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Indian Journal of Physiology and Pharmacology

Original Article

Enhancement of caudal fin regeneration in diabetic zebrafish: An insight into proangiogenic potential of bromelain

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Received : 14 February 2022
Accepted : 26 January 2023
Published : 29 March 2023

DOI
10.25259/IJPP_90_2022

Quick Response Code:

ABSTRACT

Objectives: Diabetes mellitus is a chronic metabolic disease that is characterised by hyperglycaemia, altered lipids, carbohydrates and protein metabolism, and in the long-term, with eye, kidney, cardiovascular and neurological complications. Poor wound healing is one of the major complications faced by diabetes mellitus patients. Angiogenesis is critical for tissue regeneration and wound healing. Impaired angiogenesis may lead to poor blood flow to the wound and hence delayed wound healing. Hence, it is important to find an antidote to speed up wound healing. Hence, this study focuses on the proangiogenic potential of bromelain in the caudal fin regeneration of diabetic zebrafish.

Materials and Methods: Zebrafish were divided into control, Diabetic and Diabetic + Bromelain treated groups. Diabetes mellitus was induced using multiple doses of streptozotocin (350 mg/kg b.w./i.p.) on days 1, 3, 5, 12 and 19. Glucose levels were estimated on day 21 to confirm the induction of diabetes mellitus, and then, the caudal fin was amputated. After the amputation of the caudal fin, bromelain was administered orally at a dosage of 40 mg/kg b.w. on every alternate day for 15 days. Body weight, blood glucose level, total area and percentage of fin regeneration were observed on day 36. Images were compared and areas of regeneration were analysed with the help of Image J software. One-way analysis of variance followed by Tukey's multiple comparison tests was used to analyse the data.

Results: A significant increase in the blood glucose level was observed in the diabetic group compared with control. A significant decrease in the percentage of tail regeneration and area of regeneration was observed in diabetic fishes compared to the control. Bromelain treatment has significantly increased the percentage and area of regeneration and significantly decreased the blood glucose level in the treatment group compared with the control.

Conclusion: The study confirms that bromelain can promote tissue regeneration; hence, it can be used to improve wound healing, which is one of the most common complications in diabetes mellitus.

Keywords: Diabetes mellitus, Regeneration, Bromelain, Streptozotocin

INTRODUCTION

The prevalence of diabetes in adults has been increasing in recent years in middle- and low-income countries. According to International Diabetes Federation findings, diabetes prevalence in 2017 was 451 million, and these figures are expected to increase to 693 million by 2045. It is dreadful to know that nearly 850 billion is spent on diabetes mellitus, which is almost 12%

of global health expenditure.[1] India has the largest diabetic population with 72 million people in 2017 which is about 49% of the world's diabetes burden and this figure is expected to double by 2025.[2] Chronic hyperglycaemia and altered insulin metabolism are the major biochemical diagnostic parameters of diabetes mellitus. Diabetes mellitus leads to several microvascular and macrovascular diseases such as diabetic nephropathy, neuropathy, retinopathy, coronary artery disease, peripheral arterial disease and stroke.[3] Poor healing is one of the most common problems faced by diabetes mellitus patients. Angiogenesis is crucial for tissue regeneration in wound healing, cardiovascular disease and post-operative healing. Growth factors, including vascular endothelial growth factor, growth hormone and transforming growth factor β, play important roles in angiogenesis. A poor angiogenic response to tissue injury leads to lack of oxygen and nutrients for the regeneration of damaged tissue, which may lead to scarring or loss of tissue viability. In diseases such as myocardial infarction and stroke, regeneration of the damaged tissue is essential for recovery. Many attempts have been made to speed up regeneration, particularly in ischemic tissues such as the heart and the brain. In recent decades, regeneration of the caudal fin in zebrafish has been mainly focused to study various aspects of the regenerative process.[4] This angiogenesis model of caudal fin regeneration is a simple quantitative analysis used for distinguishing total and partial inhibition of angiogenesis.[5] It is a key tool used for drug discovery and to find genes responsible for angiogenesis.[6] Knowing the role of angiogenesis in the regenerative process is of utmost importance to finding an antidote to speed up tissue regeneration in diabetes mellitus patients. One such naturally available bioactive compound is bromelain. Bromelain is a protease enzyme extract derived from fruit and the stem of pineapple. It has been used as an anti-inflammatory agent, anti-tumour agent and anti-diarrheal agent. It is widely used for removing dead and damaged tissue after a burn and treating dermatological disorders. In cardiovascular diseases, it is used for inhibiting thrombus formation and reducing the pace of clotting. Since ancient times, bromelain has been used for treating bowel conditions, relieving osteoarthritis, improving the absorption of antibiotics, shortening labour and helping the body to get rid of fat.[7] However, there is limited literature to show its effect on angiogenesis, which is important for tissue regeneration and wound healing. Zebrafish has the potential to regenerate tissues such as heart, brain and muscle, which is not possible in mammals. Therefore, zebrafish are used to study the mechanism behind regenerative angiogenesis and to find drugs that aid in or interfere with this process.[8] In recent days, zebrafish have been emerging as a powerful model for studying regeneration of the heart, spinal cord, liver, pancreas, retina, tail and other tissues.[9,10] From all of these regenerative structures, the adult zebrafish caudal fin is unparalleled for its accessibility to amputation and relatively simple cellular organisation.[11-13] Hence, our study is designed to study the effect of bromelain on impaired angiogenesis in diabetes mellitus zebrafish.

