AIMS AND OBJECTIVES

Hyperglycemia plays a key role in the development and progression of diabetic neuropathy as well as diabetes associated cognitive decline (Tomlinson and Gardiner, 2008; Piriz et al., 2009). Investigations into the molecular and biochemical pathophysiology of diabetic neuropathy and associated cognitive decline have focused on glucose metabolic pathways. Over the past 25 years, animal experiments and in-vitro studies have identified biochemical pathways likely to be important in the development of diabetic complications and have led to possible approaches of treatment. Pathways which are mainly driven by metabolism are: glucose flux through the polyol pathway; the hexosamine pathway; excess/inappropriate activation of protein kinase C isoforms and accumulation of advanced glycation end products. While each pathway may be injurious alone, collectively they cause an imbalance in the mitochondrial redox state of the cell and lead to excess formation of reactive oxygen species (Kong et al., 1999; Vinik et al., 2003; Figueroa-Romero et al., 2008). Increased oxidative stress within the cell leads to activation of the Poly (ADP-ribose) polymerase (PARP) pathway, which regulates the expression of genes involved in promoting inflammatory reactions and neuronal dysfunction. Thus, a consensus has evolved that oxidative-nitrosative stress and inflammatory cascade are the major culprits involved in the pathogenesis of diabetic neuropathy and associated cognitive decline.

The present research work was undertaken to explore whether the incidence and progression of these complications can be halted by using targeted approach against oxidative-nitrosative stress and inflammatory cascade. Tocotrienols, found in annatto sourced oil, rice bran oil and palm oil together with cereal grains, such as barley, oats, and rye; lycopene, a carotenoid mostly found in tomatoes and tomato products; and sesamol, the major constituent of sesame seed oil are known to possess strong antioxidant and anti-inflammatory activities. These molecules have been evaluated alone
Aims and Objectives

as well as in combination of insulin in experimental paradigms of diabetic neuropathy and associated cognitive decline. The present study is divided into following two chapters which are further divided into seven subparts:

CHAPTER 1: AMELIORATION OF DIABETIC NEUROPATHY: TARGETING OXIDATIVE-NITROSATIVE STRESS AND INFLAMMATORY CASCADE

CHAPTER 2: ROLE OF OXIDATIVE-NITROSATIVE STRESS AND INFLAMMATORY CASCADE IN THE DEVELOPMENT OF DIABETES ASSOCIATED COGNITIVE DECLINE
Pain is considered the third most common healthcare problem disabling more individuals than heart disease and cancer together (Zareba, 2009). Diabetic peripheral neuropathy is the most common complication of long-standing diabetes mellitus which frequently results in clinically significant morbidities e.g. pain, foot ulcers and amputations (Nicholson, 2006; Pop-Busui et al., 2006; Said, 2007). A large number of neuroanatomical, neurophysiologic and neurochemical mechanisms are thought to contribute to the development and maintenance of diabetic neuropathic pain (Gidal and Billington, 2006; Edwards et al., 2008). Hyperglycemia clearly plays a key role in the development and progression of diabetic neuropathy as well as other microvascular complications of diabetes. Understandably, then, investigations into the molecular and biochemical pathophysiology of diabetic neuropathy have focused on glucose metabolic pathways.

At present, the only agents approved for the treatment of painful diabetic neuropathy are lidocaine patches 5%, duloxetine, gabapentin, and pregabalin (Gidal and Billington, 2006; Mizoguchi et al., 2009). In the clinical setting, despite the use of these agents, the successful therapy of diabetic neuropathy remains a challenge. Due to pathogenic complexity of diabetic neuropathy, new therapeutic interventions targeting primary mechanisms contributing to nerve damage are critical for the future treatment of this complication.

Although pharmacological pain management offers a significant relief in several pain-related diseases, many patients turn to its supplementation with complementary and alternative medicine. Botanicals used in pain therapy
can contribute to restoring the quality of life of a patient and may enhance conventional pain management (Zareba, 2009). Phenolic compounds are widely present in plants and they have recently received considerable attention due to their antioxidant property. Since past decade, the scientific community has shown huge interest in the multiple activities of natural vitamin E i.e. tocotrienols, lycopene and sesamol.

Vitamin E is one of the most important phytonutrients in edible oils. It consists of eight naturally occurring isomers, a family of four tocopherols (alpha, beta, gamma and delta) and four tocotrienols (alpha, beta, gamma and delta) homologues (Sen et al., 2007). Tocotrienols possess powerful cardioprotective (Das et al., 2007, 2008), neuroprotective (Khanna et al., 2006; Shichiri et al., 2007), radioprotective (Ghosh et al., 2009), anti-angiogenic (Nakagawa et al., 2007; Shibata et al., 2009), potent natural super-antioxidant (Schroeder et al., 2006; Maniam et al., 2008; Matringe et al., 2008), anti-cancer (Nesaretnam, 2008; Wada, 2009), anti-inflammatory (Lee et al., 2009; Wu et al., 2009), cyclooxygenase-2 inhibitory (Yam et al., 2009), anti-nociceptive (Tiwari et al., 2009b), insulin sensitizing, hypoglycemic (Chen and Cheng, 2007; Budin et al., 2009), and cholesterol lowering (Chou et al., 2009) properties that often differ from the properties of tocopherols (Serbinova et al., 1991; Serbinova and Packer, 1994; Sen et al., 2007; Budin et al., 2009). The unsaturated side chain of tocotrienol allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver (Suzuki et al., 1993; Atkinson et al., 2008). Experimental research examining the antioxidant effects of tocopherol and tocotrienols has revealed that tocotrienols appear superior due to their better distribution in the fatty layers of the cell membrane (Suzuki et al., 1993; Kawakami et al., 2007; Tsuzuki et al., 2007; Maniam et al., 2008). No-observed-adverse-effect level (NOAEL) for tocotrienol was found to be 120 mg/kg body weight/day for male rats and 130 mg/kg body weight/day for female rats (Nakamura et al., 2001). It has been suggested that the safe dose of various tocotrienols for human consumption is 200-1000 mg/day (Sen et al., 2007).
Lycopene, a carotenoid, is mostly found in tomatoes and other red fruits and vegetables, such as red carrots, watermelons, pink grapefruit, pink guava, papayas and rosehip (but not strawberries or cherries). Lycopene is a powerful antioxidant with a singlet-oxygen-quenching capacity 47 and 100 times greater than that of β-carotene and vitamin E respectively (Di Mascio et al., 1989; Liu et al., 2005; Devaraj et al., 2008; Srinivasan et al., 2009; Zini et al., 2009). Lycopene is also a potent neuroprotective (Hsiao et al., 2004), cardioprotective (Riccioni et al., 2008), antiproliferative, anti-diabetic (Ali and Agha, 2009), anti-apoptotic (Markovitch et al., 2009), anticancer (Gunasekera et al. 2007; Seren et al., 2008; Tang et al., 2008), anti-inflammatory (Bignotto et al., 2009; Joo et al., 2009), anti-asthmatic (Wood et al., 2008), cognition enhancer (Akbaraly et al., 2007) and hypocholesterolemic agent (Fuhrman et al., 1997; Heber and Lu, 2002). Lycopene also modulates cyclo-oxygenase synthesis pathway (Heber and Go, 1999; Sengupta et al., 2006) and reduces mutagenesis in the Ames test (Heber and Lu, 2002; Matulka et al., 2004). Lycopene has been under considerable investigation for its anti-oxidant benefits in treating various chronic human diseases like cancer, cardiovascular diseases, osteoporosis, and diabetes (Rao et al., 2006; Rao and Rao, 2007; Riccioni et al., 2008; Seren et al., 2008; Tang et al., 2008; Ali and Agha, 2009).

Sesamol is the major constituent of sesame seed oil, which makes it more resistant to oxidative deterioration than other vegetable oils (Parihar et al., 2006). Sesamol is a powerful antioxidant (Chandrasekaran et al., 2009; Geetha et al., 2009) and inhibits UV- and Fe³⁺/ascorbate-induced lipid peroxidation in rat brain (Uchida et al., 1996; Prasada et al., 2005). Sesamol reduces ferric ions and its unique solubility in both aqueous and oily phases increases its local concentration in cell membranes and makes it a chain-breaking antioxidant (Uchida et al., 1996). Sesamol scavenges hydroxyl and lipid peroxyl radicals and reduces radiation-induced deoxyribose degradation (Joshi et al., 2005). It also inhibits the formation of single-strand DNA breaks by γ-radiation (Prasad et al., 2005). It has been shown that sesamol inhibits several steps in the generation of neoplasia and mutagenesis (Kapadia et al., 2007).
Sesamol has been shown to possess neuroprotective (Hou et al. 2006), hepatoprotective (Hsu et al. 2006a), reno-protective (Gupta et al., 2009; Kuhad et al., 2009), anti-inflammatory (Chavali et al. 2001; Hou et al. 2006), chemo-preventive (Prasad et al. 2005) and anti-ageing properties (Sharma and Kaur 2006). Recently, Sesamol has been shown to enhance antioxidant capacity of the diabetic brain and led to decreased perturbation of hyperglycemia-induced changes in blood brain barrier structure and function (VanGilder et al., 2009). However, the role of tocotrienol, lycopene and sesamol in diabetic neuropathy in human subjects or animal studies has not been investigated so far.

