Original article

Clin Ter 2010; 161 (2):117-120

The effect of Piper betel extract on the wound healing process in experimentally induced diabetic rats

E.C. Keat[1], S.S. Razak[1], N.M. Fadil[1], F.M. Yusof[1], L.H. Chah[1], F.K. Chyi[1], S.L. Teoh[2], S. Das[2], A.A. Latiff[2], M. Mazlan[3]

[1]Final year Medical students; [2]Department of Anatomy; [3]Department of Biochemistry, Universiti Kebangsaan Malaysia

Abstract

Background. Piper betel (PB) possesses antimicrobial, antifungal, antioxidant and wound healing properties due to its powerful antioxidant effect. Diabetes mellitus (DM) is a metabolic disorder which is associated with complications like impaired wound healing, nephropathy and neuropathy. The main aim of the study was to study the wound healing properties of PB.

Materials and Methods. A total of 33 male Sprague-Dawley rats (250-300g) were taken and divided into 3 groups:- Group I (control) comprising of 14 rats; Group II (diabetic untreated) comprising of 9 rats; Group III (diabetic treated) comprising of 10 rats. After 10 days of acclimatization, the animals were fasted overnight and diabetes was induced by administration of streptozotocin (45 mg/Kg body weight in a single dose, through tail vein) to group II and III animals. Four 6 mm-diameter full thickness skin excision wounds were created and PB extract (50 mg diluted in 0.1 ml of normal saline) was applied locally for 10 days in group III. The group I and II received normal saline (0.1 ml) for 10 days. The total protein content and the wound contraction rate were determined.

Results. The wound contraction rate of group III (35.03 ± 2.96) was higher as compared to group II (18.40 ± 3.87) with p = 0.014. The total protein content for group III was 106.39 ± 4.46 as compared to group II (72.86 ± 12.86) with p = 0.050.

Conclusion. PB acted as a protective agent in the early phase of wound healing by increasing total protein content and wound contraction rate. ***Clin Ter 2010; 161(2):117-120***

Key words: *antioxidant, diabetes mellitus, extract, healing, piper betel wound*

Introduction

Piper betel (PB) is a plant which belongs to the family Piperaceae, whose leaves are known to exhibit medicinal properties. The plant is green in colour and its leaves are heart shaped. The PB is widely found in the region of India, Sri Lanka, Vietnam, and Malaysia. Some of the active ingredients of PB include phenols, flavonoids, tannins and polysaccharides (1).

The PB plant has been reported to exhibit antimicrobial, antifungal, antioxidant, cardiotonic, wound healing, reversible antifertility, antimutagenecity and chemopreventive properties (1). Recent studies on PB has shown it to have positive effect in ulcer-healing due to its antioxidant and mucous protecting effect (1, 2). It has shown enhanced sensitivity of Hep G2 cell to anti cancer drug and also exhibited radioprotective action (2, 3).

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia as a result of insulin deficiency, insulin resistance, impaired insulin secretion or excessive hepatic glucose production (4). DM is known to be associated with complications such as diabetic foot, nephropathy, diabetic retinopathy, neuropathy and vascular disease (5). Impaired wound healing in diabetic patient is a matter of concern because even a minor skin wound often results in chronic, non-healing ulcers which incurs expensive treatment (6). Alarmingly, around 14-24% of patients of diabetic foot ulcer require amputation even with proper treatment and care (7).

Reactive oxygen species (ROS) are formed within the body during any metabolic process (8). These ROS are involved in a number of diseases like atherosclerosis, cancer, cirrhosis and diabetes. Some of the plant-derived antioxidants such as tannins, lignans, stilbenes, coumarins, quinones, could delay or prevent the onset of degenerative diseases because of their redox properties, which allow them to act as hydrogen donors, reducing agents, hydroxyl radicals (OH^-) or superoxide radical (O^2) scavengers (9). Interestingly, these plant-derived antioxidants are also strong chelators of metal ions (9).

Many herbs and plants have been used in traditional medicine all over the world to treat any wound and these include Amaranthaceae, Anacardiaceae, Araceae, Asclepiadaceae, Boraginaceae, Chenopodiaceae, Cruciferaceae, Capparaceae, Hydnoraceae, Iridaceae, Rhamnaceae and Sapindaceae families (9).

Streptozotocin (STZ) is a compound isolated from *Streptomyces achromogenes* which exhibits marked antileukemic activity (10). Research reports depict that STZ can induce DM in many animal species which may exactly resemble

Correspondence: Dr. Srijit Das. Associate Professor Lecturer, Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia. Tel: +603-92897263; Fax: +603-26989506. E-mail: das_srijit23@rediffmail.com

Source of funding: Grant number of FF-016–2009

human hyperglycemic nonketotic DM state (11). It has been claimed that its diabetogenic effect is due to the result of the specific damage of ß pancreatic islet cells, although some researchers have suggested that it produces a depletion of secretory granules in unaltered ß cells or suppression in insulin production and release (12).

