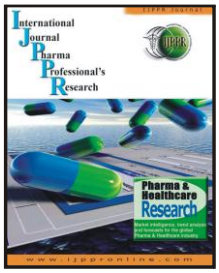
Volume1, Issue2, October 2010
Available Online at www.ijppronline.com
International Journal Of Pharma Professional's Research
Research Article



APOCYNIN SUPPLEMENTATION: COMPARISON OF THERMAL PERCEPTION AND OXIDATIVE STRESS IN DIABETIC INDUCED NEUROPATHY

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Abstract
The present study was conducted to investigate the role of Apocynin in prevention of streptozotocin induced diabetic neuropathy in wistar rats. Thirty male wistar rats (220-250g) were randomly allocated to five groups (6 rats per group). The rats were housed in individual cages in an environmentally controlled room. After one week acclimatization rats were assigned to non-diabetic control, diabetic control, two diabetic groups supplemented with Apocynin (15 and 100 mg/kg/day, *per os*) and a non diabetic control group supplemented with Apocynin (100 mg/kg/day). The Apocynin was started at end of fourth week post STZ challenge and continued for next two weeks. The development of diabetic neuropathy was assessed behaviourally by estimating hyperalgesia and allodynia. The oxidative stress in the sciatic nerve was assessed by measuring lipid peroxidation, reduced glutathione and nitroblutetrazolium (NBT) reduction. The single administration of STZ resulted in enhanced oxidative stress in the sciatic nerve and consequently diabetic neuropathy, evidenced by hyperalgesia and allodynia. The treatment with Apocynin, partially but significantly prevented the development of diabetic neuropathy by lowering ($P<0.05$) the oxidative stress. Hence, it may be concluded that diabetes-induced neuropathy is associated with an elevated oxidative stress. Apocynin delays or prevents the development of diabetes-induced neuropathy possibly by preventing the activation of NADPH oxidase and protecting the diabetic sciatic nerve from oxidative insult.

Keywords: - Apocynin, Diabetic neuropathy, Nitroblutetrazolium(NBT), Streptozotocin(STZ), Hyperalgesia and Allodynia

Introduction
Diabetes mellitus, a metabolic disorder resulting from either depletion of insulin secretion or cellular resistance to insulin, leading to hyperglycaemia and abnormalities in carbohydrate, fat and protein metabolism. The resulting persistent hyperglycaemia often results in microvascular and macrovascular complications such as nephropathy, retinopathy and neuropathy [1]. Diabetic neuropathy is a multistage clinical disorder of altered neuronal structure and function such as distal axonopathy, primary demyelination, axoglial disjunction and wallerian degeneration [2]. The hallmarks of neuropathic pain are allodynia, (pain due to a stimulus which usually not provoke pain) and hyperalgesia (increased sensitivity to pain) [3, 4, 5]. *Hyperalgesia* is an increased sensitivity to pain, caused by damage to nociceptors or peripheral nerves The mechanisms underlying chronic diabetic pain are not fully understood and several theories have been proposed. The most widely accepted states that high intracellular glucose concentration in chronic diabetes mellitus activates sorbitol-aldose reductase pathway, hexosamine biosynthetic pathway and protein kinase C enzymes. The activation of these pathways plays crucial role in several signal transduction cascades leading to development of diabetic neuropathy [6]. The elevated neuronal sorbitol and protein kinase C activation leads to depletion of neural myoinositol and activation of NADPH oxidase [7,8,9]. High glucose concentration in chronic

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diabetes mellitus induces reactive oxygen species (ROS) production subsequent to NADPH oxidase activation and consequently damages structure and function of nerve [10,11].

Apocynin has been documented to suppress NADPH oxidase. Additionally, Apocynin has been demonstrated to enhance the activity of antioxidant factors (reduced glutathione), and protects the cellular membranes against lipid peroxidation [12,13]. Apocynin has been suggested to provide neuronal protection such as nerve conduction velocity and blood flow against oxidative stress by inhibits activity of NADPH oxidase [14]. Firstly, biochemical and molecular changes in sciatic nerve, and associated oxidative stress leading to development of neuropathy were investigated. Secondly the effect of apocynin on the response to various thermal stimuli and biochemical and molecular changes were studied.