With above background, the present study was undertaken to investigate the effect of tocotrienol, lycopene, sesamol and their combination with insulin on diabetic neuropathy in rats.

1.2. MATERIALS AND METHODS

1.2.1. Animals

Male Wistar rats (200-250 g) bred in Central Animal House facility of Panjab University were used in the present study. The animals were housed under optimal laboratory conditions, maintained on 12 h light and dark cycle and had free access to food (Hindustan Lever Products, Kolkata, India) and water. Animals were acclimatized to laboratory conditions before the tests. All experiments were carried out between 0900 and 1700 h. The experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University and conducted according to the Indian National Science Academy guidelines for the use and care of animals.

1.2.2. Drugs and Reagents

Streptozotocin, lycopene and sesamol were purchased from Sigma (St. Louis, MO, USA). Tocotrienol (mixture of α-, β-, γ-tocotrienol) was received as a gift sample from Golden-Hope Bioganic, Malaysia Palm Oil Board, Malaysia. Insulin was procured from Biocon Limited, Bangalore, India. A glucose oxidase peroxidase diagnostic enzyme kit was purchased from Span...
Diagnostic Chemicals, India. ELISA kits for TNF-α, IL-1β, (R & D Systems, USA), Caspase 3 (Biovision, USA), NF-κβ (Imegenex, USA) and insulin (DRG, Germany) were purchased from different reputed international companies. All other chemicals used for biochemical estimations were of analytical grade.

1.2.3. Induction and Assessment of Diabetes

A single dose of 45 mg/kg streptozotocin prepared in citrate buffer (pH 4.4, 0.1M) was injected intraperitoneally to induce diabetes. The age-matched control rats received an equal volume of citrate buffer and used along with diabetic animals. Diabetes was confirmed after 48h of streptozotocin injection, the blood samples were collected through tail vein and plasma glucose levels were estimated by enzymatic GOD-PAP (glucose oxidase peroxidase) diagnostic kit method (Erba Glucose Kit, Transasia Bio-Medicals Ltd., Solan, India). The rats having plasma glucose levels more than 250 mg/dl (Anjaneyulu and Chopra, 2004; Kuhad and Chopra, 2007) were selected and used for the present study. Streptozotocin (STZ), a β-cytotoxic, increases pancreatic islet O-linked protein glycosylation in a dose-dependent, irreversible fashion and also inhibits GlcNAcase, the enzyme that removes O-GlcNAc from proteins which accounts for its diabetogenic toxicity (Konrad et al., 2001). Body weight, food, water intake, plasma glucose and insulin levels were measured before and at the end of the experiment to see the effect of tocotrienol, lycopene, sesamol and their combination with insulin on these parameters.

(a) Citrate buffer preparation: 1 part of citric acid (1.5 g in 100 ml of distilled water) and 1 part of trisodium citrate (1.8 g in 1000 ml of distilled water) was mixed. The final pH was adjusted to pH 4.4 using monohydrate sodium citrate solution using a pH meter.

(b) Method for glucose estimation:

Principle:

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{HBA} + \text{AAP} \rightarrow \text{Quinoneimine dye} + 2\text{H}_2\text{O}
\]

AAP: 4-Aminoantipyrine; HBA: 4-Hydroxy Benzoic acid
The intensity of the pink colour formed is proportional to glucose concentration and it was measured spectrophotometrically at 510 nm.

**Reagents:**
- Reagent 1: Glucose reagent
- Reagent 2: Glucose standard (100 mg/dl)

**Preparation of working reagent:** The vial was allowed to attain the room temperature. The content of each vial were dissolved using glucose diluent with special lipid clearing agent. The volume was made upto to 200 ml or 500 ml depending on the pack size and transferred into a clean and dry amber coloured bottle.

**Assay Procedure:**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Plasma/Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-----</td>
<td>0.01 ml</td>
<td>-----</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>-----</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
</tbody>
</table>

All the reagents were mixed well and incubated for 15 min at 37° C. The absorbance of standard and sample tubes was read against reagent blank at 510 nm.

**Calculations:**

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard (mg/dl)}
\]

**1.2.4. Treatment Schedule**

After a basal recording of nociceptive reaction at 4th week of streptozotocin injection, control and diabetic rats were randomly selected and divided in eighteen groups of 10-12 animals each. Group 1 was the non-diabetic controls and animals received a single vehicle injection of citrate buffer and received oral gavage of Tween 80 vehicle. Group 2 was the diabetic control and animals received a single intraperitoneal injection of streptozotocin (45 mg/kg) and oral gavage of Tween 80 vehicle. Group 3 was
the diabetic animals which received subcutaneous injections of insulin (10 IU/kg, s.c.). Group 4, 5 and 6 consisted of diabetic animals received oral gavage of tocotrienol 25, 50 and 100 mg/kg/day respectively. Group 7 consisted of diabetic animals treated with insulin (10 IU/kg, s.c.) + tocotrienol (100 mg/kg/day, oral gavage). Group 8 consisted of control animals which received tocotrienol (100 mg/kg, oral gavage). Group 9, 10 and 11 consisted of diabetic animals which received oral gavage of lycopene 1, 2 and 4 mg/kg/day respectively. Group 12 consisted of diabetic animals treated with insulin (10 IU/kg, s.c.) + lycopene (4 mg/kg/day, oral gavage). Group 13 consisted of control animals received lycopene (4 mg/kg, oral gavage). Group 14, 15 and 16 consisted of diabetic animals received oral gavage of sesamol 2, 4 and 8 mg/kg/day respectively. Group 17 consisted of diabetic animals treated with insulin (10 IU/kg, s.c.) + sesamol (8 mg/kg/day, oral gavage). Group 18 consisted of control animals receiving sesamol (8 mg/kg, oral gavage).
The drug treatment was started from 5\textsuperscript{th} week after STZ injection till 8\textsuperscript{th} week. Drug solutions were freshly prepared and administered in a constant volume of 10 ml/kg body weight. Tocotrienol and lycopene were freshly prepared by dissolving in double distilled water after triturating with 5\% Tween 80. Sesamol was freshly prepared by dissolving in normal saline. At the end of 8\textsuperscript{th} week, the animals were sacrificed under deep anesthesia, blood was collected by carotid bleeding and plasma separated. Sciatic nerves were rapidly removed and weighed. A 10\% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 200g for 10 min, at 4\textdegree{}C and supernatant was used for estimation of lipid
peroxidation, total nitric oxide, superoxide dismutase, catalase and non protein thiols. Cytoplasmic and nuclear fractions were prepared for the quantification of caspase-3 and p65 subunit of NFκB. Plasma was used for the estimation of TNF-α, TGF-β1 and IL-1 β. The samples were stored at -80°C until processed for biochemical estimations.

1.2.5. Behavioral Assessment

1.2.5.1. Assessment of Thermal Hyperalgesia

Thermal hyperalgesia was assessed by tail-immersion and hot plate tests. Tail flick is primarily a spinal reflex, whereas a substantial supraspinal component, which involves lifting and licking of the hind paw, is required in the hot plate assay (De Felipe et al., 1998).

1.2.5.1.1. Tail-immersion (warm water) test

Tail of rat was immersed in a warm water bath (52.5 ± 0.5°C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 12s). Shortening of the tail withdrawal time indicates hyperalgesia and is attributed to central mechanisms (Ramabadran et al., 1989; Kanaan et al., 1996).

1.2.5.1.2. Hot-plate test

The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms (Kanaan et al., 1996). In this test, animals were individually placed on a hot-plate (Eddy's Hot-Plate) with the temperature adjusted to 55 ± 1°C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10 s in order to avoid damage to the paw.

1.2.5.2 Assessment of Mechanical Hyperalgesia

Paw pressure thresholds were registered with the paw pressure analgesia meter for the Randall-Selitto test (IITC Life Science, Woodland Hills, CA) (Taiwo et al., 1989). Pressure increasing at a linear rate of 10 g/s, with the cut-off of 250 g to avoid tissue injury, was applied to the center of the hind paw. When the animal displayed pain by withdrawal of the paw, the applied paw pressure was registered by an analgesia meter and expressed in
mass units (grams). Five tests separated by at least 15 min were performed for each animal, and the mean value of these tests was calculated.

1.2.5.3. Assessment of Allodynia

Tactile responses were evaluated by quantifying the withdrawal threshold of the hind paw in response to stimulation with flexible von Frey filaments (Chaplan et al., 1994; Ilnytska et al., 2006). Rats were placed in individual plexiglass boxes on a stainless steel mesh floor and were allowed to adjust for at least 20 min. A series of calibrated von Frey filaments (range 4–28 g; IITC Life Science, Woodland Hills, CA) was applied perpendicularly to the plantar surface of a hind paw with sufficient force to bend the filament for 6 s. Brisk withdrawal or paw flinching was considered as a positive response. In the absence of a response, a filament of next-greater force was applied. In the presence of a response, a filament of next-lower force was applied. The test was repeated four to five times at 5-min intervals on each animal, and the mean value was used.