The main aim of the present study was to create an animal of DM by injecting STZ and ascertain the protective role of PB extract as a wound healing agent in DM wounds.

Materials and Methods

Prior ethical approval was obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committees (UKMAEC). The research was conducted by the final year medical students under Special Study Module (SSM) programme.

PB extract

PB extract was prepared by the Forest Research Institute of Malaysia (FRIM) (aqueous derived) was obtained in dried-powdered form. The PB extract was stored in airtight container at - 20^0 C until the time of its usage. An amount of 50mg of PB extract diluted in 0.1 ml of normal saline was applied topically at the wounds created in the experimental groups.

Animals

In this study, 33 male Sprague-Dawley rats (weighing 250-300 g) were obtained from laboratory animal resource unit of UKMMC. The rats were divided into 3 groups and acclimatized for ten days before the experiment commenced. Group I consisted of the normal control group which comprised of 14 rats. Group II was the diabetic untreated group with a total of 9 rats, and Group III consisted of diabetic treated group which comprised of 10 rats. Few rats in the diabetic groups died so it did not equal to that of the normal group. Commercial rat feed and water *ad libitium* was administered to the experimental rats. The rats were housed one per cage and the environment was controlled with 12-hours light/dark cycle during the entire experiment.

Induction of diabetes mellitus

The rats were fasted overnight and a single intravenous dose of STZ (Sigma company, Germany) in the dose of 45 mg/kg of body weight was injected for the induction of DM. The STZ was diluted in normal saline and injected into the experimental rats via the tail vein. On the third day after STZ administration, fasting blood glucose was checked with glucose reagent strip and glucose meter (Accu-Chek Advantage, Roche Diagnostics, Hamburg, Germany). As per a previous protocol, the rats with the fasting blood glucose greater than 8mmol/L were considered as diabetic (13).

Wound creation

On the tenth day after induction of DM, wound was created on all rats by punch biopsy needle (14). After general anaesthesia was administered, the hair on the dorsal aspect of the back of each rat was shaved. The area was shaved beforehand and the skin was wiped with 70% alcohol. Four 6 mm-diameter full thickness skin excision wounds were created using a punch biopsy needle (two each side of the median line, approximately 2 cm from each other).

PB extract was applied to the rats topically on the wound surface of rats in group III (diabetic, treated); while the wound of the rats in group I (control, untreated) and group II (diabetic, untreated) were treated with normal saline, once daily. All the wounds received daily standard wound cleansing prior to the next application of PB extract. Throughout the experiment, the wounds remained uncovered.

The size of the wound and total protein content in the punch biopsy wounds were determined during the progress of healing.

Wound collection

Half of the rats from each group were sacrificed on the fourth and tenth day after wound creation by cervical dislocation under anesthesia. The newly formed regenerated tissue was harvested without contamination from normal skin by using the same technique as in wound creation for total protein content determination.

Total protein content

The total protein content was determined as per an earlier method described by Bradford method (15). One wound tissue from each rat was cut into smaller pieces. The wound tissue was homogenized in 1.15% potassium chloride at a ratio of 1:5 (wt/vol). The homogenous was centrifuged at 3000G for 10 minutes at 4°C. Total of 0.01 ml of the supernatant was taken and it was mixed with 5 ml of Bradford reagent. The absorbance was read at 595 nm wavelength against normal saline, pH 7. Standard was treated similarly using Bovine Serum Albumin at concentration of 0 µg/ml, 20 µg/l,40 µg/mL,60 µg/ml,80 µg/ml and 100 µg/ml in normal saline. Total protein content reflected the cellular proliferation and migration into the wound.

Rate of wound closure

Digital photograph of the wound was taken on day 0, day 4 and 10 after the wound creation. By using image analysis software (VideoTesT-Master Morphology; VideoTesT, St Petersburg, Russia), the rate of wound closure was calculated using the following formula:

$$\text{Rate of wound closure (\%)} = \frac{\text{wound area at day 0 – day n}}{\text{Wound area at day 0}} \times 100$$

Statistical analysis

The data was expressed as mean ± SEM/SD followed by Mann-Whitney test and p value ≤ 0.05 was considered significant. All the statistical tests were performed by using SPSS software (version 12.0).