Material and methods

Animals

The experiments were carried out in adult (5 months old) wistar rats (220-250 g) . Feed and water were provided *ad libitum.* They were acclimatized in animal house for one week and were exposed to normal cycle of light and dark. All the behaviour assessments were carried between 8:00 and 14:00 hr. All procedures were performed using age-matched animals in an attempt to avoid variability between experimental groups. At the termination of study, all the rats were euthanized using gas anaesthetic and samples were taken for biochemical analysis.

Experimental Protocol

Thirty rats were randomly assigned to five groups, 6 rats per group. Diabetes was induced in group II, III and IV by single intraperitoneal injection of streptozotocin (STZ: 50 mg/kg) (Sigma-Aldrich Corporation, USA). Group I (Normal control) and Group II (Diabetic control) rats were maintained on standard food and water and no treatment (Apocynin) was administered. After 28 days of STZ administration in group III (Low dose Apocynin treated diabetic group) and group IV (high dose apocynin treated diabetic group) rats were treated orally for 14 days with 15mg/kg and 100mg/kg apocynin (Sigma-Aldrich Corporation, USA) respectively. In group V (Drug per se) the rats were treated like control group for four 28 days (i.e. normal rats with no STZ injection) and were administered apocynin (100mg/kg) for 14 days.

Assessment of induced diabetes

Estimation of serum glucose

The blood glucose was monitored once daily for first week and thereafter once weekly during the course of study in all rats. The serum glucose concentration was estimated by glucose oxidase peroxidase (GOD-POD) method [15] using the commercially available kit (Crest Biosystems, Goa, India).

Assessment of Diabetic Neuropathy

The diabetes mellitus-induced neuropathy was assessed behaviourally by cold allodynia, tail-flick assay and paw withdrawal latency as followed.

Tail withdrawal latency by cold water immersion test

Cold allodynia was evaluated as described earlier [16]. Briefly, the tail withdrawal latency (seconds) was measured at a cold temperature (10±0.5°C) that is normally innocuous. Latency of tail withdrawal to cold stimulation was measured thrice, five minute apart in each rat. Tail withdrawal latency measurements were performed once weekly for 6 weeks.

Tail withdrawal latency by warm water immersion test

The mean withdrawal latency of the rat tail was measured using a method described earlier [17]. Briefly, tail withdrawal latency (seconds) was measured by dipping the tail in water bath maintained at 46 ± 0.5°C. The tail withdrawal latency was measured thrice, five minute apart. A cut-off time of 15 seconds was imposed to avoid injury to the tail. The change in the tail withdrawal latency compared to basal response in control group was calculated as a measure of hyperalgesia.

Assessment of Sciatic nerve oxidative stress.

The assessment of oxidative stress in the sciatic nerve was performed by estimating thiobarbituric acid reactive substances (TBARS), reduced form of glutathione content (GSH) and superoxide anion generation.

Estimation of thiobarbituric acid reactive substances (TBARS)

The sciatic nerve TBARS, an index of lipid peroxidation, was estimated according to the method described earlier [18]. Briefly, a portion of left sciatic nerve was dissected out from euthanized rats and washed with ice cold isotonic saline and weighed. The sciatic nerve was then minced, and a homogenate (10% w/v) was prepared in chilled phosphate buffer (pH 7.4). 100 µL of homogenate was mixed with 2 ml of (1:1:1 ratio) thiobarbituric, trichloroacetic and hydrochloric acid (TBA-TCA-HCL) reagent (TBA 0.37%, 0.25 N HCL and 15% TCA) and placed in water bath at 80°C for 30 min. This homogenate was subsequently cooled, centrifuged and absorbance of the supernatant was measured at 532 nm (UV-1700 Spectrophotometer, Shimadhu, Japan). Finally the values were expressed as nmol/mg protein.

Estimation of superoxide anion by NBT reduction

The superoxide anion in the sciatic nerve was measured using a method described earlier [19]. Briefly, a portion of left sciatic nerve was cut into 10 mm in length and placed in 5 ml of Krebs–Henseleit solution buffer containing 100 µM of nitroblutetrazolium (NBT) and incubated at 37 °C for 1.5 h. NBT reduction was stopped by adding 0.5 N HCl. The sciatic nerve was subsequently minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg/L diethylene triamine pentaacetic acid. The mixture was centrifuged at 20,000xg for 20 min and the resultant pellets were resuspended in 1.5 ml of pyridine and kept at 80°C for 1.5h to extract formazon. The mixture was centrifuged at 10,000xg for 10 min and the absorbance of formazon was determined spectrophotometrically at 540 nm (UV-1700 Spectrophotometer, Shimadhu, Japan).

