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EXPERIMENTAL ARTICLES

Protective Effect of Lutein Supplementation on Oxidative Stress and Inflammatory Progression in Cerebral Cortex of Streptozotocin-Induced Diabetes in Rats[1]

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Abstract—Oxidative stress and inflammation are deemed to play a vital role in diabetic cerebral and neurological dysfunction. The present study was designed to investigate the protective effect of the naturally occurring antioxidant, lutein, against oxidative injury and inflammation in cerebral cortex (CCT) of diabetic animals. Using single IP injection of streptozotocin (STZ, 65 mg/kg) diabetes was induced in rats. Lutein dietary supplement was provided to diabetic animals for 5 consecutive weeks in three different doses. The extent of lipid peroxidation and cellular damage were estimated in CCT. Endogenous antioxidants molecules such as non-protein sulfhydryl groups (NP-SH) and enzymes including superoxide dismutase (SOD) and catalase (CAT) were also estimated in CCT. Levels of neurotrophic factors such as brain derived nerve factor (BDNF), nerve growth factor (NGF) and insulin growth factor (IGF) and pro-inflammatory cytokines, as markers for neural inflammation, were assessed in CCT. Lutein dietary supplement, significantly inhibited the diabetes induced increased in CCT levels of thiobarbituric acid reactive substances (TBARS), caspase-3, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6. Diabetes caused inhibition in the levels of NP-SH, DNA and RNA was significantly increased following lutein dietary supplementation to diabetic group compared to normal diet fed animals in dose dependent manner. Diabetes induced down regulation of BDNF, NGF and IGF was also attenuated by lutein dietary supplementation to diabetic model for 5 weeks. These findings suggest that lutein has the potential to ameliorate diabetes-induced oxidative and inflammatory damage and neural degeneration in the CCT.

Keywords: lutein, diabetes mellitus, oxidative stress, inflammation, streptozotocin, neural degeneration

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INTRODUCTION

The worldwide prevalence of diabetes mellitus (DM) is rising sharply and its long term complications such as nephropathy, retinopathy and central and peripheral neuropathy [1, 2] are challenges for clinicians due to their severity, chronicity and resistance to therapies. Oxidative stress is believed to be one of the major central pathophysiological factors in the DM induced cellular damages including neurons in the brain [3] along with persistent hyperglycemia that provoke free radicals generation especially reactive oxygen species (ROS) from glucose auto-oxidation and protein glycosylation [1, 4]. Lipid peroxidation (LPO) and its products namely TBARS, nitrite levels as well as total oxidants are reported to be elevated with a reduced levels and activities of total antioxidant molecules and enzymes in the brain of diabetic animals [5]. Furthermore, oxidative stress and LPO augments DM associated inflammatory response elevating the expression of proinflammatory cytokines including TNF-α and IL-6 and also increases the levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) as well as nuclear factor-kappa B (NF-κB) [6]. The central expression of neurotrophic factors in the brain plays a vital role in neurons survival, growth, and functional maintenance such as BDNF, NGF and IGF-1 were observed to have influence on cellular differentiation, synaptic connectivity, plasticity, growth and cell survival. These down-regulations of neurotrophins were explained impairments of neuronal survival of the diabetic brains [7–9].

The antioxidant content of vast verities of fruits and vegetables possess several physiological properties. These biologically active compounds are suggested to prevent oxidative stress-mediated diseases. Lutein a widely distributed carotenoids in fruits and vegetables, is one of these therapeutically active molecules [10]. Lutein is the second most prevalent carotenoid in

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human serum [11] with a suggested numerous biological actions including inhibition of cell transformation, inhibition of the monocyte-mediated inflammatory response, immune enhancement and in-vitro and in-vivo antioxidant activities [12–15]. It is an effectual quencher of singlet oxygen and the related ROS [16, 17]. Lutein also protects against LPO as well as protein and nucleic acids oxidative damage by improving other cellular antioxidant molecules and enzymes [18].

Therefore, the purpose of this study was to test the therapeutic efficacy of lutein against oxidative stress and neural inflammation and to evaluate its ability to enhance the neurotrophic support to protect the brain from diabetes deleterious effects.