Results

Rate of wound closure

The wound closure rates was measured as percentage of reduction from original wound size to the wound size on day 4 and day 10 respectively. The wound decreased in size each day after the wound creation in all groups. The rate of wound closure at day 4 in the group III i.e. diabetic treated group (35.03 ± 3.00) was significantly higher as compared to the group II i.e. DM untreated group (18.44 ± 3.87) (Table. 1 and Fig. 1).

Interestingly, at day 10, the rate of wound closure in the diabetic treated and untreated group were 61.67 ± 4.89 and 60.02 ± 3.57 respectively which meant that there was not much difference in between the two groups.

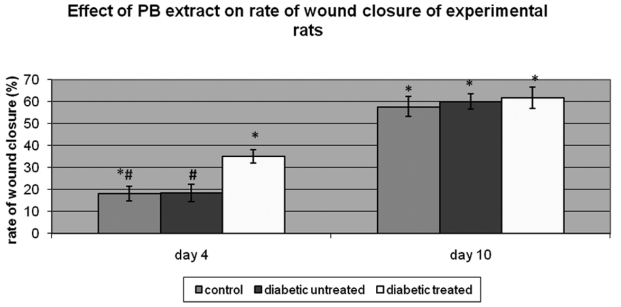

Fig.1. Bar chart showing the wound closure (in %) in the normal, diabetic treated and diabetic untreated groups. Different symbols indicate significant differences at day 4 and day 10, respectively.

Total protein content

The total protein content of the wounds collected on day 4 and 10 was tabulated (Table 1 and Fig. 2). At day 4, the total protein content of the diabetic treated group (106.39 ± 4.46) was significantly higher as compared to the diabetic untreated group (72.86 ± 12.86) ($p < 0.05$). However, the protein content of the diabetic treated and untreated groups showed no significant differences ($p > 0.05$) at day 10.

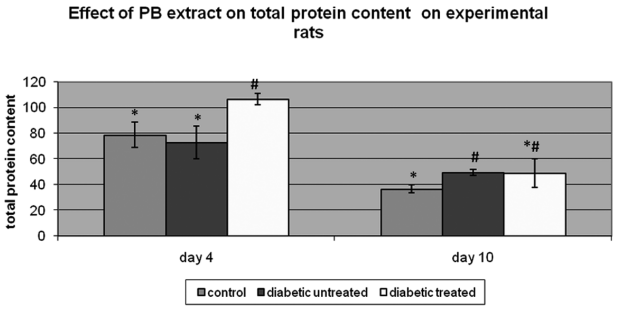

Fig. 2. Bar chart showing total protein content (in µg/ml) in the normal, diabetic treated and diabetic untreated groups. Different symbols indicate significant differences at day 4 and day 10, respectively.

Discussion

STZ has been known to cause specific damage to ß pancreatic islet cells (11). Therefore it can produce diabetogenic effect and the resultant clinical features exactly resemble those of human hyperglycemic non ketotic DM with clinical features like hyperphagia, polyuria and weight loss (16). As a result, STZ acts as an excellent model of DM for any experimental purpose. Perhaps, that was the reason why we preferred to choose STZ to create an animal model of DM.

It has been reported previously that cutaneous wounding may result in a decrease in antioxidant status as a result of production of ROS. An earlier research study reported that any diabetic ulcer that lasts for more than 4 weeks is usually an indication of worse outcome and may lead to amputation (17). Hence, the rate of wound healing plays an important role in the development of complications.

Wound healing occurs in three different phases, i.e. inflammatory phase, proliferative phase and remodeling phase. We chose to select day 4 as it would signify the late inflammatory phase or the early proliferation phase of healing.

In the study, we observed that the rate of wound closure of the diabetic treated group was significantly faster as compared to the diabetic untreated group on day 4 after wound creation. However, there was no significant increase in the rate of wound closure on day 10 in the diabetic treated group as compared to the diabetic untreated group. Increase in the wound closure at day 4 signified that PB acted better in the early phase of wound healing, i.e., at day 4.

Usually, the total protein content represents the protein level and the cellular proliferation of the wound tissues. In our study, we found out that the total protein content of the diabetic treated group only increased significantly at day 4 after wound creation as compared to the other groups. There was not much difference in the total protein content of the wound on day 10in the diabetic treated and untreated groups.

Table 1. Effect of the Piper betel on the rate of wound closure and total protein content in the control, diabetic untreated and diabetic treated group.