1.2.6 Biochemical Assessment

1.2.6.1 Assessment of Oxidative-Nitrosative Stress

Estimation of Lipid Peroxidation

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills (1965). Briefly, 0.5 ml of post mitochondrial supernatant and 0.5 ml of Tris HCl were incubated at 37°C for 2 h. After incubation, 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000xg for 10 min. To 1 ml of supernatant, 1 ml of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling 1 ml double distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56 x 10^5 M^-1 cm^-1 and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method (Lubran, 1978) and the nerve malondialdehyde content expressed as nanomoles of malondialdehyde per milligram of protein.
Estimation of Non Protein Thiols

Non protein thiols were assayed by the method of Ellman (1959). Briefly, 1.0 ml of post mitochondrial supernatant (10%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5,5, dithiobis (2-nitro benzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm.

Estimation of Superoxide Dismutase

Cytosolic superoxide dismutase activity was assayed by the method of Kono (1978). The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of above mixture was taken and to it 0.05 ml of post mitochondrial supernatant and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30/60 s intervals.

Estimation of Catalase

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml post mitochondrial supernatant (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of k minutes⁻¹ and expressed as mean ± S.E.M.

Estimation of Total Nitric Oxide

The quantification of total nitric oxide was done by the help and instructions provided by R & D Systems total nitric oxide assay kit which involves the conversion of nitrate to nitrite by the enzyme nitrate reductase. The detection of total nitrite is then determined as a colored azo dye product of the Griess Reaction. The Griess reaction is based on the two step
diazotization reaction in which acidified NO₂-produces a nitrosating agent which reacts with sulphanilic acid to produce the diazonium ions. This ion is then coupled to N-(1-naphthyl) ethylenediame to form the chromophoric azo derivative which absorbs light at 540-570 nm.

1.2.6.2. Insulin ELISA

**Principle:**

Rat insulin in the sample was bound to the rat anti-insulin antibody coated on the microplate well. Horse radish peroxidase (POD)-conjugated anti-insulin antibody was then bound to the rat anti-insulin antibody/rat insulin complex immobilized to the microplate well. The bound POD conjugate in the microplate well was detected by the addition of the 3, 3', 5, 5' - tetramethyl-benzidine (TMB) substrate solution. The insulin concentration was determined via interpolation using standard curve generated by plotting absorbance versus the corresponding concentration of rat insulin standard.

**Assay Procedure:**

1. The antibody-coated microplate was removed from the sealed foil pouch and equilibrated to room temperature. The microplate was affixed to the supporting frame.
2. In each well, 95 µL of sample diluent was dispensed.
3. 5 µL samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/mL working rat insulin standards) was pipetted into the wells.
4. The microplate was covered with the plastic microplate cover and incubated for 2 hours at 4°C.
5. The microplate was washed five times using 300 µL of wash buffer per well. After each wash, the remaining solution was removed by inverting and tapping the plate firmly on clean paper towel.
6. 100 µL per well of anti-insulin enzyme conjugate was dispensed.
7. The microplate was covered with the plastic microplate cover and incubated for 30 minutes at room temperature.
8. The microplate was washed seven times using 300 µL of wash buffer per well. After each wash, the remaining solution was removed by inverting and tapping the plate firmly on clean paper towel.
9. 100 μL per well of enzyme substrate solution was immediately dispensed and allowed to react for 40 minutes at room temperature. During the enzyme reaction, avoid exposing the microplate to light.

10. The enzyme reaction was stopped by adding 100 μL per well of enzyme reaction stop solution.

11. The absorbance of each well was read using a microplate reader set to 450 nm within 30 minutes. The concentrations of insulin were calculated from plotted standard curves.

1.2.6.3. TNF-alpha, TGF-β1, and IL-1β ELISA

Estimation of Transforming Growth Factor beta 1

Transforming Growth Factor beta1 (TGF-β1) was estimated using rat TGF-β1 immunoassay kit (R & D Systems, USA). The assay is based on the quantitative sandwich enzyme immunoassay technique (ELISA) using a microtitre plate reader at 450 nm. Serum samples were diluted 78-fold for ELISA determination of TGF-β1. Concentrations of TGF-β1 were calculated from plotted standard curves.

Principle:

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-β1 has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF-β1 bound in the initial step. The color development is stopped and the intensity of the color is measured using ELISA reader set at 450 nm.

Activation Reagent Preparation

To activate latent TGF-β1 to the immunoreactive form, the following solutions were prepared for acid activation and neutralization. The solutions were stored in polypropylene bottles at room temperature for up to one month.
Chapter 1

If any precipitate forms, the solution was gently warmed to 37° C while mixing and cooled to room temperature before use.

Reagents to activate serum/plasma samples

2.5 N Acetic Acid/10 M Urea (250 ml) - 150.2 g of Urea was added to 100 ml of deionized water and was mixed well until dissolved. 35.9 ml of glacial acetic acid was slowly added to the mixture and the final volume was made up to 25 ml with deionized water.

2.7 N NaOH/1 M HEPES (250 ml) - 67.5 ml of 10 N NaOH was added to 14 ml of deionized water. After mixing, 59.5 g of HEPES was added and the final volume was made up to 250 ml with deionized water.

TGF-β1 Sample Activation Procedure

To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the Quantikine TGF-β1 immunoassay, the activation procedure mentioned below was followed:

1. 40 μL of 2.5 N acetic acid /10 M urea was added to 40 μL of the serum/plasma and was mixed properly.
2. The solution was incubated for 10 minutes at room temperature.
3. The acidified sample was then neutralized by adding 25 μL of 2.7 N NaOH/1 M HEPES and mixed well.
4. Prior to the assay, the activated samples were diluted 30 times with the calibrator diluents.

Assay Procedure:

1. All the reagents, standard dilutions, and activated samples were prepared as directed in the kit (MB100; TGF-β1 kit).
2. 50 μL of assay diluent (for serum/plasma samples) was added to each well.
3. 50 μL of standard, control or activated samples were then added to each well and were covered with the adhesive strip. All the wells were incubated for 2 hours at room temperature on a horizontal orbit microplate shaker set at 500 ± 50 rpm.
4. The wells were aspirated and washed for a total of five washes with the wash buffer (400 μL). After the last wash, any remaining wash buffer
was decanted by inverting the plate and blotting it against a clean paper towel.

5. 100μL of TGF-β1 conjugate was then added to each well and incubated for 2 hours at room temperature on the shaker.

6. The aspiration/wash steps were repeated as in step 4.

7. 100 μL of substrate solution was then added to each well and incubated for 30 minutes at room temperature on the benchtop protected from light.

8. 100μL of stop solution was finally added to each well and the plate was gently tapped to ensure thorough mixing.

9. The absorbance of each well was read using a microplate reader set to 450 nm within 30 minutes. The concentrations of TGF-β1 were calculated from plotted standard curves.

Estimation of Tumor Necrosis Factor-alpha (TNF-α) levels

Tumor necrosis factor-alpha (TNF-α) levels were estimated using rat TNF-α kit (R&D Systems). It is a solid phase sandwich enzyme linked immunosorbent assay (ELISA), which uses a microtitre plate reader read at 450 nm. Concentrations of TNF-α were calculated from plotted standard curves.

Principle:

This assay employs the quantitative sandwich enzyme immunoassay technique. An affinity purified polyclonal antibody specific for mouse TNF-α has been pre-coated onto a microplate. Standards, Controls, and samples are pipetted into the wells and any mouse TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse TNF-α bound in the initial step. The sample values are then read off the standard curve.
Assay Procedure:

1. The reagents and standard dilutions were prepared as suggested by the manufacturer's instructions.
2. 50 µL of assay diluent was added to each well of the precoated microtitre plate.
3. 50µL of standard or sample was then added per well and were mixed gently. The plate was covered with the adhesive strip and incubated for 2 hours at room temperature.
4. Each well was then aspirated and washed with wash buffer, repeating the process four times for a total of five washes. The plate was then inverted and blotted against a clean paper towel.
5. 100 µL of rat TNF-Conjugate was added to each well and covered with a new adhesive strip. The plate was again incubated for 2 hours at room temperature.
6. Repeated the aspiration/washes as in step 5.
7. 100 µL of substrate solution was then added to each well and incubated for 30 minutes at room temperature protected from light.
8. 100µL of stop solution was finally added to each well and the absorbance of each well was read in an ELISA reader set to 450 nm.

Calculations:

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph by regression analysis. The samples which were diluted were multiplied by the dilution factor to get the exact concentration of the unknown samples.

Estimation of Interleukin 1beta

The Quantikine Rat IL-1β Immunoassay is a 4.5 hour solid phase ELISA designed to measure rat IL-1β in cell culture supernates, serum, and
EDTA plasma. It contains recombinant rat IL-1β and antibodies raised against recombinant rat IL-1β. This immunoassay has been shown to quantitate the recombinant rat factor accurately.

**Principle:**

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat IL-1β has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rat IL-1β present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-1β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat IL-1β bound in the initial step. The sample values are then read off the standard curve.

**Sample Preparation**

Rat serum and rat EDTA plasma samples require a 3-fold dilution into Calibrator Diluent RD5Y. A suggested 3-fold dilution is 50 μL sample + 100 μL Calibrator Diluent RD5Y.

**Assay Procedure:**

1. All reagents and samples were brought to room temperature before use. All samples, standards, and control were assayed in duplicate.
2. Reagents, standard curve dilutions, and samples were prepared.
3. 50 μL of assay diluent RD1-21 was added to each well.
4. 50 μL of standard, control, or sample were added per well and mixed by gently tapping the plate frame for 1 minute and covered with the adhesive strip. Incubated for 2 hours at room temperature.
5. Each well aspirated and washed, repeating the process four times for a total of five washes.
6. 100 μL of Rat IL-1β conjugate was added to each well and incubated for 2 hours at room temperature.
7. The aspiration/wash was repeated as in step 5.
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8. 100 µL of substrate solution was added to each well and incubated for 30 minutes at room temperature.