MATERIALS AND METHODS

Animals

Male Wistar albino rat, 12 to 13 weeks old and approximately 250 g of weight were received from Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. They were maintained under controlled conditions of temperature (22 ± 1°C), humidity (50–55%), light (12 h light/12 h dark cycle) with free access to food and drinking water. They were acclimatized for 7 days for to the laboratory conditions. Animals handling, treatment, euthanasia and other experimental procedures were in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications no. 80-23; 1996) as well as the obtained approval (238-EACC-2014) from the Ethical committee of Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Diets

Experimental diets were prepared in pellet form by adding lutein (Carbone Scientific Co., Ltd., London, UK) in three different doses 40, 80 and 160 mg/kg in rat chow powder following shade dry method. During whole experimental period, all groups of animals were kept on free access to food and water.

Diabetes Induction

DM was induced by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in citrate buffer (pH 4.5), while Control animals received the same injection volume of citrate buffer without STZ. Two days later, the blood glucose values of the fasted animals were determined using an Accu–Chek Compact Plus glucose meter system (Roche Diagnostics, Meylan, France) and animals with blood glucose levels >250 mg/dL were considered as diabetic model.

Study Design

Control and diabetic animals were randomly divided in to five groups by taking six in each group, as follow; (1) Control (vehicle) (C), (2) Diabetic (STZ), (3) Diabetic rats were supplemented with lutein content (40 mg/kg) diet (STZ + L40), (4) Diabetic rats were supplemented with lutein content (80 mg/kg) diet (STZ + L80) and (5) Diabetic rats were supplemented with lutein content (160 mg/kg) diet (STZ + L160). Diets were fed at free access to diabetic model for 5 consecutive weeks. Body weights of the rats were recorded at the beginning and every week throughout the study period. Animals' general health was observed during treatment periods. At the end of treatment period, blood samples were collected through the cardiac puncture under light anesthesia and then sacrificed cerebral cortex (CCT) was dissected and stored at –80°C till analysis. Using a glass homogenizer, CCT tissues were homogenized by in 50 mM phosphate-buffred saline (pH 7.4) and then centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was used for the biochemical analyses.

Estimation of TBARS Levels in Brain Cells

Brain values of the LPO byproduct, malondialdehyde (MDA) was estimated using biochemical assay kit (ZeptoMetrix Corporation, Buffalo, NY, USA) by measuring the TBARS levels. In brief, 0.1 mL of CCT homogenate was added to 2.5 mL reaction buffer (provided by the kit) then the mixture was heated for 60 min at 95°C. After cooling down at room temperature, the color absorbance of the supernatant was measured. The quantified MDA levels were expressed as nmole/mg protein.

Estimations of NP-SH Levels in Brain Cells

Non-protein sulfhydryl group levels were estimated following the method described by Sedlak and Lindsay (1968). In brief, CCT homogenate was mixed with 50% trichloroacetic acid solution (TCA). Then after shaking intermittently for 10–15 min, samples were centrifuged for 15 min and 2 mL of the supernatant was mixed with 4 mL of 0.4 M Tris buffer (pH 8.9) and 0.1 mL DTNB. The absorbance was read within 5 min at 412 nm.

Estimations of SOD and CAT Activities

Enzymatic activity of the antioxidant enzyme SOD was assayed by the method described by Kono, (1978) [19]. The generated superoxide anions by oxidation of hydroxylamine hydrochloride reduced nitrobluetetrazolium to blue formazan mediated. The extent of this reduction was measured at spectrophotometrically under aerobic conditions. The enzymatic activity of the antioxidant enzyme CAT was assayed by the method described by Aebi, (1978) [20]. In brief,

0.5 mL of the post-mitochondrial supernatant of the CCT homogenate was mixed with 50 mM phosphate buffer (pH 7.0) and 20 mM H_2O_2. The produced color was recorded using spectrophotometer following the decrease in absorbance. All absorbencies were measured by a Pharmacia-LKB UVM II spectrophotometer (GE Healthcare Life Sciences, Marlborough, MA, USA).

Estimations of Caspase-3 Activity in Brain

Caspase-3/CPP32 activity was estimated by the commercially available colorimetric kit (Biovision Inc, Milpitas, CA, USA). Pre-chilled CCT homogenates were transferred to 96 well plates with lysis buffer. Reaction buffer and DEVD-*p*NA substrate was added to the samples and mixed well. Immediately after 2 h of incubation at 37°C in darkness, the plates were read for absorbance at 405 nm.