MATERIALS AND METHODS

Experimental framework

The experimental procedures described herein were performed in accordance with the Ethical Principles in Animal Research adopted by the Committee for Control and Supervision of Experiments on Animals. The animals used in this experiment were approved by the Institutional Animal Ethical Committee (IAEC) of Institute of Basic Medical Sciences, University of Madras, Chennai (IAEC No: 205/GO/ReBi/SL/2000/CPCSEA, Dated 04/05/2018). The experiment was carried out with 3–6-month-old adult zebrafish which were obtained from a commercial aquarium. Healthy zebrafish were selected for the study, for which the inclusion criterion was weight of the fish. Zebrafish weighing above 0.5 g and below 1 g were included in the study. Fishes were housed in properly aerated fish tanks with a 12 h light/12 h dark cycle at 26–28°C under optimum conditions. The fish were fed twice a day with commercially available fish pellets. Total of 120 adult male and female wild type strains of zebrafish were randomly divided into three groups. Group I: Control, Group II: Diabetes mellitus (streptozotocin [STZ] 350 mg/kg b.w./i.p. on day 1, 3, 5, 12, 19, 26 and 33), and Group III: Diabetes mellitus + Bromelain treatment (STZ 350 mg/kg b.w./i.p. on day 1, 3, 5, 12, 19, 26 and 33; Bromelain 40 mg/kg b.w./oral from day 21 to day 36 on alternate days). On day 21, the tail fin was amputated in all the groups. Preliminary study conducted by us showed no significant change between control and bromelain alone administered group, so bromelain alone group was not included in the study.

Induction of diabetes mellitus

Fishes were anesthetised for giving intraperitoneal dosage of STZ. According to Institutional Animal Care and use committee, University of Georgia guidelines, 0.4% Tricaine solution was used to anesthetise the zebrafish. Tricaine solution was prepared by adding 4.2 mL of Tricaine stock solution to 100 mL of fish tank water. Tricaine stock preparation: To prepare 50 mL of stock solution, add 200 mg of Tricaine powder and 2 mL of 1M Tris base (pH 9) to 48 mL of d.H_2O. Adjust to pH 7.

An adult zebrafish model of diabetes mellitus was developed using the diabetogenic drug, STZ. According to Intine *et al.*,[14] 'hyperglycaemia was induced and sustained in the zebrafish by intraperitoneal injection of 0.3% STZ solution on day 1, 3, 5, 12, 19, 26 and 33. 0.3% of STZ solution was prepared by dissolving 6 mg of STZ in 2 mL of 0.09% Sodium

chloride. STZ was delivered in accordance with the body weight; intraperitoneally using super-fine needle at a dosage of 350 mg/kg body weight from the 0.3% STZ solution'. The same procedure is followed in our study to induce diabetes mellitus. According to this procedure, hyperglycaemia is detected within 24 h of the first injection, but to induce chronic hyperglycaemia, the zebrafish were administered with STZ on alternative days for the 1st week followed by weekly maintenance dosage.

To induce chronic hyperglycaemia, zebrafish were treated with STZ for 3 weeks. On day 21, blood glucose estimation was done to confirm the induction of diabetes mellitus, and then, the caudal fin was amputated. Till the completion of experiments, zebrafish were maintained in the hyperglycaemic state by giving weekly maintenance doses of STZ.[14] Once a week throughout the completion of experiment, body weight and blood glucose were monitored.

