and wet weight was recorded. The tissue was dried in an oven at 60ºC and the dry weight noted again. Acid hydrolysate of the dry tissue was used for the determination of hydroxyproline[14]. Additional sections will be processed by routine methods for histological examination. Simultaneously blood sample was also collected to determine blood glucose level.

Wound area measurement: The measurements of the wound areas were taken on the day 1, 5 and 11 using transparency paper and a permanent marker. The wound areas were recorded and measured on graph paper.

Histological study: The healing tissues were obtained on the day 11 from all the animals were used for histological study. The amount of collagen was quantified using Van Geison stain.

Statistical analysis: Results, expressed as mean ± SEM. One-way analysis of variance was used to examine differences in wound healing between the groups. Data was analyzed using the statistical package for SPSS and P-value that is less than 0.05 i.e. P<0.05 was taken as the cut off level for significance.

RESULTS AND DISCUSSION

The topical application of the extract to the wound of diabetic experimental animals (group 4) significantly increased the rate of wound contraction (P<0.001) when compared with the diabetic (group 3) and normal controls (group 1). By day 11 wounds in diabetic animals treated with the experimental extract had closed by 44.4% as compared with wounds treated with the standard formulation of mupirocin. (P<0.001). Granulation tissue had proliferated demonstrating a significant increase (P<0.001) in dry weight of 31.6% in the diabetic experimental test group (group 4) compared with standard test group animals (group 5). Granulation tissue obtained from the diabetic experimental animals that were treated with *Vinca rosea* extract showed the highest (P<0.001) hydroxyproline content (199.0 ± 2.7) when compared with the control and mupirocin treated animals. Overall the weights of the animals did not differ for any of the study groups.

Animals that did not receive the *Vinca rosea* extract treatment, showed wounds which appeared to be hard and crusty with undermined margins and were generally unclean with a biofilm glaze on the surface (Fig. 1). In contrast the wounds in animals treated with *Vinca rosea* were clean and showed bright red healthy granulation tissue (Fig. 2).

The histological study of the healing tissue obtained from the diabetic animals of extract treated (Fig. 3) showed the fast lay-down of collagen when compared to diabetic control (Fig. 4) and mupirocin.

Normal wound healing occurs in three stages: inflammation, proliferation and remodeling. The wound healing process depends on a given provision of local circulation, as well as the formation and deposition of collagen. The wound healing is impaired in the diabetic state because of, at least in part, low growth factors.

Wound healing deficits in diabetes are diverse, multifactorial, complex and inter related[15] and are believed to be caused by impaired blood flow and oxygen release from increased blood sugar, decreased collagen and fibronectin synthesis from protein malnutrition, impaired local immune and cell defenses and decreased anabolic activity with decreased insulin and growth hormone. Collagen, fibrin and keratin

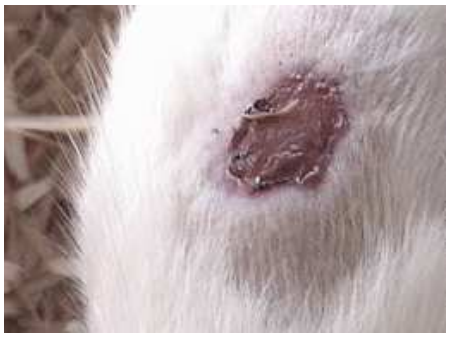

Fig. 1:Diabetic controls treated withVaseline (Day 11)

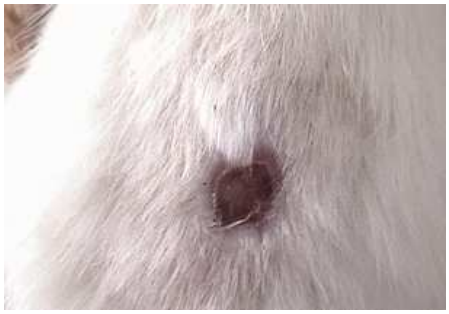

Fig. 2: Diabetic experimental treated with *Catharanthus* extract (Day 11)

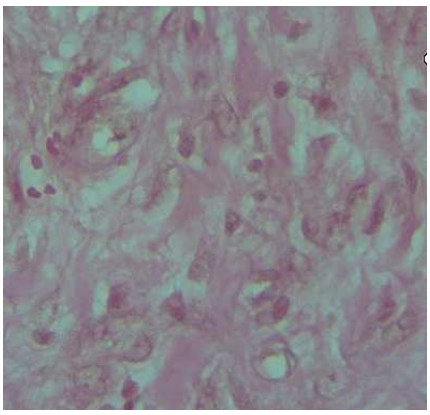

Fig. 3: Granulation tissue with more collagen (diabetic experimental group)