Estimations of Pro-Inflammatory Cytokines Levels in Diabetic Brain

Levels of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 were estimated in CCT homogenates using enzyme-linked immunosorbent assay (ELISA) technique following instruction provided by the kits (R&D Systems, Inc., Minneapolis, MN, USA). Values were expressed as pg/mg protein.

Estimations of Neurotrophic Factors Levels in Diabetic Brain

Protein expression of neurotrophic factors including brain derived nerve factor (BDNF), nerve growth factor (NGF) and insulin growth factor (IGF) were and quantified in normal and diabetic CCT homogenates using ELISA technique following instructions provided by the kits (RayBiotech, Inc., Norcross, GA, USA).

Estimations of Nucleic Acid Levels in Brain Cells

Nucleic acids concentrations in CCT were determined using Bregman, (1983) method [21]. The CCT homogenates were suspended in 10% ice-cold TCA and centrifuged. The developed pellets were extracted two times with 95% ethanol. For DNA quantification, portion of the extract was treated with diphenylamine reagent, while for RNA quantification the remaining portion of the extract was treated with orcinol reagent. The adapted Lowry method by Schacterle and Pollack, (1973) [22] was employed to estimate total protein (TP) levels in CCT using bovine plasma albumin as a standard. All absorbencies were measured by a Pharmacia–LKB UVM II spectrophotometer (GE Healthcare Life Sciences, Marlborough, MA, USA).

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) and analyzed statistically using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test. P values ≤ 0.05 was considered statistically significant. Graph Pad prism program (version 5) was used as analyzing software (Graph Pad Software, Inc., La Jolla, CA, USA).

RESULTS

Diabetes significantly ($P < 0.01$) elevated the brain values of TBARS, while inhibited the brain sulfhydryl's as compared to normal rats. All doses of lutein in diets reduced ($P < 0.01$) the level of brain TBARS in diabetic model as compared to untreated diabetic animals. The inhibited values of NP-SH due to diabetes showed significant and dose dependent increase in lutein diet supplemented rats when compared to normal diet fed diabetic animals. Diabetic model showed a significant ($P < 0.01$) inhibition in brain SOD and CAT activities as compared to control animals. The reduced SOD activities were significantly and dose dependently corrected by the lutein supplementations to diabetic model compared to STZ group. While the improvement of CAT activities in brain of diabetic animals was only noticed at the middle and high doses ($P < 0.05$ and $P < 0.01$) of lutein diets fed animals when compared to normal diet fed diabetic model (Fig. 1).

Caspase-3/CPP32 activity was significantly ($P < 0.01$) increased in CCT of diabetic brains compared to normal animals. The higher dose of lutein diet markedly ($P < 0.05$) decreased the caspase-3/CPP32 activity in diabetic animals compared to normal diet fed diabetic animals. The levels of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 were significantly ($P < 0.01$) increased in diabetic CCT homogenate compared to control ones. Both doses of lutein diets supplementation (80 and 160 mg/kg) to diabetic model markedly ($P < 0.05$) inhibited the elevated level of TNF-α in CCT of diabetic brain compared to normal diet fed animals. However, only the high dose of lutein content diet significantly ($P < 0.05$) the elevated levels of IL-1β and IL-6 in the CCT of diabetic model, while compared to STZ group values (Fig. 2).

Levels of BDNF, NGF and IGF in CCT of diabetic animals were significantly ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively) down regulated as compared to control group. The higher dose of lutein diet supplementation significantly ($P < 0.05$) increased the levels of BDNF and IGF in the CCT of diabetic brains compared to the normal diet fed diabetic model. However, the inhibited CCT levels of NGF in diabetic animals were significantly ($P < 0.05$) improved with lutein diets (80 and 160 mg/kg) as compared to normal diet fed rats (Fig. 3).

