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***Vinca rosea* Normalizes Oxidative Stress and Inhibits Hyperglycemia Induced Increase in VEGF in Zebrafish Retina.**

Jayshree Nellore *, Cynthia Pauline P, Surya Prabha Mohanan, Ramya Ravikumar, and Ragavi Chidambarathanu Pillai

Department of Biotechnology, Sathyabama University, Chennai, Tamilnadu, India

ABSTRACT

This study evaluated the effect of ethanolic extracts of *vinca rosea* leaves (VRE) on hyperglycemia induced increase in VEGF by normalizing oxidative stress in adult zebra fish retina. Hyperglycaemia was accomplished by alternately immersing in glucose solution or water in zebrafish model of diabetic retinopathy. A group of fishes received normal powdered diet or powdered diet supplemented with 0.1% VRE soon after induction of hyperglycaemia. Age-matched normal fishes served as control subjects. At 28 days of hyperglycaemia, oxidative stress and vascular endothelial cell growth factor (VEGF) were quantified in the retina. The levels of lipid peroxide, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and reduced glutathione (GSH) were similar in the retinas of VGE-treated hyperglycaemic zebrafish and normal control fishes, and these values were significantly different from those obtained from hyperglycaemic fishes without any supplementation. In the same fish, VGE also prevented hyperglycaemic -induced increases in retinal VEGF mRNA expression. These findings indicate that ethanolic extracts of *vinca rosea* leaves effectively reversed the induction of VEGF mRNA expression by high glucose via ameliorating oxidative stress. *Vinca rosea* ethanolic extract can potentially be an effective antioxidant therapy for the treatment of diabetic vascular endothelial death.

Keywords: Hyperglycemia, Diabetes, *vinca rosea*, Zebrafish, retinas, retinal oxidative damage

**Corresponding author*

INTRODUCTION

Diabetic retinopathy (DR) is one of the micro-vascular complications of diabetes which leads to blindness, is still an important research issue. It has been reported that high glucose treatment (hyperglycemia) stimulated endothelial cell death in zebrafish [1], rats [2] and patients [3].

The development of diabetic chronic vascular disease is predisposed by multiple factors. Clinic studies support the important role of increased oxidative stress as a mechanism of the endothelial dysfunction in diabetes mellitus [4]. Further findings strengthen the hypothesis that an increased presence of oxidative species contributes to membrane lipid peroxidation, oxidative damage to DNA (indicated by 8-hydroxy-2'-deoxyguanosine, 8-OHdG) and compromised activities of antioxidant defense enzymes responsible for scavenging free radicals and maintaining redox homeostasis such as SOD, glutathione reductase, glutathione peroxidase, and catalase stimulate the endothelial cell death in retinas of diabetic patients.

Vascular endothelial growth factor (VEGF) is one of biomarkers for the progression of vascular diseases [5]. It will increase vascular permeability and promote angiogenesis [6] and this increase is associated with the manifestation of diabetic retinopathy. Clinical studies have shown increased levels of VEGF in patients with proliferative diabetic vascular disease and in children with microvascular complications and type I diabetes in comparison to the healthy controls [7]. The induction of VEGF by hyperglycemia can be reversed by overexpression of Mn-SOD in diabetic mice [8]. Studies have also shown that oxidative stress is required for the stimulation of VEGF expression [9]. Therefore an approach that can reverse the induction of VEGF by hyperglycemia via reduction of oxidative stress may potentially benefit the outcome of diabetic chronic vascular disease.

Due to this multifactorial nature the treatment of this complex disease is difficult and limited treatment options are available. The surgical procedures-laser photocoagulation and vitrectomy are currently the primary treatment options available for diabetic retinopathy. Furthermore, the present demand to use natural products in the treatment of diabetes and its complications including retinopathy is increasing worldwide and this is largely due to the reason that the herbal medicines and nutraceuticals possess multi-target, multi-channel and synergistic properties in their mechanisms of action due to which they may be beneficial in dealing with diabetes itself, as well as its complications, due to the fact that various mechanisms are involved in diabetic vascular complications [10].