9. 100 µL of stop solution was added to each well and gently tapped the plate to ensure thorough mixing.

10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

**Calculations:**

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph by regression analysis. The samples which were diluted were multiplied by the dilution factor to get the exact concentration of the unknown samples.

1.2.6.4. Quantification of NF-κB p65 unit

The nuclear levels of p65 may correlate positively with the activation of NF-κB pathway. The NF-κB/p65 ActivELISA (Imgenex, San Diego, USA) kit was used to measure NF-κB free p65 in the nuclear lysate. The NF-κB ActivELISA is a sandwich ELISA. Free p65 was captured by anti-p65 antibody coated plates and the amount of bound p65 was detected by adding a second anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405 nm.

**Preparation of lysates from Tissue**

1) Cytoplasmic Fraction Collection (Tissue Homogenization based on 1 gram rat tissue)

1. Sciatic nerve/brain was weighed and cut into small pieces using clean razor blade and wash in 5 ml of cold 1X PBS-PMSF. Collect cut pieces in a clean homogenizer.

2. 5 µl of 1M DTT and 500 µl of 10% Detergent Solution to 4.495 ml of ice cold 1 X Hypotonic Buffer per gram of tissue was added and
homogenized. The homogenate was incubated on ice for 15 to 30 min (whole cell lysate).

3. The homogenate was centrifuged for 10 min at 10,000 rpm at 4°C and supernatant (cytoplasmic fraction) was transferred into a 15 ml tube and stored at 4°C. The pellet comprised the nuclear fraction.

**ii) Nuclear Fraction Collection**

1. The nuclear pellet was resuspended in 500 μl nuclear lysis buffer by pipetting up and down. The suspension was vigorously vortexed and incubated at 4°C, for 30 min on a rocking platform.

2. The suspension was centrifuged at 14,000 rpm for 10 min at 4°C in a microcentrifuge.

3. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C until further use.

4. The protein concentration in the nuclear extract was determined using a detergent compatible assay technique.

**Assay Procedure:**

1. **Coating:** 100 μl of capture antibody (IMK-503-01) was diluted in 10 ml of coating buffer (KC-104). 100 μl of diluted antibody was pipetted into each well and incubated the plate overnight (12-24 h) at 4°C. The coated wells were washed twice with 300 μl of 1X wash buffer.

2. **Blocking:** 200 μl of prepared blocking buffer was added to each well to block the remaining reactive surface and incubated for 30 min to 1 h at room temperature.

3. **Prepare p65 Standard Curve:** Quick spin down the Recombinant p65 Standard vial and 420 μl of sterile deionized H₂O was added and vortexed to dissolve. A standard curve was set up in duplicate using the following concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.0 (blank) ng/well.

4. **Samples:** 100 μl of positive and negative controls and 100 μl test samples was pipetted into the appropriate wells. Incubate plate at 4°C overnight or 4 h at room temperature. Samples might be diluted or serially diluted using blocking buffer.
5. **Washing:** The samples and control lysates were removed and washed with wash buffer. The plate was tapped several times upside down to remove residual wash buffer after final wash.

6. **Detecting Antibody:** 100 µl of detecting antibody (IMK-503-02) was diluted in 10 ml of blocking buffer and 100 µl diluted detecting antibody was added to each well and incubated for 1 h at room temperature.

7. **Washing:** The antibody solution was removed and washed wells 4 times with wash buffer. The plate was tapped several times upside down to remove residual wash buffer after final wash.

8. **Secondary Antibody:** 5 µl of AKP-Conjugated secondary Ab (KC-130) was diluted in 10 ml of blocking buffer. 100 µl of diluted secondary antibody was added to each well and incubated for 1 h at room temperature.

9. The secondary antibody was removed and washed thoroughly with wash buffer letting the solution sit briefly between each wash. During the last wash, pNPP substrate was prepared. The plate was tapped several times upside down to remove residual wash buffer after final wash.

10. **pNPP Substrate:** 10 mg pNPP was dissolved into 10 ml of pNPP substrate buffer and mixed. (Note: Prepare substrate mix just before use). 100 µl of pNPP substrate was added to each well and incubated the plate at room temperature for 30 min. The color development was read at 405 nm.

### 1.2.6.5. Caspase-3 Colorimetric Assay

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. The Caspase-3/CPP32 Colorimetric Assay Kit provides a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD. The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400- or 405-nm.
**Assay Procedure:**

Aliquot enough 2X Reaction Buffer for the number of assays to be performed. Add DTT to the 2X Reaction Buffer immediately before use (10 mM final concentration: add 10 µl of 1.0 M DTT stock per 1 ml of 2X Reaction Buffer). Protect DEVD-pNA from light.

1. Apoptosis was induced in cells by desired method. Concurrently incubated a control culture without induction.
2. Cells and pellet 1-5 x 10⁶ cells were counted.
3. Cells were suspended in 50 µl of chilled cell lysis buffer and incubated on ice for 10 minutes.
4. Centrifuged for 1 min in a microcentrifuge (10,000 x g).
5. Supernatant (cytosolic extract) was transferred to a fresh tube and put on ice for immediate assay or aliquot and store at −80°C for future use.
6. Protein concentration was assayed.
7. Diluted 50-200 µg protein to 50 µl cell lysis buffer for each assay.
8. 50 µl of 2X reaction buffer (containing 10 mM DTT) was added to each sample.
9. 5 µl of the 4 mM DEVD-pNA substrate (200 µM final conc.) added to each well and incubated at 37°C for 1-2 hour.
10. Samples were analyzed at 400- or 405-nm in a microtiter plate reader, or spectrophotometer using a 100-µl micro quartz cuvet (Sigma), or dilute sample to 1 ml with dilution buffer and using regular cuvet.
11. Fold-increase in CPP32 activity was determined by comparing the results with the level of the un-induced control.

1.2.7. **Statistical Analysis**

Results were expressed as mean ± SEM. The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Tukey's test to assess the significance. Statistical significance was considered at $p<0.05$. The statistical analysis was done using the SPSS Statistical Software version 15.
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1.3. RESULTS

1.3.1. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on blood glucose, body weight, water intake, food intake and insulin level in diabetic rats

Four weeks after STZ injection, diabetic animals exhibited increased blood glucose levels (402 ± 10.52 mg/dl vs 111 ± 7.94 mg/dl (p<0.001)) and decreased body weight (182 ± 1.32 g vs 304 ± 2.15 g) (p<0.01)) compared with control rats (Table 1.1). Chronic treatment with tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) in diabetic rats from 5th to 8th week significantly ameliorated plasma glucose levels as well as increased the body weight as compared with vehicle treated diabetic rats (p<0.05). Further, a significant increase in food (59 ± 2.69 g vs 28 ± 1.05 g) and water (108 ± 1.65 ml vs 35 ± 1.23 ml) intake in STZ injected rats were significantly attenuated with tocotrienol, lycopene and sesamol treatment. Plasma insulin levels were significantly decreased in diabetic (25 ± 18.45 pmol/l) rats as compared to control (102 ± 5.51 pmol/l) rats (p<0.01). However, insulin levels were not significantly altered in tocotrienol, lycopene and sesamol treated diabetic rats. Insulin (10 IU/kg) alone improved body weight, plasma glucose, insulin, food and water intake in diabetic rats. However, insulin (10 IU/kg) in combination of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) produced more marked effect on body weight, plasma glucose, food and water intake as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05). In addition, tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) per se did not affect body weight, plasma glucose, insulin, food and water intake in control rats.

1.3.2. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on thermal hyperalgesia

The nociceptive threshold was significantly lower in diabetic rats as compared with the basal value tested in both the tail-immersion (1.9 sec vs
10.5 sec) (Fig. 1.1.1.A, Fig. 1.1.2.A, Fig. 1.1.3.A) and hot-plate (1.5 sec vs 9.7 sec) (Fig. 1.1.1.B, Fig. 1.1.2.B, Fig. 1.1.3.B) assays. Hyperalgesia was evident in the tail-immersion and hot-plate after 1 week and 2 weeks respectively, and the maximum decrease in pain threshold was observed at 4 weeks after streptozotocin injection in rat as compared to non-diabetic control rat (p<0.001). Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) administration to diabetic rat produced a dose and time dependent increase in pain threshold as compared to untreated diabetic rat. The maximum increase in pain threshold and that too in progressive manner was observed with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) in both tail immersion (Fig. 1.1.1.A, Fig. 1.1.2.A, Fig. 1.1.3.A) and hot-plate (Fig. 1.1.1.B, Fig. 1.1.2.B, Fig. 1.1.3.B) assays. Insulin (10 IU/kg) alone partially improved thermal hyperalgesia in diabetic rats. However, insulin (10 IU/kg) in combination of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly enhanced the paw withdrawal threshold (thermal hyperalgesia) as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).

1.3.3. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on mechanical hyperalgesia and tactile alldynia

Diabetic rats with 4-week duration of STZ-induced diabetes also had mechanical hyperalgesia detected with paw pressure Randall-Selitto test (Fig. 1.2.). In particular, paw-withdrawal threshold in the Randall-Selitto test was reduced by 76% in diabetic rats compared with controls (p<0.001). Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) significantly and dose-dependently corrected this variable in diabetic rats (p<0.05).

