Changes in the brain cortex of rabbits on a cholesterol-rich diet following supplementation with a herbal extract of *Tribulus terrestris*

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Summary. Extracts of the medicinal herb *Tribulus terrestris* (TT) are used for treating various diseases. The saponins, a component of TT, play a role in regulating blood pressure and in treatment of hyperlipidemia. The aim of the study was to investigate the immunohisto-chemical and ultrastructural alterations in the cerebral cortex of experimental rabbits on a cholesterol rich diet treated with TT. The rabbits were divided into three groups and followed for 12 weeks as control group (CG); experimental group I (EG-I), fed with a cholesterol-rich diet; experimental group II (EG-II), treated with an extract of TT (5mg/kg/day) after a cholesterol-rich diet of 4 weeks. In EG-I there were ultrastructural changes, including mitochondrial degeneration, increased lipofuscin pigments, myelin sheath damage with axoplasmic shrinkage and electron dense granules in the neurovascular unit. The number of synapses apparently decreased in both experimental groups. Administration of TT extract in EG-II led to marked ultrastructural alterations in neurons, including decreased mitochondrial degeneration (P<0.001) and extensive oedematous areas in the neurovascular unit. However, in EG-II, lamellar myelin, axonal structures and mitochondria were well protected. These alterations possibly indicate that saponins have an effect on the neurons either directly or by its conversion to steroidal saponins. Therefore, these findings add further evidence supporting the protective claims of TT in cerebral architecture in dietary induced hyperlipidemia.

Key words: *Tribulus terrestris*, Cerebral cortex, Rabbit, Ultrastructure, Saponin

Introduction

Although the brain constitutes a very small portion of the body weight, it contains 25% of the total body cholesterol. As cholesterol is a large molecule, it is believed that under normal conditions it is very difficult to pass the blood-brain barrier (BBB), and is synthesized *in situ*. Excess cholesterol is exported from the brain in oxysterol and steroid forms that can pass the BBB (Lutjohann and Von Bergmann, 2003; Vaya and Schipper, 2007; Wang et al., 2007). Cytochrome P450 enzymes are responsible for the oxidative metabolism of a plethora of endogenous and exogenous substrates. Usually they form part of multi-component electron transfer chains, known as P450-containing system. This system, which converts the cholesterol of the central nervous system to oxysterols is mainly localized in the neuronal mitochondria. Both oxysterols and steroids are biologically active and interact with ion channels to modulate neural transmission (Vaya and Schipper, 2007; Wang et al., 2007).

Cholesterol and phospholipids are found to be significantly different in synaptic mitochondria compared to non-synaptic mitochondria (Ruggiero et al., 1992; Brown et al., 2006), however, the composition of lipids is highly variable according to the brain region. Total lipid content is highest in the medulla oblongata, followed by hippocampus, cerebellum, basal ganglia, and frontal cortex. This regional distribution may be attributed to their relative gray/white matter content (Chavko et al., 1993), and this is also valid for lipid sub-fractions.

It is well known that hypercholesterolemia affects brain functions in various ways. Hyperlipidemia, besides its atherogenic effects on the vascular system, may also activate hemocoagulation processes by increasing factor Xa (Cipolli et al., 1991) and lead to cerebrovascular

ischemia. However, high serum cholesterol may exert additional local effects in the brain. Agar et al., (1994) showed that hypercholesterolemia significantly depressed brain waves in the spectral analysis of EEG recordings from parietal lobes of rats (Agar et al., 1994). It was also reported that high cholesterol diet could lead to segmental atrophy and gliosis in optic nerves (Yanko et al., 1983) and to cause ultrastructural damage and severe oedema in the cochlea (Satar et al., 2001). The cerebellum, however, is not affected by a cholesterol-rich diet in terms of formation of reactive microglial cells and perivascular cholesterol rich phagocytes (Lafarga et al., 1994). There is growing evidence to suggest that cellular cholesterol homeostasis may be causally involved in different pathological change events in the brain, leading to Alzheimer's disease (AD) (Kirsch et al., 2003). Furthermore, serum hypercholesterolemia may be an early risk factor for the development of AD amyloidal pathology (Pappolla et al., 2003). Although still controversial, some cross-sectional observations have reported that the use of statins may potentially suppress the development of AD (Rockwood, 2006). Statins are widely used to control hyperlipidemia and for cardioprotective purposes. It was previously reported that different statins, regardless of their brain availability, induce alterations in cellular cholesterol distribution in the brain (Kirsch et al., 2003).