Vinca rosea (*C. roseus*) Linn. (Apocynaceae) is an herbaceous subshrub also known as Madagascar periwinkle, *Vinca rosea*, or *Lchnera rosea* worldwide. The leaves are used traditionally in various regions of the world including India, West Indies as well as Nigeria to control diabetes [11]. The leaves have been known to contain 150 useful alkaloids among other pharmacologically active compounds. Significant antihyperglycemic and hypotensive activity of the leaf extracts (hydroalcoholic or dichloromethane-methanol) have been reported in laboratory animals [12]. Fresh leaf juice of *Vinca rosea* has been reported to reduce blood

glucose in normal and alloxan diabetic rats [13]. However, further studies are required to generate a matrix of scientific evidence at the pre-clinical levels, including chemical, cellular and animal studies, in order to develop effective medicines for the prevention and treatment of diabetic complications.

The existence of an experimental animal model of a disease helps not only in the understanding of the pathophysiology of such disease, but also in the development of drugs for its treatment. The retinal vasculature of adult zebrafish shares some similarities with that of humans. For example, mural cell coating, endothelial cell junctions, and basement membrane composition are similar in humans and zebrafish [14]. The retinas of hyperglycaemic zebrafish recapitulate the morphological and/or functional changes that are associated with early-stage human DR. Thus, a retinal neovascularization disease model in adult zebrafish would be invaluable for defining novel therapeutic targets and drug validation.

Here we present data demonstrating the utility of the zebrafish model in empirically assessing the therapeutic potential of by testing *Vinca rosea* extract on hyperglycemia induced increase in VEGF by normalizing oxidative stress in adult zebra fish retina.

MATERIALS AND METHODS

Chemicals and Plants:

Ethanol, tricine, glucose, Paraformaldehyde, Phenyl methyl sulfonyl fluoride, 5,5'-dithio-bis (2 nitro benzoic acid) , Pyrogallol, 1-chloro-2,4-dinitrobenzene (CDNB) substrate, Reduced glutathione and Trizol Reagent was purchased from Sigma Corporation, India. *Vinca rosea* leaves were procured from, V. Chelladurai, 476F, first south street, T.Nagar, Tirunelveli-11, Tamil nadu, India.

Preparation of ethanolic extracts of *vinca rosea* leaves (VRE):

The *Vinca rosea* leaves (100g) were cleaned with water following which the leaves were ground into solution using an electric blender and successfully extracted with 200 ml of ethanol (80%). 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.

Selection of animals:

18-month-old adult zebra fishes were maintained according to standard procedures on a 14-hour light/10-hour dark cycle at 28°C [15] essentially following the protocol of Gleeson *et. al* [1] . Animals were acclimated for at least 2 weeks to acclimate before the experiments. The procedures were approved by the Institutional animal ethics committee.

Induction of Hyperglycaemia in Zebrafish

The zebrafish were “hyperglycemic” – for 28 days prior to their sacrifice, the zebrafish were alternated every 24 hours between a water environment and a 2% glucose environment [1]. The water in the control group was also changed every 24 hours. Every week, 2-3 fish from each group were anesthetized in 0.02% tricaine solution and decapitated using a sharp blade behind the eyes, where the heart is located. After decapitation, blood was collected on a strip directly from the punctured heart, and the blood glucose level was measured using glucometer strip (Accu-Chek Aviva Test strip).

Dosage optimization of ethanolic extracts of *vinca rosea* leaves (VRE) and Experimental Design:

Six groups of 20 fishes per 2-litre tank were subjected to 28 days treatment as follows:

Group I: Normal control (water).

Group II: 24-hour treatment with 2% glucose (~110 mM), followed by 24 hours in freshly prepared system water.

Group III: Alternated every 24 hours between a 2% glucose environment and 0.02% *Vinca rosea*.