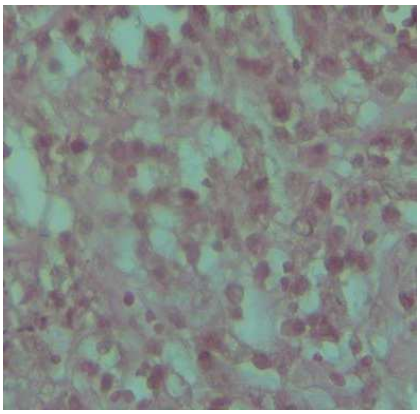

Fig. 4: Granulation tissue with less collagen (diabetic control group)

Table 1: Diabetic wound healing activity of the *Vinca rosea* in streptozotocin induced rats

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Wound area (mm^2):					
Day 1	190.1 ± 2.52	190.8 ± 2.28	190.80 ± 21.8	199.8 ± 7.9	190.33± 2.7
Day 5	135.5 ± 2.9	137.1 ± 2.6	146.50 ± 8.1	82.1 ± 0.65**	168.30± 0.21
Day 11	51.0 ± 1.3	52.0 ± 1.7	58.50 ± 6.0	23.6 ± 1.80 ***	42.5 ± 2.01
Wet granulation weight (mg)	101.16 ± 6.16*	152.6 ± 4.92	88.50 ± 4.89	160.83 ±25.93	134.2 ± 6.4
Dry granulation weight (mg)	31.50 ± 2.77	45.25 ± 3.40	28.83 ± 2.38	46.30 ± 4.40**	35.34 ±4.9
Hydroxyproline (mg g^{-1})	116.60± 23.20	184.0 ± 12.1	135.00 ± 2.0	199.00 ± 2.7***	104.0 ± 12.9

Values are mean ± SEM of 6 animals in each group **P. < 0.05, ***P<0.001
Comparison between groups: 1 and 2; 1 and 3; 3 and 4; 3 and 5.
Group 1- Normal control, Group 2- Normal experimental, Group 3- Diabetic control, Group 4- Diabetic experimental and Group 5- Reference standard.

accumulate advanced glycation Amadori end products which affect binding of regulatory molecules, susceptibility to proteolysis and decrease the ability for protein cross-linkage[16]. Di Girolamo et al postulated that defects in wound healing are caused by the hyperglycosylation of the locally synthesised cellular fibronectin[17]. Hyperglycaemia affects the whole range of neutrophil functions, including migration, chemotaxis, adherence and phagocytic and bactericidal activity[18].

We have demonstrated wound healing properties of *Vinca rosea* after topical application in streptozotocin-induced diabetic rats. Animals with induced diabetes had reduced wound size as early as day 5 and till the end of the experiment compared with the wounds in control animals as well as those treated with topical mupirocin. The researchers showed that the combination of transforming growth factor–β1 and fibroblast growth factor had marked positive effects on biochemical parameters of wound healing and reversed the tensile strength deficit of diabetic wounds[19]. The presence of *Vinca rosea* seemed to reduce the amount of dead tissue at the wound site and provide better wound healing. The observed increase in hydroxyproline content of the granulation tissue of the excision wounds indicated rapid collagen turnover resulting in rapid healing of the wounds. During the early wound-healing process, the epithelial cells proliferate, migrate from the edges of the wound and eventually cover the wound with skin. Although the earlier studies showed the presence of several phytochemicals[20], it is not yet clear which constituents of the extract of *Vinca rosea* are responsible for its diabetic wound healing properties. Little research has been done to determine individual phytochemicals in *Vinca rosea*. In general, it is known to contain glycosides, lipids, alkaloids, flavonoids, tannins, cardenolids, triterpenes, polyphenols, steroids and resins.

Possibly, the constituents like alkaloids, triterpenoids and tannins of *Vinca* may play a major role in the process of wound healing in diabetic rats. Tannins[21] and triterpenoids[22] are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation. The vascular and lymphatic systems are of primary importance during the process of wound healing. Failure or delay of vascular regeneration decrease oxygen transport to the wound, which subsequently depresses the mobilization of excessive fluids from the wound site. The wound becomes edematous, leading to further damage, infection and eventually cell death. In wound healing, new blood vessels sprout up from the platelets or macrophages to keep the wound open-ended. Hypoxia may be the stimulant to revascularization. *Vinca* may, thus achieve the following effects to improve tissue healing: an increased blood supply which increases the oxygen supply to the wound by blocking vasoconstructive compounds; greater migration of epidermal cells and extensive reorientation of collagen fibers caused by a stronger cross-linking[23]. Several authors also reported this type of pro-healing action with the extract of many indigenous medicinal plants[24,25].

Our histologic work indicates that *Vinca rosea* stimulates and enhances the faster lay down of collagen fibers with the extract received diabetic animals than the untreated diabetic control wound.

CONCLUSION

The present study suggested that the topical administration of ethanol extract of *Vinca rosea leaves* plays a major role in diabetic wound healing. Our present documented findings may suggest the use of *Vinca rosea* to treat and management of diabetic wounds.

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