Estimation of reduced glutathione

The reduced glutathione (GSH) level in the sciatic nerve was estimated by the method as described earlier [20]. Briefly, a portion of left sciatic nerve from each rat was weighed and homogenized in cold 10 % trichloroacetic acid (TCA) and centrifuged at 3000xg for 15 min. 100 µL of supernatant was mixed with 890 µL of 1 M Tris HCL and 0.02 M EDTA (pH 8.2). To this, 10 µl of DTNB 5,5'-dithiobis (2-nitrobenzoic acid) solution (99 mg in 25 ml of methanol) was added and the absorbance was measured spectrophotometrically at 412 nm (UV-1700 Spectrophotometer, Shimadhu, Japan). A standard curve was plotted using reduced form of glutathione and the results were expressed as nmol/mg of protein.[21]

Statistical Analysis:

The data for behaviour parameters, serum glucose, TBARS and GSH were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison test using sigmaplot and sigmastat programs (Jandel, San Rafael, CA, version 3.02). The p value <0.05 was considered to be statistically significant. All values were expressed as mean ± S.D.

Results

Effect of Acetyl-l-carnitine on body weigh

The diabetic rats had lower (P<0.05) body weight compared to non diabetic rats (Table 1). The body weight was not different (P<0.05) in diabetic groups supplemented with Apocynin at 15 and 100mg/kg.

Table 1. Initial and final body weight in non diabetic rats (normal control), diabetic control rats, non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

Groups	Initial body weight (gm)	Final body weight (gm)
Normal control	216.36 ±14.22	255.26 ± 13.10[b]
Diabetic control	225.73 ±13.55	189.73 ± 14.92[a]
Apocynin per se	229.56 ± 16.36	269.38 ± 12.45[b]
D + Apocynin (15mg/kg)	236.25± 13.98	181.27 ± 15.76[ab]
D + Apocynin (100mg/kg)	219.40 ±11.26	172.38 ± 17.23[b]

[a,b] Values within column with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Effect of STZ administration on serum glucose

Serum glucose concentration was significantly higher (P<0.05) in streptozocin induced diabetic rats compared with age matched normal rats in all groups irrespective of the treatment with Apocynin (Figure 1). Furthermore, no significant (P>0.05) effects were observed on serum glucose level in Apocynin supplemented groups (15 mg/kg and 100 mg/kg).

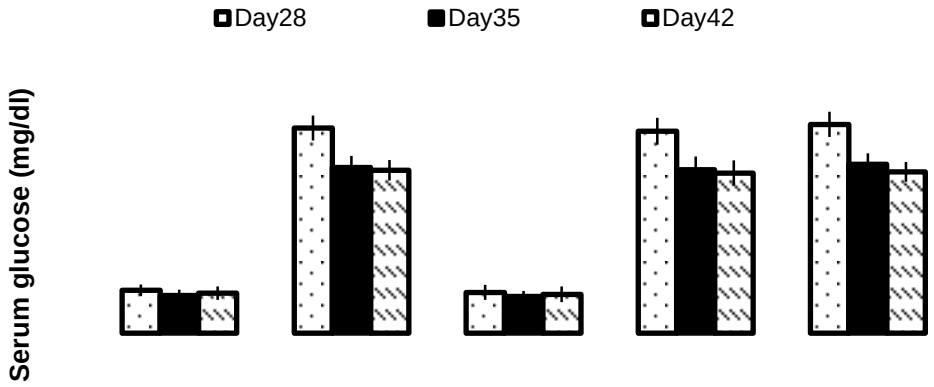

Figure 1. Serum glucose level (gm/dl) in non diabetic rats (normal control), diabetic control rats, non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

[a,b] Adjacent bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats)

Behavioural Parameters

Effect of Apocynin on tail withdrawal latency in response to cold stimuli

Cold water immersion test on day 28th revealed that there was significant reduction (P<0.05) in tail withdrawal latency in streptozocin induced diabetic rats compared with the non-diabetic control rats (Figure 2). On day 42th, diabetic rats supplemented with Apocynin at 100 mg/kg resulted in increased (P<0.05) tail withdrawal latency compared with diabetic control rats.