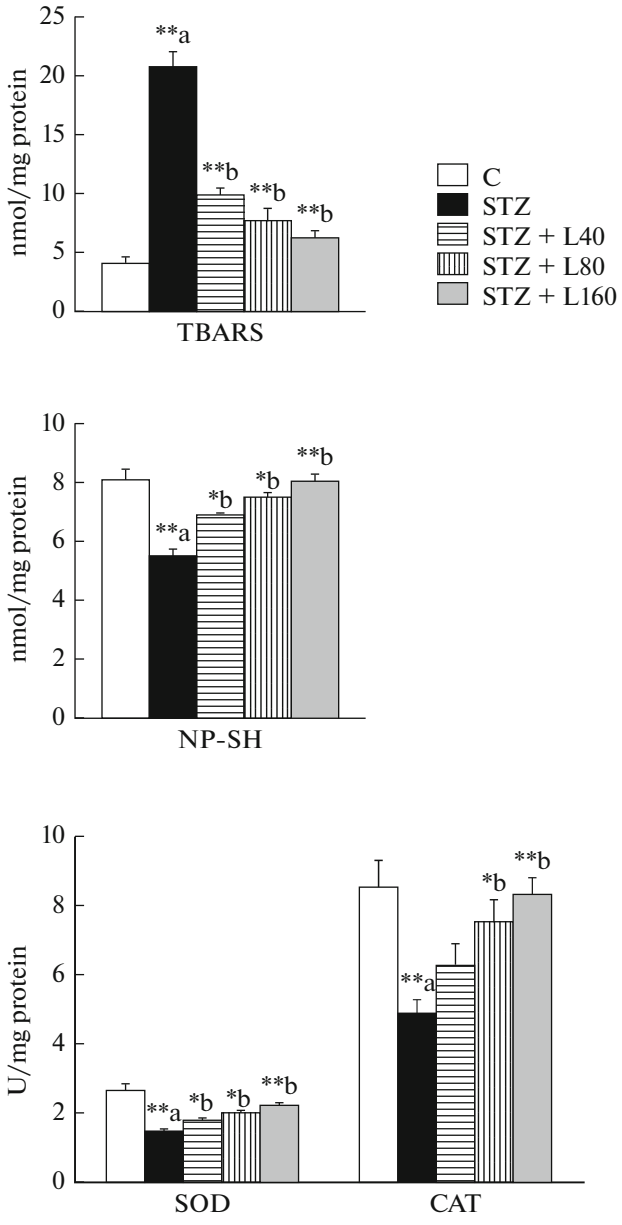

Fig. 1. Effects of lutein on CCT levels of TBARS, NP-SH and activities of SOD and CAT in diabetic model. All data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test. Six rats were used in each group. "a" STZ group was compared with C group and "b" lutein supplemented groups were compared with STZ group. *P* values were considered significant at $*P < 0.05$ and $**P < 0.01$.

Nucleic acids, DNA and RNA levels were significantly ($P < 0.05$ and $P < 0.01$ respectively) reduced in the CCT of diabetic animals compared to control rats. The DNA decreased values in the diabetic brain of CCT were restored by higher dose of lutein diet while the RNA values were corrected significantly ($P < 0.05$ and $P < 0.01$, respectively) with the medium and higher doses of lutein diet compared to normal diet fed diabetic group (Fig. 4).

DISCUSSION

Several cellular, molecular and functional reports suggested that DM and the associated hyperglycemia can induce brain injury in diabetic animals [7, 23, 24]. In present study, we tried to explain part of the brain damage mechanisms as well as therapeutic and protective potential of lutein dietary supplementation in attenuating this damage in the CCT of diabetic model. Lutein showed a significant ability to attenuated DM induced oxidative injury via restoring the endogenous antioxidants levels such as NP-SH and enzyme activities including CAT and SOD. Lutein also prevented LPO and cellular damage and down regulated TBARS and nucleic acids levels and caspase-3 activities. Furthermore, we explored the anti-inflammatory properties of lutein in diabetic brain region of CCT using the proinflammatory cytokines including TNF-α, IL-β and IL-6 as biomarkers. Finally, the potential protective effects of lutein against DM induced alteration in neurotrophic factors (BDNF, IGF and NGF) in the diabetic brain of CCT as a marker for neurodegeneration were reported. Our findings clearly demonstrated that, lutein therapeutic value to the diabetic animals as a beneficial drug with antioxidant, anti-inflammatory and neurotrophic support protective properties.