Another sensory abnormality developing in diabetic rats was tactile alldynia. Tactile withdrawal threshold in response to light touch with flexible von Frey filaments was reduced by 64% in diabetic rats compared with controls (p<0.01) (Fig. 1.3).
Table 1.1. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on various parameters (mean ± SEM)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma Glucose (mg/dl)</th>
<th>Plasma Insulin (pmol/l)</th>
<th>Body Weights (g)</th>
<th>Food Intake (g)</th>
<th>Water Intake (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111 ± 7.94</td>
<td>102 ± 5.51</td>
<td>304 ± 2.15</td>
<td>28 ± 1.05</td>
<td>35 ± 1.23</td>
</tr>
<tr>
<td>STZ</td>
<td>402 ± 10.52*</td>
<td>25 ± 18.45*</td>
<td>182 ± 1.32*</td>
<td>59 ± 2.69*</td>
<td>108 ± 1.65*</td>
</tr>
<tr>
<td>STZ + Insulin (10)</td>
<td>199 ± 10.34#</td>
<td>65 ± 12.56#</td>
<td>215 ± 4.35#</td>
<td>33 ± 1.29#</td>
<td>45 ± 3.02#</td>
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<tr>
<td>STZ + Toco (25)</td>
<td>313 ± 7.18#</td>
<td>44 ± 23.48</td>
<td>179 ± 8.22</td>
<td>49 ± 1.08#</td>
<td>96 ± 3.96#</td>
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<tr>
<td>STZ + Toco (50)</td>
<td>270 ± 9.43#</td>
<td>41 ± 25.89</td>
<td>187 ± 4.54</td>
<td>43 ± 1.11#</td>
<td>79 ± 2.05#</td>
</tr>
<tr>
<td>STZ + Toco (100)</td>
<td>201 ± 7.89#</td>
<td>32 ± 11.47</td>
<td>209 ± 1.89#</td>
<td>37 ± 1.12#</td>
<td>57 ± 1.11#</td>
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<tr>
<td>STZ + Insulin (10) + Toco (100)</td>
<td>136 ± 9.83#</td>
<td>52 ± 9.04#</td>
<td>243 ± 2.11#</td>
<td>32 ± 1.06#</td>
<td>41 ± 1.00#</td>
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<tr>
<td>Toco (100)</td>
<td>99 ± 8.84</td>
<td>100 ± 6.33</td>
<td>312 ± 5.07</td>
<td>27 ± 1.02</td>
<td>36 ± 1.94</td>
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<tr>
<td>Treatment</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
<td>Value 5</td>
</tr>
<tr>
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<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>STZ + Lyco (1)</td>
<td>355 ± 7.67&quot;</td>
<td>39 ± 12.92</td>
<td>197 ± 5.64</td>
<td>51 ± 1.00&quot;</td>
<td>97 ± 1.11&quot;</td>
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<tr>
<td>STZ + Lyco (2)</td>
<td>293 ± 10.07&quot;</td>
<td>42 ± 19.07</td>
<td>209 ± 3.11&quot;</td>
<td>44 ± 1.12&quot;</td>
<td>85 ± 3.89&quot;</td>
</tr>
<tr>
<td>STZ + Lyco (4)</td>
<td>210 ± 8.37&quot;</td>
<td>36 ± 13.97</td>
<td>227 ± 2.36&quot;</td>
<td>36 ± 1.00&quot;</td>
<td>61 ± 1.07&quot;</td>
</tr>
<tr>
<td>STZ + Insulin (10) + Lyco (4)</td>
<td>152 ± 11.73&quot;</td>
<td>69 ± 10.88&quot;</td>
<td>262 ± 2.79&quot;</td>
<td>30 ± 1.00&quot;</td>
<td>44 ± 2.01&quot;</td>
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<tr>
<td>Lyco (4)</td>
<td>101 ± 5.23</td>
<td>103 ± 10.76</td>
<td>296 ± 7.09</td>
<td>28 ± 1.01</td>
<td>32 ± 1.01</td>
</tr>
<tr>
<td>STZ + SML (2)</td>
<td>337 ± 7.94&quot;</td>
<td>33 ± 16.43</td>
<td>191 ± 6.73</td>
<td>52 ± 1.21&quot;</td>
<td>92 ± 1.42&quot;</td>
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<tr>
<td>STZ + SML (4)</td>
<td>286 ± 7.18&quot;</td>
<td>37 ± 17.93</td>
<td>201 ± 3.87</td>
<td>43 ± 1.61&quot;</td>
<td>81 ± 3.12&quot;</td>
</tr>
<tr>
<td>STZ + SML (8)</td>
<td>212 ± 8.46&quot;</td>
<td>34 ± 12.91</td>
<td>226 ± 2.41&quot;</td>
<td>34 ± 1.09&quot;</td>
<td>54 ± 2.16&quot;</td>
</tr>
<tr>
<td>STZ + Insulin (10) + SML (8)</td>
<td>142 ± 7.96&quot;</td>
<td>75 ± 16.79&quot;</td>
<td>289 ± 2.73&quot;</td>
<td>25 ± 1.10&quot;</td>
<td>39 ± 2.87&quot;</td>
</tr>
<tr>
<td>SML (8)</td>
<td>98 ± 4.98</td>
<td>101 ± 3.87</td>
<td>322 ± 4.09</td>
<td>30 ± 1.37</td>
<td>34 ± 2.46</td>
</tr>
</tbody>
</table>

*(p<0.01) different from control; #(p<0.05) different from diabetic group. Toco (25) = Tocotrienol 25mg/kg, Toco (50) = Tocotrienol 50mg/kg, Toco (100) = Tocotrienol 100mg/kg, Lyco (1) = Lycopene 1 mg/kg, Lyco (2) = Lycopene 2 mg/kg, Lyco (4) = Lycopene 4 mg/kg, SML (2) = Sesamol 2mg/kg, SML (4) = Sesamol 4mg/kg, SML (8) = Sesamol 8mg/kg.
Figure 1.1.1. Effect of tocotrienol (25, 50 and 100 mg/kg) and its combination with insulin (10 IU/kg) on pain threshold in tail-immersion (A) and hot-plate (B) assays in control and streptozotocin-injected diabetic rat. Values are expressed as mean ± S.E.M. (n=10 in each group). a (p<0.001) different from control; b (p<0.05) different from diabetic group, c (p<0.05) different from one another, d (p<0.05) different from diabetic rats treated with either tocotrienol or insulin. Toco (25) = Tocotrienol 25mg/kg, Toco (50) = Tocotrienol 50mg/kg, Toco (100) = Tocotrienol 100mg/kg.
Figure 1.1.2. Effect of lycopene (1, 2 and 4 mg/kg) and its combination with insulin (10 IU/kg) on pain threshold in tail-immersion (A) and hot-plate (B) assays in control and streptozotocin-injected diabetic rat. Values are expressed as mean ± S.E.M. (n=10 in each group). a (p<0.001) different from control; b (p<0.05) different from diabetic group, c (p<0.05) different from one another, d (p<0.05) different from diabetic rats treated with lycopene or insulin. Lyco (1) = Lycopene 1 mg/kg, Lyco (2) = Lycopene 2 mg/kg, Lyco (4) = Lycopene 4 mg/kg.
Figure 1.1.3. Effect of sesamol (2, 4 and 8 mg/kg) and its combination with insulin (10 IU/kg) on pain threshold in tail-immersion (A) and hot-plate (B) assays in control and streptozotocin-injected diabetic rat. Values are expressed as mean ± S.E.M. (n=10 in each group). a (p<0.001) different from control; b (p<0.05) different from diabetic group, c (p<0.05) different from one another, d (p<0.05) different from diabetic rats treated with sesamol and insulin alone. SML (2) = Sesamol 2 mg/kg, SML (4) = Sesamol 4 mg/kg, SML (8) = Sesamol 8 mg/kg.
Figure 1.2 Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on mechanical hyperalgesia in the diabetic rats after 8 weeks. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tocotrienol</th>
<th>Lycopene</th>
<th>Sesamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+T1</td>
<td>25mg/kg</td>
<td>1mg/kg</td>
<td>2mg/kg</td>
</tr>
<tr>
<td>D+T2</td>
<td>50mg/kg</td>
<td>2mg/kg</td>
<td>4mg/kg</td>
</tr>
<tr>
<td>D+T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
</tr>
<tr>
<td>D+I+T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
</tr>
<tr>
<td>T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
</tr>
</tbody>
</table>
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Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) significantly and dose-dependently corrected diabetes-induced decrease in tactile withdrawal thresholds in diabetic rats. Insulin (10 IU/kg) alone partially improved allodynia and mechanical hyperalgesia in diabetic rats. However, insulin (10 IU/kg) in combination of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly enhanced the withdrawal threshold (alldynia and mechanical hyperalgesia) as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05) (Fig. 1.3). In addition, tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) per se did not affect allodynia and mechanical hyperalgesia in control rats.