Group IV: Alternated every 24 hours between a 2% glucose environment and 0.1% *Vinca rosea*

Group V: Alternated every 24 hours between a 2% glucose environment and 0.15% *Vinca rosea*

Group VI: *Vinca rosea* (0.15%) in water

Every week, 2-3 fish from each group were anesthetized in 0.02% tricaine solution and sacrificed, and blood glucose values were measured as described in the above method. Based on the assessment made for 28 days of hyperglycaemia the Effective inhibitor dosage 0.1% of VRE which reduced the blood glucose level significantly in comparison to other dosages was fixed as the optimum dosage, with which the further studies were carried out. Following the treatment the fishes were dissected and the retina was prepared for further studies.

Preparation of retina

Following the treatment the fishes were killed, followed by decapitation. Fish heads were immersed immediately in 4% PFA, incubated at 4°C overnight and eyes were enucleated. Retinas were isolated from the other tissues and flat-mounted onto glass slides as described previously [16].

Preparation of retinal homogenates:

The retina was weighed and homogenised using ice cold homogenising buffer made of 20mM Tris-HCl pH 7.4, 10mM MgCl₂, 0.6mM CaCl₂, 0.5mM EDTA, 0.005% TritonX 100 and 1mM phenyl methyl sulfonyl fluoride (2ml/100mg) by sonication. The homogenate was then centrifuged at 3000rpm for 45minutes in the cooling centrifuge. Similarly the body of the fishes

were also homogenised and the supernatant was collected. The supernatant was stored at -20°C and used for further investigations.

Estimation of Lipid peroxidation and Antioxidant enzymes (SOD, Catalase, GSH and GPx) activities in retina

The retinal supernatants were then subjected to the measurement of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) by spectrophotometric methods. The Thiobarbituric Acid Reactive Substance (TBARS) was measured to analyse the MDA levels [17]. GSH content was measured according to the method of Moron *et al* (1979) [18] based on the reacting with 5, 5'-dithio-bis (2 nitro benzoic acid) (DTNB or Ellman's reagent) to give a yellow colour compound that absorbs at 412 nm. Activity of SOD was assayed by monitoring the inhibition of the reduction of nitro blue tetrazolium by the sample at 560nm [19]. Catalase was analysed as the rate of decrease in absorbance of H₂O₂ at 240 nm/min/mg protein [20]. Glutathione peroxidase was detected with 5-5'-dithiobis-p-nitrobenzoic acid [21].

Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted from fresh-frozen retina using the Trizol Reagent (USA Invitrogen). cDNA was synthesized with oligo-dT by use of reverse transcriptase (Super Script II) at 42°C for 1 hour. cDNA (0.5 µg) was subjected to VEGF PCR with 50 fmol of primers. VEGF: sense: 5'-GTGGACATCTTCCAGGAGGAGTA-3'; antisense: 5'-CTCTGAACAAGGCTCACAGT-3'. Cycling parameters were as follows: 30 s for annealing at 56°C, PCR amplification for 30 cycles. Eight microliter of the PCR products was analyzed by electrophoresis on a 1.5% agarose gel.

RESULTS AND DISCUSSION

Induction of Hyperglycaemia in Zebrafish and evaluation of ethanolic extracts of *vinca rosea* leaves (VRE):

The average glucose levels measured every week in zebrafish exposed to 2% glucose over a period of 28 days was 397.5 mg/dl. The blood glucose levels after 7 days treatment alternatively every 24 hours between a water environment and a 2% glucose environment was 420 mg/dl, 375 mg/dl after 14 days, 395 mg/dl after 21 days and 400 mg/dl after 28 days respectively as shown in figure 1. With reference to Gleeson *et al*. [1] we define hyperglycaemia as the values above the resting blood glucose levels i.e., 200mg/dl thus our data demonstrate that hyperglycaemia was induced and maintained in zebrafish.