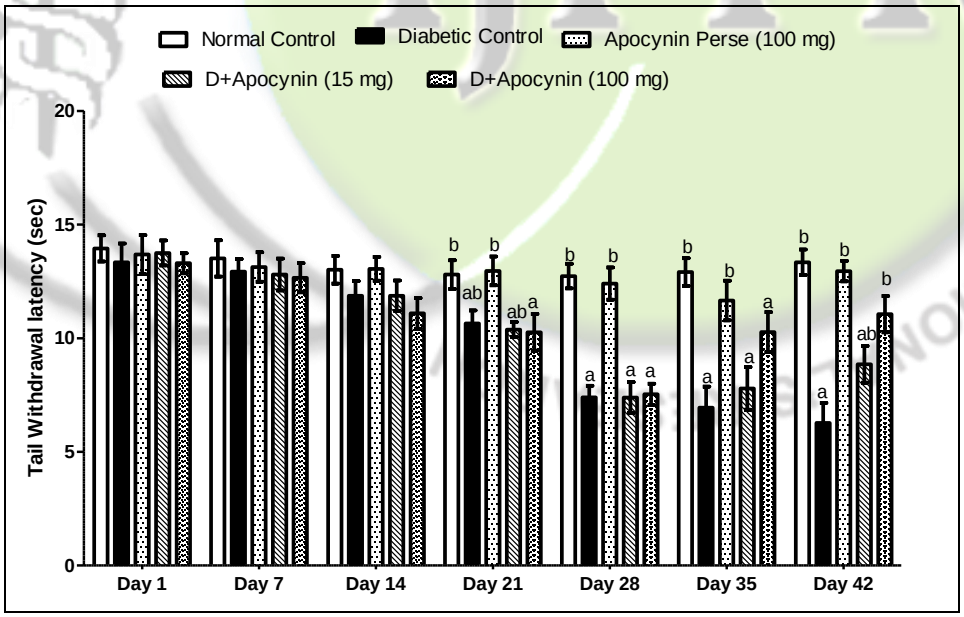

Figure 2. Tail withdrawal latency to cold stimuli (indicative of allodyania) in non diabetic control rats (NC), diabetic control rats (DC), non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin per se) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

a,b Adjacent bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Effect of Apocynin on tail withdrawal latency in response to hot stimuli

In hot water immersion test, on day 28th there was significant reduction (P<0.05) in tail withdrawal latency in streptozocin induced diabetic rats compared with the non-diabetic rats (Figure 3). On day 42th, diabetic rats supplemented with Apocynin at 15 and 100 mg/kg resulted in increased (P<0.05) tail withdrawal latency compared with diabetic control rats. Furthermore, there was no (P>0.05) difference in tail withdrawal latency of normal rats exposed to Apocynin at 100 mg/kg compared with non-diabetic control rats.

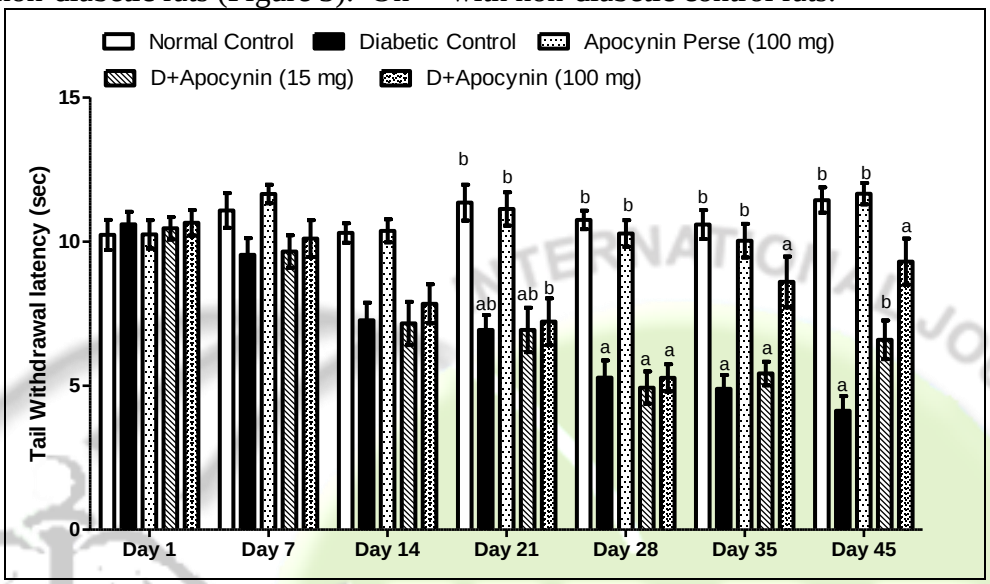

Figure 3. Tail withdrawal latency to hot stimuli (indicative of allodyania) in non diabetic control rats (NC), diabetic control rats (DC), non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