Oxidative stress and generation of ROS are suggested to be the fundamental mechanisms in the pathogenesis of tissue damage including brain during the burden of DM [3]. Several studies have shown that hyperglycemia induces oxidative tissue damage in various organs including retina, sciatic nerves, brain and bone of diabetic rodents [24–27]. DM is coupled with a reduction in the general antioxidant status of the body leading to increased risks of free radicals' deleterious effects [28]. Hyperglycemia can provoke ROS production from glucose auto-oxidation and protein glycosylation causing alteration in normal cellular defense mechanisms including sulfhydryl groups, which eventually leads to oxidative tissue damage. Furthermore, the increased levels of ROS in diabetes has been linked to decreased enzymatic antioxidant activities [29]. In the current study, the levels of NP-SH and activities of the antioxidant enzymes SOD and CAT were markedly reduced in the CCT of diabetic model. Lutein dietary supplementations to diabetic rats for 5 weeks attenuated the altered endogenous antioxidant molecules and enzymes in the CCT towards their normal control values. These reported properties are in agreement with earlier investigations, where lutein was proposed to have benefit effects following its intake due to its antioxidative potentials [15, 16]. It is also considered effective quenchers of singlet oxygen and related ROS [16, 17]. The chemical structure of lutein elucidates its antioxidant properties by showing two hydroxyl groups, one on each side of

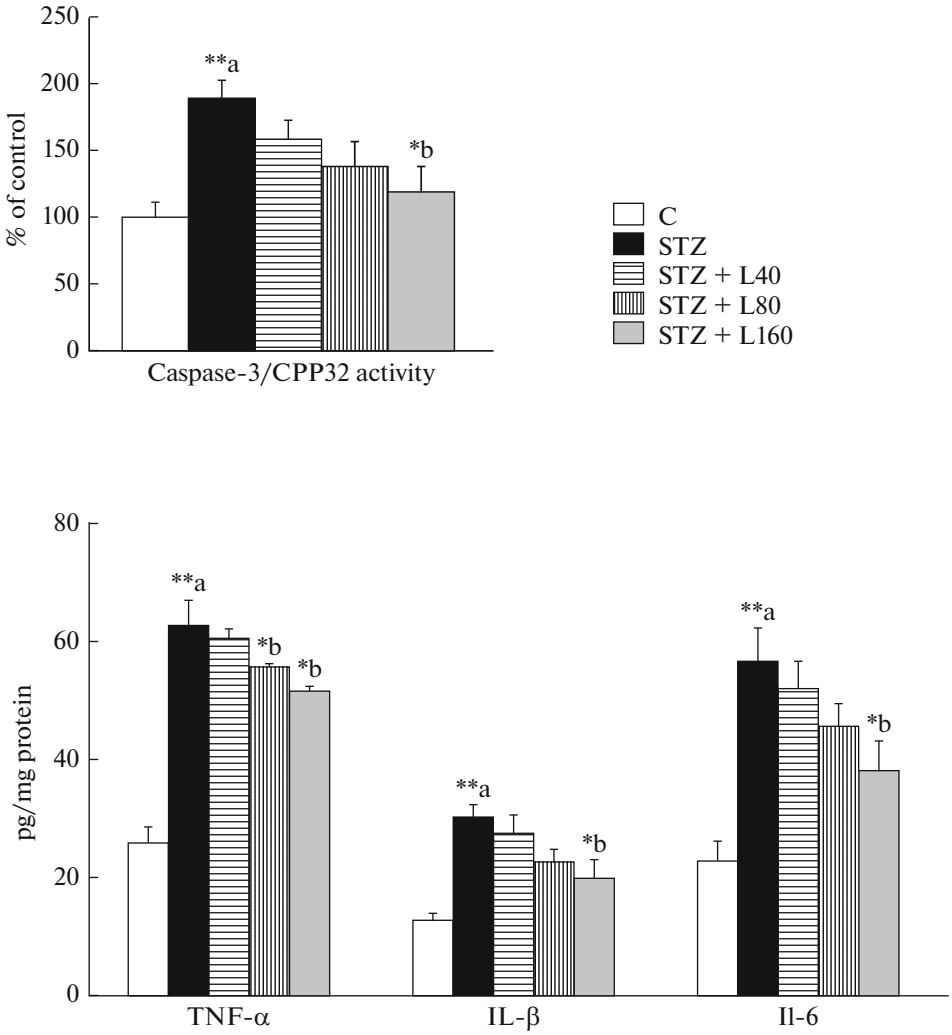

Fig. 2. Effects of lutein on caspase-3/CPP32 activity and on levels of TNF-α, IL-1β and IL-6 and in CCT of diabetic model. All data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test. Six rats were used in each group. "a" STZ group was compared with C group and "b" lutein supplemented groups were compared with STZ group. *P* values were considered significant at $*P < 0.05$ and $**P < 0.01$.

the molecule, which are believed to play a critical role in its biologic function [30, 31].