The zebrafish administered with alternate treatment every 24 hours between a 2% glucose environment and various concentrations of *Vinca rosea* (0.02%, 0.1% and 0.15%) resulted in a significant reduction in blood glucose levels in a dose dependent manner. The reduction of blood glucose levels of zebrafish treated with 0.02% of VRE was 305 mg/dl, 0.1%

was 198 mg/dl and 0.15% was 190 mg/dl respectively. Based on the assessment made for 28 days of hyperglycaemia the effective inhibitor dosage was 0.1% of VRE which reduced the blood glucose level significantly in comparison to other dosages. Amilcar Arenal et al [22] were also able to reduce blood glucose levels of hyperglycemic tilapia by administering aqueous leaf extract of *O. tenuiflorum*. Mohammed Fazil Ahmed et al demonstrated anti-hyperglycemic activity of Alcoholic extracts of *Vinca rosea* in alloxan-induced hyperglycaemic rats [13].

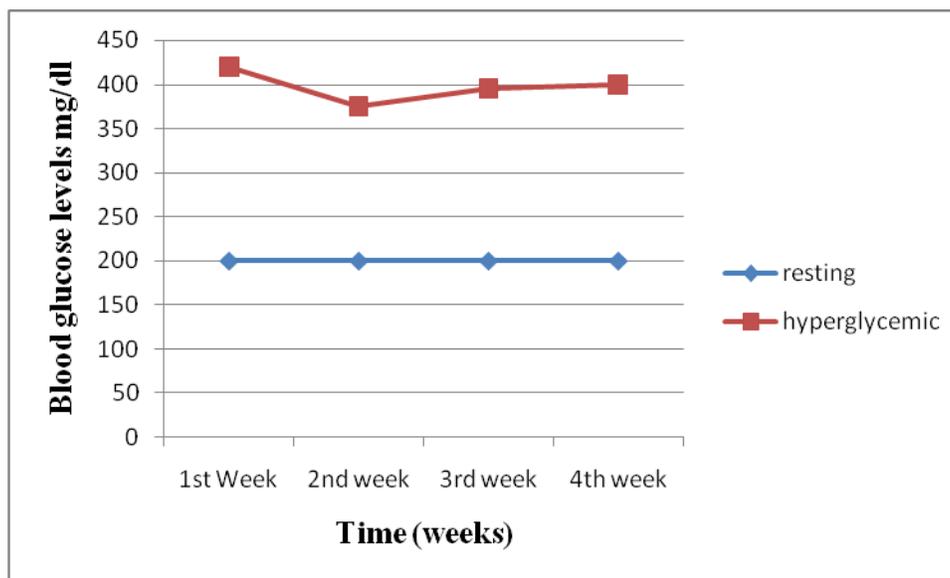


Figure 1: Blood glucose levels measured every week in zebrafish exposed to 2% glucose over a period of 28 days

Effects of VRE on MDA levels, Enzymatic and Non-enzymatic antioxidant enzyme activity in hyperglycaemic zebrafish retina.

Previously chronic hyperglycaemia resulted in significant thinning of the inner plexiform layer (IPL) and inner nuclear layer (INL) in zebrafish retina, consistent with onset of diabetic retinopathy [1]. Various studies support the important role of increased oxidative stress as a mechanism of the endothelial dysfunction in diabetes mellitus. Hence in the present study MDA, the end-product of oxidative stress-induced lipid peroxidation was employed to measure the oxidative stress induced damage. As shown in Figure 2A after 28 days the “hyperglycemic” zebrafish retina demonstrated significant increase in the levels of lipid peroxides [MDA levels], while those of hyperglycaemic+0.1% VRE group fishes were lower than those of hyperglycaemic group fishes, with no significant differences compared with those of control group rats. This may be attributed to the better radical scavenging activity of *vinca rosea* as reported previously by Jayakumar et al [23].

In hyperglycemic zebrafish, the activities of antioxidant defense enzymes responsible for scavenging free radicals and maintaining redox homeostasis such as SOD, glutathione reductase, glutathione peroxidase, and catalase are diminished in the retina as shown Figure 1 B, C, and D respectively). However 0.1% VRE could restore the activities of SOD, CAT, and GPx (Figure 1 B,C and D respectively) and thus attenuates lipid peroxidation in retina as *vinca rosea*

has been reported to be rich in alkaloids and terpenoids, well-known antioxidants [24], which scavenge the free radicals generated during hyperglycemia. The increase in SOD activity may protect CAT and GPx against inactivation by O^{2-} anions as these anions have been shown to inactivate CAT and GPx [25].