1.3.4. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on diabetes-induced changes in lipid peroxidation

Thiobarbituric acid reactive substance levels were increased significantly in the sciatic nerves of diabetic (3.58 ± 0.11 nmol/mg protein) rats as compared to control (1.44 ± 0.04 nmol/mg protein) rats (p<0.05) (Table 1.2). Chronic treatment with tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) produced a significant and dose-dependent reduction in thiobarbituric acid reactive substance levels in STZ-treated rats (p<0.05). Insulin (10 IU/kg) alone partially inhibited lipid peroxidation in diabetic rats. However, diabetic rats treated with insulin (10 IU/kg) in combination of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly prevented this rise in lipid peroxidation as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05). In addition, tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) per se did not lipid peroxidation in the sciatic nerves of control rats.

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Figure 1.3 Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on allodynia in the diabetic rats after 8 weeks. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tocotrienol</th>
<th>Lycopene</th>
<th>Sesamol</th>
</tr>
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<tr>
<td>D+T1</td>
<td>25mg/kg</td>
<td>1mg/kg</td>
<td>2mg/kg</td>
</tr>
<tr>
<td>D+T2</td>
<td>50mg/kg</td>
<td>2mg/kg</td>
<td>4mg/kg</td>
</tr>
<tr>
<td>D+T3</td>
<td>100mg/kg</td>
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<td>D+I+T3</td>
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<tr>
<td>T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
</tr>
</tbody>
</table>
Table 1.2. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on lipid peroxide, non protein thiols, superoxide dismutase (SOD), catalase and total nitric oxide levels (mean ± SEM)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO (nmol/mg protein)</th>
<th>Non protein thiols (moles/mg protein)</th>
<th>Superoxide Dismutase (units/mg protein)</th>
<th>Catalase (k/min)</th>
<th>Total Nitric Oxide (umol)</th>
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<tr>
<td>Control</td>
<td>1.44 ± 0.04</td>
<td>28.56 ± 1.00</td>
<td>16.44 ± 0.09</td>
<td>2.89 ± 0.07</td>
<td>49 ± 1.48</td>
</tr>
<tr>
<td>STZ</td>
<td>3.58 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.14 ± 1.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.32 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>197 ± 2.17&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>STZ + Insulin (10)</td>
<td>2.97 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.79 ± 1.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.99 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.89 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>172 ± 1.28&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>STZ + Toco (25)</td>
<td>2.84 ± 0.08&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>11.42 ± 1.12&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>5.63 ± 0.11&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.08 ± 0.13&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>151 ± 1.40&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>STZ + Toco (50)</td>
<td>2.29 ± 0.11&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>15.67 ± 1.08&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>6.79 ± 0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.58 ± 0.05&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>111 ± 1.31&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>STZ + Toco (100)</td>
<td>1.88 ± 0.06&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>20.51 ± 1.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>9.23 ± 0.10&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.16 ± 0.10&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>87 ± 1.82&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>STZ + Insulin (10) + Toco (100)</td>
<td>1.57 ± 0.04&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>27.87 ± 1.00&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>13.64 ± 0.11&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>2.68 ± 0.11&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>66 ± 1.46&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<td>Toco (100)</td>
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<td>27.78 ± 1.01</td>
<td>16.67 ± 0.12</td>
<td>2.91 ± 0.09</td>
<td>50 ± 2.07</td>
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<tr>
<td>Treatment</td>
<td>2.89 ± 0.08&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>10.46 ± 1.08&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>5.37 ± 0.17&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>0.95 ± 0.10&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>158 ± 1.11&lt;sup&gt;b,e&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>STZ + Insulin (10) + SML (8)</td>
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<td>Lyco (4)</td>
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<td>STZ + Insulin (10) + SML (8)</td>
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<td>SML (8)</td>
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</table>

a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. Toco (25) = Tocotrienol 25mg/kg, Toco (50) = Tocotrienol 50mg/kg, Toco (100) = Tocotrienol 100mg/kg, Lyco (1) = Lycopene 1 mg/kg, Lyco (2) = Lycopene 2 mg/kg, Lyco (4) = Lycopene 4 mg/kg, SML (2) = Sesamol 2mg/kg, SML (4) = Sesamol 4mg/kg, SML (8) = Sesamol 8mg/kg.
1.3.5. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on diabetes-induced changes in the antioxidant profile

The non protein thiols (8.14 ± 1.24 mol/mg protein vs 28.56 ± 1.00 mol/mg protein) and enzyme activity of superoxide dismutase (4.32 ± 0.11 units/mg protein vs 16.44 ± 0.09 units/mg protein) and catalase (0.45 ± 0.08 k/min vs 2.89 ± 0.07 k/min) significantly decreased in the sciatic nerves of diabetic rats as compared to control group (p<0.05) (Table 1.2). This reduction in the sciatic nerve antioxidant profile of STZ-treated rats was significantly and dose dependently improved by the treatment with tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg). Insulin (10 IU/kg) alone partially increased endogenous antioxidant levels in diabetic rats. However, diabetic rats treated with insulin (10 IU/kg) in combination of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly restored the endogenous antioxidant profile as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05). In addition, tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) per se did not alter endogenous antioxidant levels in the sciatic nerves of control rats.

1.3.6. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on diabetes-induced nitrosative stress

Total nitric oxide levels were significantly elevated in the sciatic nerves of diabetic rats (197 ± 2.17 µmol) as compared to control rats (49 ± 1.48 µmol) (p<0.01) (Table 1.2). Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) treatment significantly and dose dependently inhibited this rise in nitric oxide levels (p<0.05). Insulin (10 IU/kg) alone partially reduced total nitric oxide levels in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly decreased total nitric oxide levels as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).
1.3.7. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on the plasma TNF-α levels

TNF-α level was markedly increased in the plasma of diabetic (804 ± 56.29 ng/ml) rats as compared to control (76 ± 4.53 ng/ml) rats (p<0.01) (Fig. 1.4). Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) produced a dose-dependent decrease in TNF-α levels (p<0.05). Insulin (10 IU/kg) alone partially decreased TNF-α level in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly reduced TNF-α level as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).

1.3.8. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on the plasma IL-1β levels

Plasma IL-1β level was significantly increased in diabetic (748 ± 33.91 ng/ml) as compared to control (110 ± 6.38 ng/ml) rats (p<0.01) (Fig. 1.5). Tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) significantly and dose-dependently decreased IL-1β levels (p<0.05). Insulin (10 IU/kg) alone did not decrease IL-1β level in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly inhibited IL-1β levels as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).

1.3.9. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on the plasma TGF-β1 levels

TGF-β1 level was significantly increased in the plasma of diabetic (265 ± 17.93 ng/ml) rats as compared to control (33 ± 2.1 ng/ml) rats (p<0.01) (Fig. 1.6). Tocotrienol (25, 50 and 100 mg/kg), lycopene (2 and 4 mg/kg but not 1 mg/kg) and sesamol (2, 4 and 8 mg/kg) produced a dose-dependent decrease in TGF-β1 levels (p<0.05) in diabetic rats.
Figure 1.4: Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on TNF-α levels in the diabetic rats. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tocotrienol</th>
<th>Lycopene</th>
<th>Sesamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+T1</td>
<td>25mg/kg</td>
<td>1mg/kg</td>
<td>2mg/kg</td>
</tr>
<tr>
<td>D+T2</td>
<td>50mg/kg</td>
<td>2mg/kg</td>
<td>4mg/kg</td>
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<tr>
<td>D+T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
</tr>
<tr>
<td>D+I+T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
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<td>T3</td>
<td>100mg/kg</td>
<td>4mg/kg</td>
<td>8mg/kg</td>
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</tbody>
</table>
Figure 1.5: Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on IL-1β levels in the diabetic rats. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.
Figure 1.6: Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on TGF-β1 levels in the diabetic rats. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.
Insulin (10 IU/kg) alone did not reduce TGF-β1 levels in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly inhibited TGF-β1 levels as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).

1.3.10. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on p65 subunit of NF-κβ

p65 subunit of NF-κβ was significantly increased in the sciatic nerve of diabetic (85 ± 17.93 pg/mg protein) rats as compared to control (15 ± 2.1 pg/mg protein) rats (p<0.01) (Fig. 1.7). Tocotrienol (50 and 100 mg/kg but not 25 mg/kg), lycopene (1, 2 and 4 mg/kg) and sesamol (2, 4 and 8 mg/kg) produced significant decrease in p65 subunit of NF-κβ (p<0.05) in diabetic rats. Insulin (10 IU/kg) alone did not reduce p65 subunit of NF-κβ in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly and dose-dependently decreased p65 subunit of NF-κβ as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).

1.3.11. Effect of tocotrienol, lycopene, sesamol and their combination with insulin on caspase-3 levels

Caspase-3 level was found to increase significantly in the sciatic nerves of diabetic as compared to control rats (p<0.01) (Fig. 1.8). Tocotrienol (25, 50 and 100 mg/kg), lycopene (2 and 4 mg/kg but not 1 mg/kg) and sesamol (2, 4 and 8 mg/kg) significantly and dose-dependently decreased caspase-3 levels (p<0.05) in diabetic rats. Insulin (10 IU/kg) alone did not reduce caspase-3 levels in diabetic rats. However, insulin (10 IU/kg) in combination with higher doses of tocotrienol (100 mg/kg), lycopene (4 mg/kg) and sesamol (8 mg/kg) significantly reduced caspase-3 levels as compared to diabetic rats treated with either insulin (10 IU/kg) or tocotrienol (100 mg/kg) or lycopene (4 mg/kg) or sesamol (8 mg/kg) alone (p<0.05).
Figure 1.7. Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on p65 subunit of NF-κβ in the diabetic rats. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.