[a,b] Adjacent bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Assessment of Sciatic nerve oxidative stress

Effect of Apocynin on lipid peroxidation

Diabetic control rats had significantly higher (P<0.05) TBARS level in sciatic nerve compared to control rats. In this study, two weeks post treatment with Apocynin at 100 mg/kg dose in non-diabetic rats did not alter sciatic nerve TBARS level compared to non-diabetic control rats (Figure 4). However, administration of Apocynin at 15 and 100 mg/kg dose significantly reduced (P<0.05) TBARS level in sciatic nerve of diabetic rats compared to the levels observed in diabetic control rats.

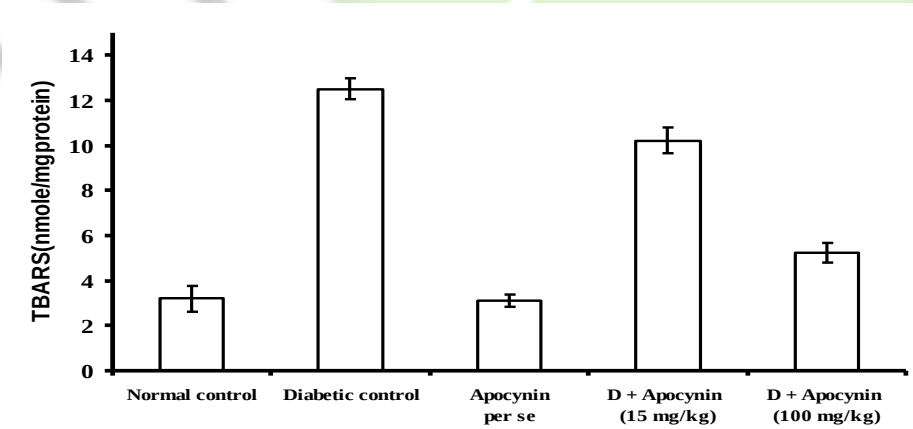

Figure 4. Thiobarbuturic acid reactive substance (TBARS) measured in sciatic nerves of non diabetic control rats (NC), diabetic control rats (DC), non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

[a,b,c] Bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Effect of acetyl-l-carnitine on super oxide anion

Streptozocin induced diabetic control rats showed higher (P<0.05) superoxide anion production compared to normal control rats as measured by reduced nitroblue tetrazolium (NBT) in sciatic nerve. Apocynin at 100 mg/kg dose in non-diabetic. rats did not alter reduced NBT level compared to control rats (Figure 5). However, administration of Apocynin at 15 mg/kg and 100 mg/kg significantly lowered (P<0.05) elevated reduced NBT in diabetic rats compared to the levels observed in diabetic control rats. There was significant decrease (P<0.05) in reduced NBT in Apocynin at 100 mg/kg dose compared with Apocynin at 15 mg/kg dose in diabetic rats

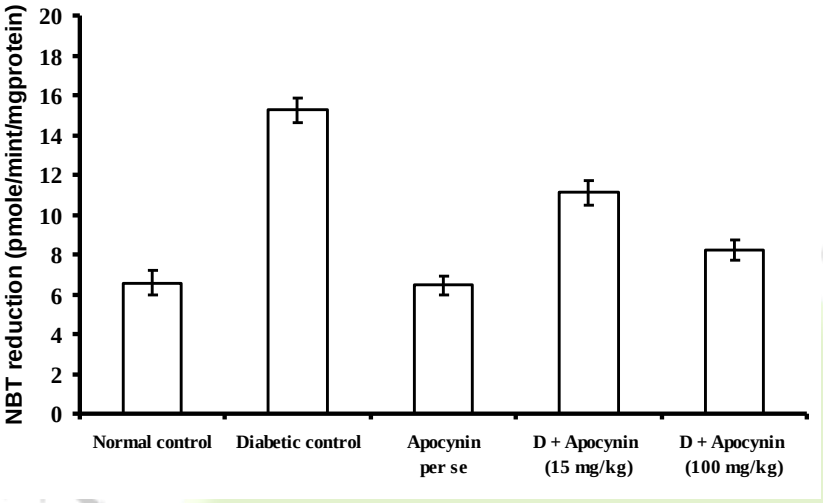

Figure 5. Reduced nitroblue tetrazolium (NBT), an indicator of superoxide generation in sciatic nerves of non diabetic control rats (NC), diabetic control rats (DC), non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