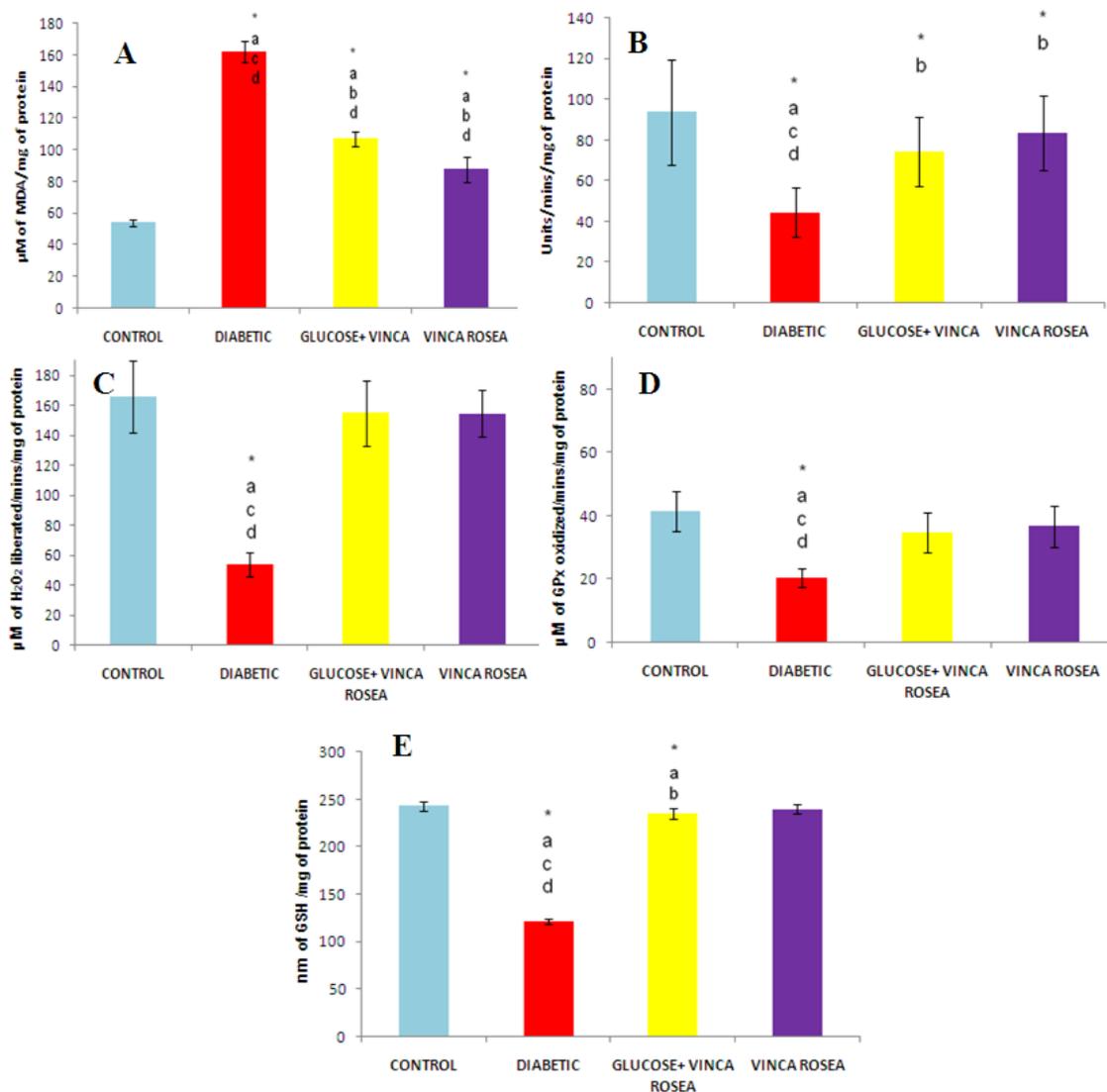


Figure 2: Effect of 0.1% VRE on the levels of A) lipid peroxide (LPO), B) superoxide dismutase (SOD), C) catalase (CAT), D) glutathione peroxidase (GPx) and E) reduced glutathione (GSH) . Data represents the mean of 6 repeats for each treatment (Mean ± SD, * p< 0.05, compared to control).

Further, the levels of the intracellular antioxidant, GSH; are decreased in the hyperglycemic zebrafish retina (Figure 1E). The decrease in GSH levels represents increased utilization due to oxidative stress [26]. The depletion of GSH content may also lower the GPx activity [27]. GPx has been shown to be an important adaptive response to condition of increased peroxidative stress. Zebrafish treated with VRE increased the GSH content in the

retina which may be the responsible aspect for inhibition of lipid peroxidation. The increased levels of GSH rescue the cellular proteins against oxidation through glutathione redox cycle and also directly detoxify ROS generated due to hyperglycemia. The noteworthy raise in GSH content and GPx in hyperglycemic zebra fish treated with VRE indicates an adaptive mechanism of retina in response to oxidative stress.

Prevention of hyperglycemia induced VEGF expression in zebrafish retinas

The retina responds to hyperglycemic by means of a number of biochemical changes. Dysregulated retinal VEGF production during diabetic retinopathy is among the most devastating responses to oxidative stress [28]. Agents that antagonize VEGF via reduction of oxidative stress may potentially benefit the outcome of diabetic chronic vascular disease. As demonstrated in Figure 2A-E, 0.1% of ethanolic extract of *vinca rosea* significantly attenuated hyperglycemia induced MDA, the end-product of oxidative stress-induced lipid peroxidation. This finding paved us to further explore the effect of 0.1% VRE on VEGF expression in hyperglycemic zebrafish retinas.