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<th>Sesamol</th>
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<tr>
<td>D+T2</td>
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<td>2mg/kg</td>
<td>4mg/kg</td>
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Figure 1.8. Effect of tocotrienol (25, 50 and 100 mg/kg), lycopene (1, 2 and 4 mg/kg), sesamol (2, 4 and 8 mg/kg) and their combination with insulin (10 IU/kg) on caspase-3 levels in the diabetic rats. Data are expressed as mean ± S.E.M. a (p<0.01) different from control; b (p<0.05) different from diabetic group, c,e,g (p<0.05) different from one another; d (p<0.05) different from tocotrienol and insulin alone groups; f (p<0.05) different from lycopene and insulin alone groups; h (p<0.05) different from sesamol and insulin alone groups. C = control, D = diabetic, D+I = diabetic rats treated with insulin. T1, T2 and T3 refer to three different doses of tocotrienol, lycopene and sesamol.

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Table: 1.3. Pearson’s correlation analysis of mechanical hyperalgesia (MH) at different doses of tocotrienol, lycopene, sesamol and their combination with insulin with various oxidative, inflammatory and molecular markers

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<tr>
<th>Study Group</th>
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<td>LYCO</td>
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<tr>
<td>SML</td>
<td>-0.993</td>
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LPO: Lipid peroxidation; LYCO: Lycopene; MH: Mechanical Hyperalgesia (Randall Sellito Test); NO: Nitric oxide; SML: Sesamol; SOD: Superoxide dismutase; TOCO: Tocotrienol

The pair(s) of variables with positive correlation coefficients tends to increase together. The limit of significance is p<0.005.

1.4. DISCUSSION

Hyperglycemia and inflammation unleash a cascade of events that affect cellular proteins, gene expression and cell surface receptor expression leading to progressive pathologic changes and subsequent diabetic complications (Pop-Busui et al., 2006). Pathogenetic mechanisms underlying the progressive nerve fiber loss in diabetes seem to be multifactorial, including polyol pathway, glycation, reactive oxygen species and altered protein kinase C activity (Brownlee et al., 2001; Yagihashi et al., 2001). In the present study, streptozotocin-injected mice had significantly high blood glucose levels, increased food and water intake, decreased body weight and the nociceptive threshold was significantly lower than non-diabetic rats, indicating that diabetic animals exhibited allodynia, thermal and mechanical hyperalgesia. This is in line with various other observations that streptozotocin-induced mice exhibited thermal allodynia and hyperalgesia.
tested on exposure of tail to noxious heating (Ohsawa and Kamei, 1999; Kuhad et al., 2008). In the present study, chronic treatment with tocotrienol, lycopene and sesamol significantly increased pain threshold in diabetic rats. This was coupled with increased body weight and decreased plasma glucose levels, food and water intake in diabetic rats. However, plasma insulin levels remained unchanged in the diabetic rats. Tocotrienol has been recently reported to increase nociceptive threshold as evident from increased tail flick latency (thermal hyperalgesia) and increased paw-withdrawal threshold in Randall-Selitto test (mechanical hyperalgesia) and von-Frey hair test (mechanical allodynia) in chronic ethanol treated rats (Tiwari et al., 2009b). Lycopene and sesamol have been shown to enhance pain threshold via modulating cyclo-oxygenase synthesis pathway in few studies (Chavali et al., 1999; Heber and Go, 1999; Sengupta et al., 2006) but their role in neuropathic pain has not been reported as yet.

The progression of diabetic neuropathy in a distal–proximal axon length-dependent manner suggests that damage is initiated in the axon (Leinninger et al., 2006b). Axons are susceptible to hyperglycemic damage both due to their direct access to nerve blood supply and their large population of mitochondria. Mounting evidence suggests that the hyperglycemic environment coupled with a compromised blood supply overloads the metabolic capacity of the mitochondria, producing oxidative stress (Brownlee, 2001, 2005; Perez-Matute et al., 2009). This oxidative stress leads to mitochondrial damage followed by axonal degeneration and death coupled with decreased pain threshold in diabetics.

Mitochondrial damage occurs due to excess formation of ROS and reactive nitrogen species (Nishikawa et al., 2000; Obrosova et al., 2005b, 2007). ROS, such as superoxide and hydrogen peroxide, are produced under normal conditions through the mitochondria electron transport chain and are normally removed by cellular detoxification agents such as superoxide dismutase, catalase, and glutathione (Leinninger et al., 2006b). Hyperglycemia leads to increased mitochondrial activity; raising ROS production in the mitochondria. Peroxynitrite, the primary RNS, is formed by
the reaction of superoxide and nitric oxide. RNS induces a number of cytotoxic effects including protein nitrosylation and activation of PARP (Obrosova et al., 2005a; Obrosova and Julius, 2005). Excessive ROS/RNS formation eventually overloads the natural antioxidant capacity of the cell, resulting in injuries to lipids, proteins and DNA. This damage ultimately compromises cellular function and integrity. As mitochondria are the origin of ROS/RNS generation, they are most susceptible to damage. Cellular oxidative stress is further enhanced when excessive glucose leads to overproduction of superoxide as a byproduct of mitochondrial oxidative phosphorylation (Vincent and Feldman, 2004). Overproduction of superoxide also markedly inhibits GAPDH, causing accumulation of upstream glycolytic intermediates. These intermediates further enhance AR, hexosamine, PKC and AGE production.

Experimental support for this unifying hypothesis derives from studies demonstrating that inhibition of superoxide accumulation by overexpression of superoxide dismutase prevents hyperglycemia-induced increases of AR (Nishikawa et al., 2000), hexosamine pathway products (Du et al., 2000), PKC activation and AGE formation. Thus, there exists a vicious feed-forward system in cells prone to diabetic complications, where glucose-activated metabolic pathways converge to produce cellular oxidative stress. Decreased nerve blood flow and ischemia, resulting from the processes described above, further exacerbate tissue injury. In summary, oxidative stress and ROS link the metabolic initiators and physiological mediators implicated in progressive nerve fiber dysfunction in diabetic neuropathy. The generation of ROS may initiate a feed-forward cycle in which oxidative stress itself impairs antioxidative defense mechanisms.

In our study, lipid peroxidation was significantly reduced by tocotrienol, lycopene and sesamol treatment. Non protein thiols, superoxide dismutase and catalase activities were significantly improved following treatment with tocotrienol, lycopene and sesamol. Tocotrienol has been reported to inhibit oxidative stress in plethora of disease conditions like ischemia-reperfusion injury, alcoholic complications, depression, dementia.
Exogenous administration of individual gradual doses of lycopene to hyperglycaemic rats causes a dose-dependent decrease in glucose level, a decrease of H$_2$O$_2$ and TBARS levels, as well as increased total antioxidant status with increased antioxidant enzyme activities (CAT, SOD and GPx) with improvement in serum lipid profile (Ali and Agha, 2009). Lycopene also acts as a potential antiatherogenic agent by preventing 7-ketocholestrol-induced oxidative stress and apoptosis in human macrophages (Palozza et al., 2009). Sesamol treatment enhanced antioxidant capacity of the diabetic brain and led to decreased perturbation of hyperglycemia-induced changes in BBB structure and function (VanGilder et al., 2009; Jan et al., 2009). Hsu et al. (2008) reported that sesamol attenuated oxidative stress by reducing xanthine oxidase. Sesamol also protected gastric mucosa against diclofenac-induced injury by inhibiting hydroxyl radical-associated lipid peroxidation. Therefore, tocotrienol, lycopene and sesamol might protect diabetic neuropathy by reducing oxidative stress in rats.

Inflammatory agents including C-reactive protein and TNF-α are present in the blood of both T1DM and T2DM patients (Gomes et al., 2003; Gonzalez-Clemente et al., 2005). Higher levels of these proteins correlate with the incidence of neuropathy (Gonzalez-Clemente et al., 2005). Recent data from the Eurodiab Prospective Complications Study demonstrates a correlation between diabetic neuropathy and plasma levels of heat shock protein (HSP) 27 (Gruden et al., 2008). HSP 27 is a required intermediate in the pathway of TNF-α induction of the inflammatory mediators as cyclooxygenase-2 (COX-2), IL-6, and IL-8. The production of the initiating inflammatory mediators TNF-α and TGF-β results from several of the glucose-induced pathways (Vincent and Feldman, 2004; Brownlee, 2005). When excess glucose is shunted through alternative metabolic pathways such as the fructose-6-phosphate or diacylglycerol, the signaling intermediates and modified transcription factors lead to increases in TGF-β and NF-κβ (Brownlee, 2001). Similarly, breakdown of glycolytic triose phosphates forms methylglyoxal, an AGE, that covalently modifies transcription factors (Yao et
One specific consequence of these modifications is decreased binding of a repressor of angiotensin II, known as Sp3. Thus, angiotensin II increases and leads to activation of vascular endothelial cells (Yao et al., 2007). In the endoneurium, this activation leads to inflammatory cell recruitment, local generation of cytokines, and reduced blood flow that leads to further generation of ROS (Coppey et al., 2006). Other extracellular AGEs that activate RAGE also lead to intracellular inflammatory signaling to upregulate NF-κB (Toth et al., 2008).