[a,b,c] Bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Effect of Apocynin on reduced glutathione

Diabetic control rats had significantly lower (P<0.05) reduced GSH level in sciatic nerve compared to non-diabetic rats (Figure 6). Apocynin (100 mg/kg) administration in non-diabetic rats did not alter reduced GSH level compared to non-diabetic control rats. On the other hand, administration of Apocynin (100 mg/kg) significantly improved (P<0.05) reduced GSH level of the sciatic nerve in diabetic rats compared to the levels observed in diabetic control rats.

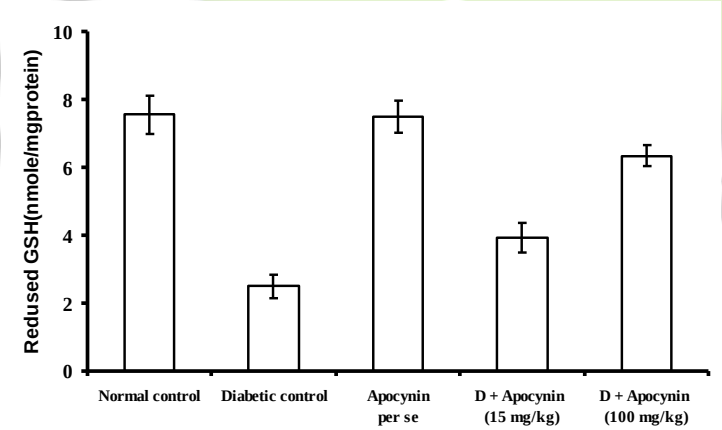

Figure 6. Reduced glutathione (GSH) levels in non diabetic control rats (NC), diabetic control rats (DC), non diabetic rats supplemented with Apocynin at 100mg/kg (Apocynin *per se*) and in diabetic rats supplemented with Apocynin (D + Apocynin) at 15 and 100mg/kg body weight.

[a,b] Bars with different superscripts are significantly different (P< 0.05). All Values are means ± SD (n=6 rats).

Discussion

The results obtained in the present study provide evidence that Apocynin administration in rats with streptozocin induced diabetic neuropathy results in lowered oxidative stress (elevated glutathione level, decrease in TBARS level and decrease in superoxide generation) and reduces sensitivity of nerve towards pain stimuli. Apocynin supress NADPH oxidase and reduces overall neuronal oxidative stress, a crucial step in pathogenesis of diabetic neuropathy.

The present study confirms previous reports demonstrating that Apocynin does not significantly affect blood glucose level and body weight in diabetic rats However, STZ induced hyperglycemia and resulting diabetic neuropathy have been documented in the literature. STZ induced diabetic neuropathy is associated with hyperalgesia and allodynia, increased sensitivity of nerves towards pain stimuli. In the present study, Apocynin improved these established symptoms of diabetic-induced neuropathy pain.

There is strong correlation between STZ induced hyperglycemia and oxidative stress in diabetic neuropathy. Hyperglycemia results in a series of events leading to lowered total antioxidant status and hence progressive functional and structural alterations of peripheral nerves in diabetes mellitus. In the present study, hyperglycemia induced oxidative stress was demonstrated by increased lipid peroxidation, increased generation of superoxide anions and reduced glutathione levels in diabetic neuropathy. The lowered reduced glutathione and increased lipid peroxidation and superoxide anions are considered as markers of oxidative stress . Apocynin has been demonstrated to enhance the activity of antioxidant factors and protect the cellular membranes against lipid peroxidation , a finding confirmed in the present study.

Lowered Na^+/K^+ ATPase activity is well documented in STZ induced neuropathy . This lowered Na^+/K^+ ATPase activity may be further complicated by lowered antioxidant level, leading to altered neuronal function as evidenced by tail flick response to warm and cold stimuli. Apocynin administration improves neuronal conduction function by either increasing Na^+/K^+ ATPase activity or/and by improving antioxidant level, resulting in improved nerve conduction function and hence lowered hyperalgesia and allodynia in neuropathic rats. On the basis of results obtained in present study, it may be concluded that Apocynin improves neuronal function by its direct effect on NADPH oxidase level and prevent reduction in total antioxidant status, reported to be major contributor for development of diabetic neuropathy. The demonstrable benefits of Apocynin supplementation in diabetic neuropathy rats suggests that clinical studies are required to investigate the therapeutic potential of Apocynin in diabetes patients.