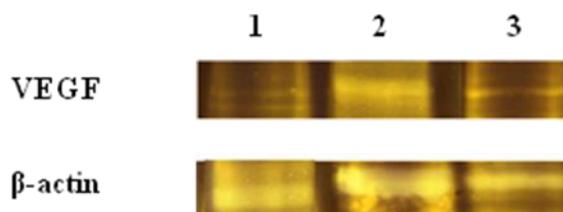


Figure 3 Expression of VEGF, and β -actin genes 1) Control, 2) glucose, or 3) glucose with *vinca rosea* extract

Vinca rosea has been studied for its wide-ranging effects on angiogenesis [29], apoptosis and signal transduction pathways [30]. *Vinca rosea* is known to exert its effects through modulation of gene expression [30] and thus we investigated its effect on the expression of VEGF. The RT-PCR-amplified products were showed in Figure. 3. These data were quantified by densitometry and normalized to β -actin. As shown in Figure 3 we found that VEGF mRNA expression increased significantly in hyperglycemic zebrafish retina, but decreased after 0.1% VRE intervention, which significantly decreased the expression of VEGF mRNA ; this finding is consistent with those of Fang Chang et al [31].

CONCLUSION

The present study clearly demonstrates that both of controlling of hyperglycaemia and catalytic scavenging of reactive oxygen species are effective approaches for the prevention of diabetic retinopathy. Ethanolic extracts of *vinca rosea* leaves supplementation has beneficial effects, based on its ability to reverse the induction of VEGF by hyperglycemia via reduction of oxidative stress, this concept appears attractive for the prevention or delay of diabetic chronic vascular disease. Further investigation is necessary to determine the exact phytoconstituents (s) responsible for this rescue effect on hyperglycemic induced diabetic retinopathy. The

zebrafish an animal model to study diabetic retinopathy may help to accelerate these studies and test therapeutic potentials of plant extracts.

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