COX-2 is an important enzyme that is upregulated by NF-κB (Lee et al., 2004a). This upregulation is observed in peripheral nerves and vascular tissues in experimental diabetes (Kellogg and Pop-Busui, 2005). COX-2 activity appears to drive a feed-forward loop since COX-2 is upregulated by NF-κB and in turn it generates prostaglandin E2 and ROS that activate NF-κB. Pharmacological blockade or gene ablation of COX-2 prevents diabetes-induced changes in peripheral nerves including depletion of GSH, increases in TNF-α, and blood flow and nerve conduction deficits (Kellogg et al., 2007; Matsunaga et al., 2007). In diabetic DRG and nerve, TGF-β isoforms applied directly in-vitro reduce neurite outgrowth, and this effect is partially reversed by TGF-β neutralizing antibody. These findings implicate upregulation of TGF-β in experimental diabetic peripheral neuropathy and indicate a novel mechanism of cellular injury related to elevated glucose levels (Anjaneyulu et al., 2008).

IL-1β, IL-6, and TNF-α in the dorsal horn are increased after nerve lesion and have been implicated in contributing to nerve-injury pain, presumably by altering synaptic transmission in the CNS, including the spinal cord (Inoue, 2006). In the present study, a marked rise in the inflammatory cytokine (TNF-α, TGF-β1 and IL-1β) release was observed in the diabetic rats. Chronic treatment with tocotrienol, lycopene and sesamol significantly and dose dependently inhibited production and release of these cytokines. These results are in line with the observation that ghrelin, a potent anti-inflammatory agent; by inhibiting proinflammatory cytokines such as TNF-α and IL-1β and interleukin-6 (IL-6), ameliorated neuropathic pain (Guneli et al.,
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Tocotrienols have been shown to inhibit inflammatory cytokines such as C-reactive protein, IL-8, PAI-1 levels (Singh and Devaraj, 2007), TNF-α, and cytokine-induced neutrophil chemoattractant-1 mRNA levels (Hybertson et al., 2005). TNF-α and IL-1β levels were significantly reduced by tocotrienol in both serum and sciatic nerve of ethanol-treated rats (Tiwari et al., 2009b). Lycopene is reported to inhibit TNF-α-induced NF-κβ activation, ICAM-1 expression, and monocyte-endothelial interaction (Hung et al., 2008). Recently, we have reported that sesamol inhibited inflammatory cytokines in diabetic nephropathy (Kuhad et al., 2009). Thus inhibition of these inflammatory cytokines could be another possible mechanism involved in antinociceptive effects of tocotrienol, lycopene and sesamol.

Another inflammatory enzyme regulated by NF-κβ is inducible nitric oxide synthase (iNOS) (Kim et al., 2008a). Like COX-2, iNOS both induces and is induced by NF-κβ, leading to a vicious cycle of inflammation (Hasnis et al., 2007; Kim et al., 2008a). The NO generated by iNOS directly modulates the blood supply to nerves and participates in microvascular changes following injury (Levy and Zochodne, 2004; Zochodne and Levy, 2005). NO has direct roles in axon and myelin breakdown following an injury and also contributes to the development of neuropathic pain (Levy and Zochodne, 2004; McDonald et al., 2007). Excessive local levels of NO during inflammation may damage axons and growth cones (Zochodne and Levy, 2005). Total nitric oxide, an indicator of nitrosative stress, is increased in the experimental model of diabetic neuropathy (Kuhad et al., 2008). Tocotrienol, lycopene and sesamol treatment at varying doses attenuated the increased nitric oxide levels, may be, due to its iNOS inhibitory potential (De Stefano et al., 2007; Sengupta et al., 2006; Wu et al., 2008; Yam et al., 2009). Lycopene inhibited the inflammatory response of RAW 264.7 cells to lipopolysaccharide through inhibiting ERK/p38 MAP kinase and the NF-κβ pathway (Feng et al., 2009). Zhou et al. (2008) reported that the suppression of oxidative stress, the reduction of plasma TNF-α and NO levels, and the down-regulation of TNF-α in lungs contributed to the alleviation of pulmonary fibrosis in rats administered lycopene. Sesamol was also demonstrated to be effective in the
focal cerebral ischemia model of Sprague-Dawley rat, where sesamol dose-dependently (10-100 microM) attenuated nitrite production, iNOS mRNA, NF-κβ and p38 MAPK activation in lipopolysaccharide-stimulated murine BV-2 microglia (Hou et al., 2006).

The cytokines induced by NF-κβ in endothelial cells, Schwann cells and neurons also lead to macrophage recruitment in diabetic nerves (Yamagishi et al., 2008). Macrophages promote diabetic neuropathy through a variety of mechanisms, including production of ROS, cytokines and proteases, which result in myelin breakdown and cellular oxidative damage (Conti et al., 2002; Tesch, 2007; Kawamura et al., 2008). Excessive macrophage recruitment likely impairs nerve regeneration in diabetic neuropathy (Conti et al., 2002; McDonald et al., 2007). Thus it is proposed that enhanced oxidative-nitrosative stress and inflammatory cytokines triggered NF-κβ in the sciatic nerves of diabetic rats which led to nerve damage and precipitated neuropathic pain in diabetic rats (Ledeboer et al., 2005). Chronic treatment with tocotrienol, lycopene and sesamol significantly suppressed NF-κβ in the sciatic nerves of diabetic rats and alleviated pain threshold in diabetic rats via inhibiting the vicious cycle. Tocotrienol treatment caused a large decrease in NFκβ transcriptional activity, apparently by suppressing I kappa B-kinase (IKK)-alpha/beta activation, an enzyme associated with inducing NFκβ activation (Sylvester et al., 2005). Lycopene also inhibited the inflammatory response of RAW 264.7 cells to LPS by inhibiting ERK/p38 MAP kinase and the NF-κβ pathway (Feng et al., 2009).

Oxidative stress not only damages mitochondria, DNA, proteins, and membranes, but it also initiates signaling pathways that result in localized mitochondrial destruction called mitoptosis. One pathway essential to mitoptosis, and subsequently apoptosis, involves mitochondrial division via the dynamin related protein 1 (Drp1) (Frank et al., 2001; Lee et al., 2004b). Mitochondria normally undergo an equilibrium-driven process of fission and fusion. In times of stress, Drp1 translocates from the cytosol to the mitochondria so as to increase mitochondria fission events (Arnoult et al., 2005). Aberrant mitochondria fission is associated with mitoptosis and
implicated in apoptosis. Increased levels of Drp1 are found in in-vitro and in-vivo models of diabetic neuropathy (Leinninger et al., 2006a). This implicates mitochondrial fission in diabetic neuropathy and renders Drp1 a potential therapeutic target. As such, neurons in a hyperglycemic environment display signs of both oxidative stress and apoptosis (Russell et al., 1999).

To better understand the pathway leading to apoptosis in diabetes, we also investigated the expression of apoptosis-related protein, caspase-3, in the sciatic nerves of diabetic rat. In dorsal root ganglion cultured in defined medium, addition of moderate glucose levels results in neurite degeneration and apoptosis. These changes are coupled with activation of caspase-3, dependent on the concentration of glucose (Russell et al., 1999; Gou et al., 2004; Sharifi et al., 2009). In high glucose conditions, combination with TGF-beta2>beta1 increases the percent of cleaved caspase-3 (Anjaneyulu et al., 2008). In the present study, diabetes produced marked increase in caspase-3 activity in the nerves, this effect being amenable to reversal by tocotrienol, lycopene and sesamol. Very recently, we have reported that tocotrienol and sesamol inhibited caspase 3 activity in the kidneys of diabetic rats (Kuhad and Chopra, 2009; Kuhad et al., 2009). Lycopene has also been shown to prevent 7-ketocholestrol-induced oxidative stress and apoptosis in human macrophages (Palozza et al., 2009).

It has been observed that even with strict glycemic control, the onset and severity of diabetic complications is not affected. The concept of “metabolic memory,” that is of diabetic vascular stresses persisting after glucose normalization, has been supported both in the laboratory and in the clinic and in both type 1 and type 2 diabetes. The mechanisms for propagating metabolic memory appear focused on the nonenzymatic glycation of cellular proteins and lipids and on an excess of cellular reactive oxygen and nitrogen species, in particular originating at the level of glycated mitochondrial proteins and perhaps acting in concert with one another to maintain stress signaling independent of glucose levels (Brownlee, 2005; Ceriello et al., 2009). Therefore, “switching off” the metabolic memory, could be an important strategy for the prevention of diabetic complications. This
concept is supported by the results of the present study. Insulin could produce 50% reduction in blood glucose levels but failed to alter the biochemical and molecular indices of oxidative-nitrosative stress, inflammation and apoptosis. However, tocotrienol, lycopene and sesamol reduced blood glucose levels and also markedly reduced oxidative-nitrosative stress, proinflammatory cytokines and apoptosis. This led to a marked amelioration of accompanying neuropathic pain.

Therefore, the major finding of the study is that insulin alone reversed the hyperglycemia but partially reversed the neuropathic pain in diabetic rats. However, insulin in combination with tocotrienol, lycopene and sesamol not only attenuated the diabetic condition but also reversed neuropathic pain by “switching off” the metabolic memory phenomenon through modulation of oxidative-nitrosative stress, inflammatory cytokine release, NF-κβ and caspase-3 in the diabetic rats and thus these interventions may find clinical application to treat neuropathic pain in the diabetic patients.