Drug treatment

Bromelain was administered orally for 15 days (every alternative day) at a dosage of 40 mg/kg b.w. after the caudal fin amputation. Bromelain was delivered to each fish using oral gavage – 22G needle with 22G flexible tubing so that bromelain is delivered directly into the gut of zebrafish. Fishes were made vertically immobilised in a damp sponge with the gills exposed. Then, using the gavage apparatus, bromelain was delivered to the vertically positioned zebrafish.

Euthanisation of zebrafish

On 36th day, zebrafish were euthanised by rapid chilling in ice cold water (2–4°C) for 10 min until the fishes become immobilised.

Determination of fasting blood glucose

After euthanisation on the 36th day, the fish was placed on a tissue paper to absorb any excess water, and then, it was placed on a Petri dish. Using a scalpel a sharp cut was made at the base of the operculum, the blood that is released from the fish was collected and kept on glucometer strip to get the blood glucose values. Glucometer kit was used to analyse the blood glucose concentration.

Caudal fin amputation

After the confirmation of diabetes mellitus in the afore-mentioned groups, caudal fin was amputated to analyse the regenerative changes. Caudal fin was amputated in a straight line using a sterile scalpel proximal to the first lepidotrichia branching point. Fishes were allowed to recover and, then, shifted to their respective tanks. Fishes were routinely examined for the regenerative growth at 24, 48 and 72 h post amputation.

Analysis of caudal fin regeneration

Photos of caudal fin were taken before amputation, immediately after amputation and after 15 days of amputation, because the complete regeneration of caudal fin occurs within 15 days in a normal adult zebrafish. These photos were analysed for area of caudal fin regeneration and percentage of growth using image J software.

RESULTS

Statistical analysis was done using SPSS 20.00. Data were analysed using one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant. Data were represented as bar charts.

Blood glucose analysis

A significant increase in the blood glucose level was observed in diabetes mellitus group when compared with the control group. Bromelain treatment has markedly decreased the blood glucose level compared to diabetes mellitus group, but it remained higher than the control [Figure 1].

Body weight analysis

No significant change in the body weight was observed in all the groups studied [Figure 1b].

Area of caudal fin regeneration

In caudal fin regeneration, diabetes mellitus-induced fishes showed a significant decrease in area of tail regeneration compared with control. Bromelain administration has significantly increased the tail regeneration back to the control level [Figures 2 and 1c].

Area of percentage of fin growth

In the area of percentage of fin growth, diabetes mellitus-induced fishes showed a significant decrease in the area percentage of fin growth compared with control. Bromelain administration has significantly increased the percentage of fin growth to the control level [Figure 2].

DISCUSSION

Considerably, plant proteases have received increased attention from medicinal areas due to their remarkable properties. One of the most recognised plant proteases with superior value is Bromelain from pineapple (*Ananascomosus*).[15] Bromelain is a combination of cysteine proteases containing dissimilar thiolendopeptidases and other components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates and

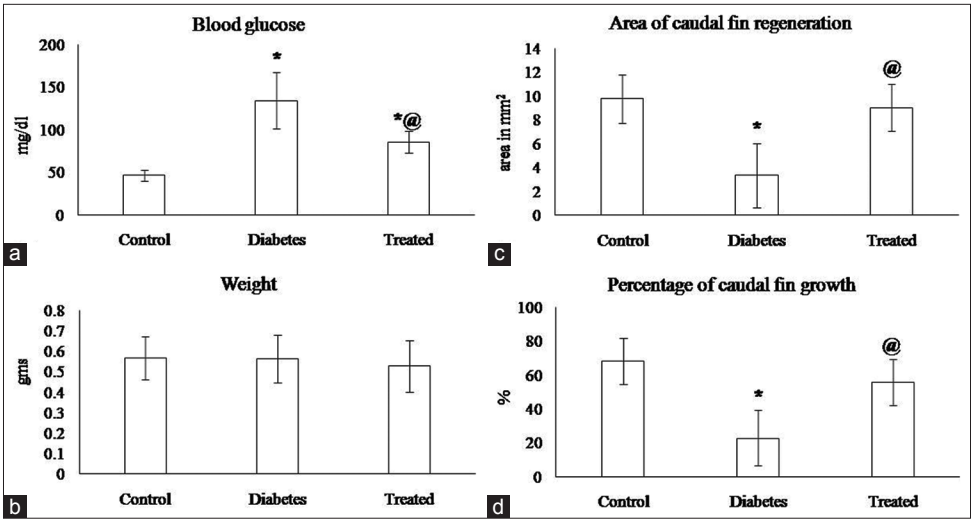

Figure 1: *Indicates significance compared with control; @ indicates significance compared with diabetes mellitus group. $P < 0.05$ is considered significant. (a) Blood glucose; (b) body weight; (c) area of caudal fin regeneration; and (d) percentage of caudal fin growth.