Day on which wound was measured	Group	Rate of wound closure (Mean ± SEM)	Total protein content (Mean ± SEM)
4	Control	18.14 ± 33.30^{ab}	78.64 ± 9.71^{a}
	Untreated	18.44 ± 3.87^{b}	72.86 ± 12.86^{a}
	Treated	35.03 ± 3.00^{a}	106.39 ± 4.46^{b}
10	Control	57.70 ± 4.56^{a}	36.58 ± 3.12^{a}
	Untreated	60.02 ± 3.57^{a}	49.41 ± 2.30^{b}
	Treated	61.67 ± 4.89^{a}	49.03 ± 11.26^{ab}

This suggested that the PB extract may stimulate the cellular proliferation in the early stage of the wound healing process as observed on day 4.

In conclusion, the present study looked into the biochemical parameters of wound healing i.e. wound contraction rate and total protein content of DM wounds treated with or without PB extract.. Based on the results of our study, we found out that the PB extract exerted a protective role wound healing rate more in the acute stage of the wound healing (at day 4) as compared to the later stage (day 10). Considering the cost effectiveness and absence of toxic effects, the PB may be actively used as a supplement in treating DM wounds.

Acknowledgement

This study was supported by Special Study Module grant received from the Universiti Kebangsaan Malaysia. The authors sincerely thank Prof. Madya. Dr. Faizah Othman and all Anatomy Department supporting staff members, Prof. Dr. Yasmin Anum, Prof. Dr. Ruszymah Idrus, Mr. Michael of Animal House, UKM and Miss Khadijah Adam for their kind assistance.

References

1. Kanjwani DG, Marathe T, Chiplunkar SV, et al. Evaluation of immunomodulatory activity of methanolic extract of Piper betel. Scand J Immunol 2008; 67:589-93
2. Bhattacharya S, Banerjee D, Bauri AK, et al. Healing property of the Piper betel phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action. World J Gastroenterol 2007; 13:3705-13
3. Young SC, Wang CJ, Lin JJ, et al. Protection effect of piper betel leaf extract against carbon tetrachloride-induced liver fibrosis in rats. Arch Toxicol 2007; 81:45-55
4. Kasper DL, Braunwald E, Fauci AS, et al. Harrison's Manual of Medicine. Boston, McGraw-Hill, 2005
5. Longmore M, Wilkinson IB, Rajagopalan S. Oxford Handbook of Clinical Medicine. Oxford University Press, 2004
6. Li H, Fu X, Zhang L, et al. Research of PDGF-BB-gel on the wound healing of diabetic rats and its pharmaodynamics. J Surg Res 2008; 145:41-8
7. Trousdale RK, Jacobs S, Simhaee DA, et al.. Wound closure and metabolic parameter variability in a db/db mouse model for diabetic ulcers. J Surg Res 2009;151:100-7
8. Parihar A, Parihar MS, Milner S, et al. Oxidative stress and anti-oxidative mobilization in burn injury. Burns 2008; 34: 6-17
9. Marwah RG, Fatope MO, Al Mahrooqi R, et al. Antioxidant capacity of some edible and wound healing plants in Oman. Food Chem 2007; 101:456-70
10. Bolzán AD, Bianchi NO, Bianchi MS. Effects of antioxidants on streptozotocin-induced clastogenesis in mammalian and insect cells. Mutation Res 1998; 418:35-42
11. Mir SH, Baqui A, Bhagat RC, et al. Biochemical and histomorphological study of streptozotocin-induced diabetes mellitus in rabbits. Pak J Nutri 2008; 7:359-64
12. Aughsteen AA. An ultrastructural study on the effect of streptozotocin on the islets of Langerhans in mice. J Electron Microsc (Tokyo) 2000; 49:681-90
13. Musalmah M, Nizrana MY, Fairuz AH, et al. Effects of palm vitamin E and alpha-tocopherol on wound healing and wound tissue antioxidant enzyme levels in diabetic rats. Lipids 2005; 40:575–80
14. Teoh SL, Latiff AA, Das S. The effect of topical extract of Momordica charantia (bitter gourd) on wound healing in nondiabetic rats and in rats with diabetes induced by streptozotocin. Clin Exp Dermatol 2009 Mar 23 Early online DOI: 10.1111/j.1365-2230.2008.03117. 2009; 34(7):815-22
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 1976; 72:248–54
16. Qiu Z, Kwon AH, Kamiyama Y. Effects of plasma fibronectin on the healing of full-thickness skin wounds in streptozotocin-induced diabetic rats. J Surg Res 2006; 138:64–70
17. Jeffcoate WJ, Price P, Harding KG. International Working Group on Wound Healing and Treatments for People with Diabetic Foot Ulcers. Wound healing and treatments for people with diabetic foot ulcers. Diabetes Metab Res Rev 2004; 20 Suppl 1:S78-89
18. Wound healing and treatments for people with diabetic foot ulcers. Diabetes Metab Res Rev 2004; 20:S78-89