The present study clearly indicated that STZ induces a significant central LPO and tissue damage as the CCT levels of LPO product (TBARS) were markedly increased. LPO is known to induce cellular apoptosis, cytotoxicity, formation of DNA adducts and strand breaks [32, 33]. As a result, the levels of cellular nucleic acids (DNA and RNA) were reduced, while caspase-3 activity was markedly higher in brains of diabetic animals. The elevated levels of TBARS and caspase-3 activity were markedly lowered following lutein diet supplementations to the diabetic group. Moreover, estimated levels of nucleic acids (DNA and RNA) in CCT revealed that lutein can prevent cytotoxic and oxidative damage induced by STZ. This may be due to the inhibitory effects of lutein against LPO, DNA adduct formation and cellular apoptosis. Present results are further justified with earlier study that showed lutein supplementation to lens epithelial cells blocked H_2O_2-induced protein oxidation LPO and DNA damage and increased the levels of GSH and GSSG, particularly after H_2O_2 challenge [18].

Oxidative stress and inflammation are interrelated as oxidative stress can trigger inflammatory responses [34, 35], and also inflammation may enhance ROS production [36, 37]. In support of this idea, several experimental studies suggested that antioxidants administration effectively may prevent or treat inflammatory response in several metabolic syndromes including rheumatoid arthritis [38], arteriosclerosis [39] and vascular changes in diabetes [40]. Diabetic associated neuropathological state is characterized by inflammation and elevation of proinflammatory cytokines in brain. Among these proinflammatory bio-