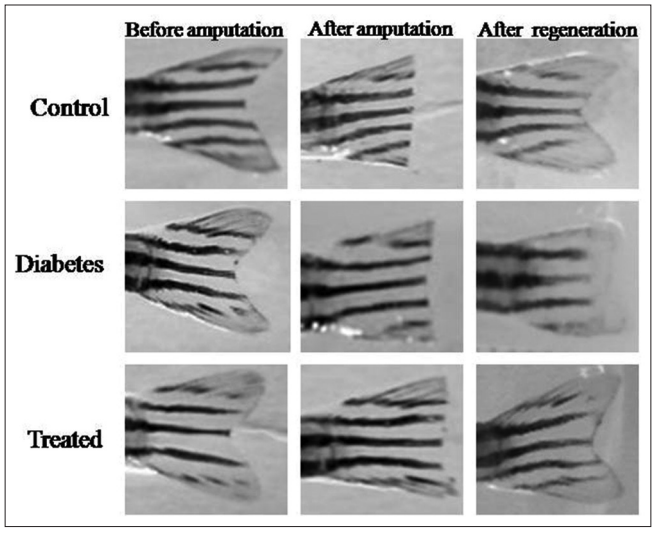

Figure 2: Caudal fin of zebra fish before amputation, after amputation and after regeneration.

several protease inhibitors.[16] Bromelain has been used as a drug orally for the systemic treatment of inflammatory, blood-coagulation-related and malignant diseases. Since the 1980s, bromelain has been widely used in the debridement of burns.[17-19] Toxic and other side effects of bromelain were rarely reported. Bromelain has been shown to get absorbed in human intestines without degradation and without losing its biological activity.[20] In the present study, we measured the area of tail regeneration, percentage of tail growth, body weight and blood glucose level in STZ-induced diabetic zebrafish and bromelain-treated zebrafish.

Sufficient perfusion of tissue is essential for wound healing and regeneration. Blood perfusion provides the oxygen and nutrition that cells require for tissue repair and removing metabolic products.[21] In a healthy individual when there is a cut in the skin, the angiogenic switch is on and it leads to increased production of proangiogenic factors.[22] These proangiogenic factors stimulate angiogenesis and improve blood supply to the healing wound which is necessary for immune response, proper oxygen and nutrient supply, fibroblast proliferation and collagen formation.[23]

In the present study, decrease in the area of tail regeneration and percentage of tail growth was observed in diabetic fishes. This decrease in the regeneration might be due to neuropathy the most common clinical manifestation seen in diabetes mellitus. Neuropathy when coupled with an impaired ability to fight infection, suffer from inadequate inflammatory response.[24] The decreased regeneration seen in diabetes may also be due to insufficient angiogenesis, decreased vascularity and capillary density which could be improved by bromelain. The enhanced angiogenesis in bromelain-treated zebrafish might be due to the escharase present in bromelain, which has the potential to accelerate the wound healing. Furthermore, bromelain is shown to decrease the interleukin (IL)1β, IL6 and tumour necrosis factor (TNF)-α[25] and increase the secretion of TGF-β.[26,27] The combined effects of these two could have been the reason for enhanced wound healing and hence the tail fin regeneration in zebrafish.

CONCLUSION

Our study has shown that there is impaired caudal fin regeneration in the diabetes mellitus group. Whereas when these diabetic zebrafish were treated with bromelain, they showed enhanced caudal fin regeneration compared with non-treated diabetic fishes. This study presented evidence

that bromelain is a novel antidote which enhances tissue regeneration and wound healing in diabetes mellitus. The decreased blood glucose level observed in the diabetic fish after bromelain administration needs further investigation. Hence, it is clear from the present study that bromelain improves caudal fin regeneration which is crucial for tissue regeneration and proper wound healing in diabetes mellitus.

Acknowledgment

We thank University of Madras for providing us with research facilities and financial support.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

Financial support and sponsorship

University of Madras.

Conflicts of interest

This paper was presented at APPICON 2018 and the abstract was published in conference proceedings.

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How to cite this article: Parasuraman R, Govindarajulu SN. Enhancement of caudal fin regeneration in diabetic zebrafish: An insight into proangiogenic potential of bromelain. Indian J Physiol Pharmacol 2023;67:3-7.