Acknowledgement

Authors are thankful to Mr. G.S.Gil, the Chairman, Global Memorial College of Pharmacy, Ropar (Punjab) for invaluable support and encouragement. Authors also express their thankfulness to his guide Prof. R.D.budhiraja Director academics, ISF College of Pharmacy, Moga (Punjab).

Reference

1. Fowler, M.J., Microvascular and macrovascular complications of diabetes. Clin Diab, 216: 77–82,2008.
2. Yagihashi, S., Pathology and pathogenetic mechanisms of diabetic neuropathy. Diabetes Metab Rev, 11:193-225,1995.
3. Xie, W., Strong, J.A., Meij, J.T.A., Zhang, J., Yua, L., Neuropathic pain: Early spontaneous afferent activity is the trigger. Pain, 116:243-256, 2005.
4. Meeus, F.C., Nijs, J., Central sensitization: a biopsychosocial explanation for chronic widespread pain in patients with fibromyalagia and chronic fatigue syndrome. Clin. Rheumatol, 26:465-473,2007.
5. Suter, M.R., Wen, Y., Decosterd, R., Ji, R., Do glial cell control pain? Neuron Glia Biol, 3:255-268,2007.
6. Maria, S., Yavuz, D., Diabetic Neuropathy: Pathogenesis and Treatment. Journal of Reconstructive Microsurgery, 212:584-662,2004.
7. Koya, D., King, G.L., Protein kinase C activation and development of diabetic complications. Diabetes, 46(6):859-66,1998.
8. Burt, D.J., Gruden, G., Thomas, S.M., P 38 mitogen activated protein kinase mediates hexosamine induced TGA 1 m RNA expression in human mesangial cells. Diabetologia , 46:531-537,2003.
9. Brownlee, M., The pathpphysiology of diabetic complication: a unifying mechanism. Diabetes, 54: 1615-1625,2005.
10. Bayens, J.W., Thrope, S.R., Role of oxidative stress in diabetic complication a new prospective on an old paradigm. Diabetes, 48:1-9,1999.
11. Ceriello, A., Post prandial hyperglycemia and diabetes complications is it time to treat?.Diabetes, 54:1-7,2005.
12. Sheetz, M.J., King, G.L., Molecular understanding of hyperglycemia's adverse effects for diabetic complications. AMA, 49(5):232-243,2002.

13. Bril, V., Buchanan, R., The AS-3201 study group, sural nerve poloyl pathway inhibition by AS-3201,an aldose reductase inhibitor in patients with diabetic poly neuropathy Presented at International Poloyl Pathway conference, 13-17, Kona Hawai, 2004.

14. Cotter, M.A., Cameron, N.E., Effect of the NADPH oxidase inhibitor, Apocynin on peripheral nerve perfusion and function in diabetic rats. Life.Sci, 73(14): 1813-24,2003.

15. Courteix, C., Eschalier, A., Lavarenne, J., 1993. Streptozotocin –induced diabetic rats: behaviour evidence for a model of chronic pain. Pain 53, 81-88.

16. Attal, N., Jazat, F., Kayser, V., Guilbaud, G., 1990. Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy. Pain. 41, 235-251.

17. Yalcin, I., Charlet, A., Freund-Mercier, M.J., Barrot, M., Poisbeau, P., 2009. Differentiating thermal allodynia and hyperalgesia using dynamic hot and cold plate in rodents. J Pain. 10, 767-73.

18. Niehaus, W.G. Jr., Samuelsson, B., 1968. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem. 6, 126-30.

19. Lou, M.F., Dickerson, J.E.Jr., Garadi, R., York BM, J.r., 1988. Glutathione depletion in the lens of galactosemic and diabetic rats. Exp. Eye. Res. 46, 517-530.

20. Wang, H.D., Pagano, P.J, Antonio, J.D., Cayatte, M.T., Quinn, PB., Richard, A., 1998. Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. Circ. Res. 82, 810-818.

21. Teerlink, T., Hennekes, M., Bussemaker, J., Groeneveld, J., 1993. Simultaneous determination of creatine compounds and adenine nucleotides in myocardial tissue by high-performance liquid chromatography. Anal. Biochem. 214, 278–283.