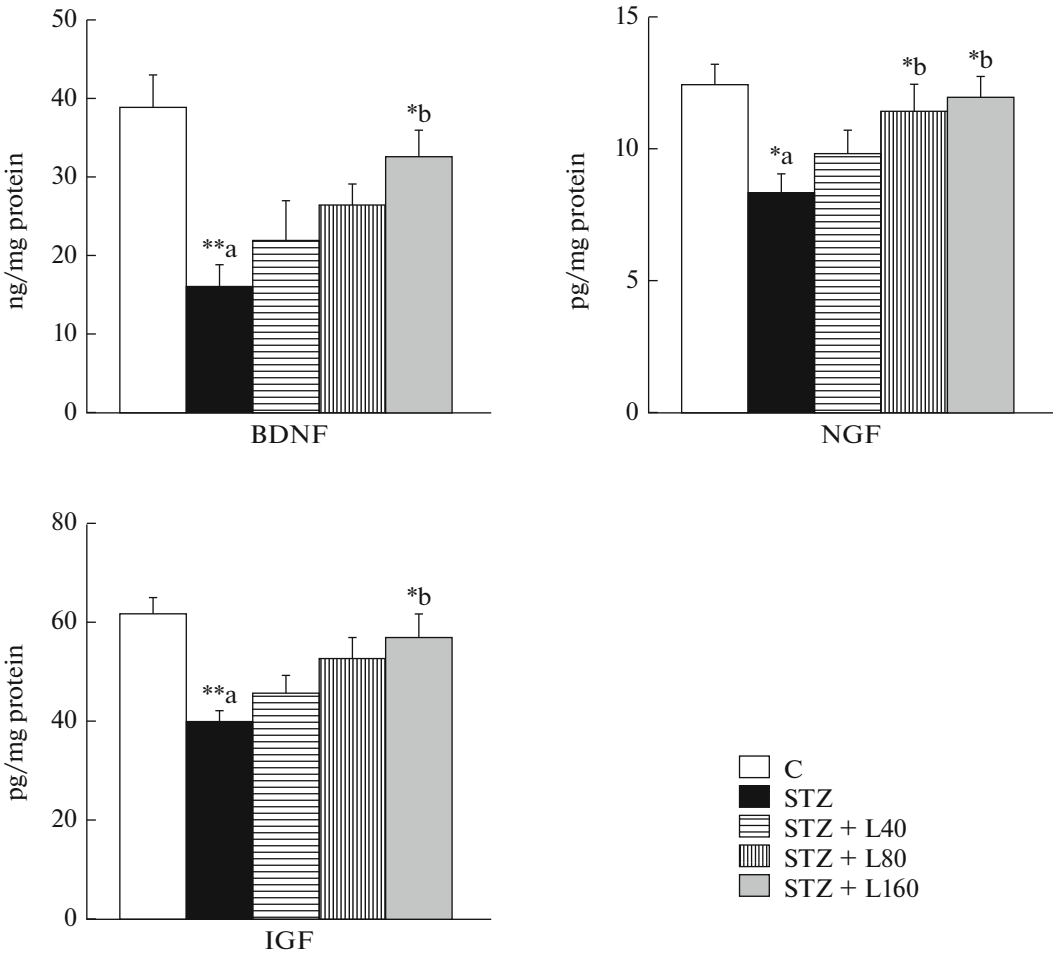

Fig. 3. Effects of lutein on CCT levels of BDNF, NGF and IGF in diabetic model. All data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test. Six rats were used in each group. "a" STZ group was compared with C group and "b" lutein supplemented groups were compared with STZ group. *P* values were considered significant at $*P < 0.05$ and $**P < 0.01$.

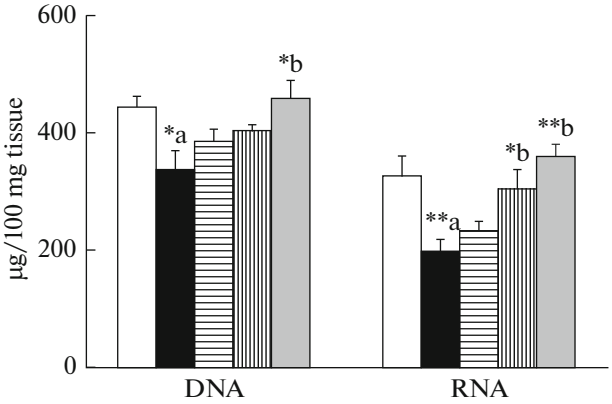

Fig. 4. Effect of lutein on nucleic acids (DNA and RNA) levels in CCT of diabetic model. All data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons test. Six rats were used in each group. "a" STZ group was compared with C group and "b" lutein supplemented groups were compared with STZ group. *P* values were considered significant at $*P < 0.05$ and $**P < 0.01$.

markers, TNF-α, IL-1β, and IL-6 are the major cytokines that set off the inflammatory response to hyperglycemia [41]. Elevation of the levels of these cytokines in diabetic brains rats may contribute to glial-induced neuronal death and degeneration. In present study, CCT levels of TNF-α, IL-1β, and IL-6 were markedly elevated in diabetic animals. However, lutein dietary supplementation with different doses showed significant reduction those cytokines in CCT. Such anti-inflammatory effects of lutein also reported earlier in experimental studies [15, 16]. Rising evidences showed that, lutein produced anti-inflammatory functions may include a reduction in serum levels of C reactive protein and soluble intercellular adhesion molecule [42, 43]. It also modulates the inflammatory reactions via preventing the inactivation of proteasome by oxidative stress [44] as well as suppressing the activation of NF-κB, one of the downstream effectors of inflammatory cytokine signaling [45]. Therefore, lutein may exert cerebroprotective action via its anti-inflammatory properties.

Neurotrophic factors including BDNF, IGF and NGF have a crucial role in the survival and maintenance of neuronal cells. BDNF promotes neurons survival via modulating their growth, maturation and maintenance. It also regulates neural circuit structure and synaptic plasticity in the adult brain [7]. IGF has a structure and functional anabolic similarity with insulin. It prevents neural cells apoptosis and regulate its growth and proliferation as well as DNA synthesis [46]. NGF also has a vital neuro-protective function as it potentiates axonal development. Pathological conditions that alter the expression of NGF may lead to neuronal functional failure and death [47]. In the present study, experimental diabetes markedly decreased the levels of BDNF, NGF and IGF in CCT by indicating neurodegeneration occurred in the diabetic brains, which is in consistent with other studies [7, 9]. Lutein dietary supplementation to the diabetic animal's markedly ameliorated diabetes induced reduction in neurotrophic factors levels. Similarly, several natural compounds showed the ability to induce synthesis and secretion of these factors in glial cells and brain [48, 49]. Therefore, lutein might demonstrate cerebroprotective action under diabetic conditions through induction of neurotrophic support to the neuronal cells.

CONCLUSIONS

In conclusion, findings of the present study suggested that lutein owns a clear restorative potential against neuronal injury and degeneration via its antioxidant, antiapoptotic, anti-inflammatory and neurotrophic supportive actions in the diabetic-induced neurological disorders. Finally, lutein may represent an important therapeutic molecule with a potential to limit neuro-deterioration and prevent or reverse diabetic-induced encephalopathy.

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