There are several medical herbs in traditional medicine, used alone or in combination, to treat various cardiovascular diseases. One such traditional herb is Tribulus terrestris (TT), which is a member of the Zygophyllaceae family. Extracts of this herb are widely used in traditional Chinese and Indian medicine (Anand et al., 1994; Shi et al., 1998) for cardiovascular diseases (Adaikan et al., 2000; Adimoelja, 2000; Al-Ali et al., 2003; Phillips et al., 2006) and also to treat sexual dysfunction (Gauthaman et al., 2003;Gauthaman and Adaikan, 2005).

Pharmacologically this plant has several different active compounds which have been classified into various groups (Li et al., 2002; Chu et al., 2003; Yang et al., 2005; Zhang et al., 2005). The basic active compound of TT is the steroidal saponin (Phillips et al., 2006), which includes different terrestrosin types (Yan et al., 1996) and has a hypolipidemic effect (Yang et al., 1999; Chu et al., 2003).

The biochemical, pharmacological, and physiological effects of TT on the central nervous system are less clear. The first report about the influence of TT on the brain in sheep showed development of symptoms such as asymmetrical pelvic limb paresis and spontaneous rotational behaviour (Bourke, 2006). Moreover, these clinical signs did not respond to a single high dose of levo-dopa administration. Histological examination revealed that nigrostriatal levels of dopamine and 3,4-dihydroxyphenylacetic acid were decreased significantly, suggesting a presynaptic dopaminergic disorder in the nigrostriatal region (Bourke, 1987). It was later suggested that TT toxicity in

sheep could induce motor neuron disease with scattered areas of mild Wallerian degeneration throughout the spinal cord (Bourke, 2006). In addition small numbers of degenerated astrocytes containing novel cytoplasmic pigment granules were observed (Bourke, 2006). Despite the neurotoxic effects on the brain and spinal cord, TT caused no apparent change in the sheep peripheral nervous system even after long-term administration (Bourke et al., 1992). Another study indicated that TT may contain active components that affect the normal functional development of fetal brain in sheep (Walker et al., 1992).

The effects of chronic high cholesterol and TT extract on the brain need further elucidation to reveal the pathogenetic mechanisms. It is not known whether TT administration is beneficial in treating damage caused by high serum cholesterol. The aim of the present study was to investigate the ultrastructural alterations in the cerebral cortex due to high cholesterol diet, and to see whether TT extract treatment can ameliorate possible damage.

Materials and methods

Experiments and light microscopy procedures

Eighteen adult New Zealand male albino rabbits (3.5-4.0kg) obtained from Experimental Animal Center of Cerrahpasa Medical Faculty were used and randomly divided into three groups (n=6 per group): control group (CG), and two experimental groups I and II (EG-I, EG-II). The animals were housed individually in stainless steel cages under standard conditions (21 ± 2°C and 50 ± 5% humidity) with a 12 hour light-dark cycle as described previously (Demir et al., 2005). The animals were kept in the laboratory for two weeks prior to the experiments to allow for acclimatization. The experimental protocol was reviewed and approved by the Committee for Ethics in Animal Care and Experiments, Istanbul University, Cerrahpasa Medical Faculty.

The control group (CG) was treated with a standard rabbit diet (composed mainly of vegetables together with 25% saturated and 75% unsaturated fat) and tapwater ad libitum. The experimental group-I (EG-I) was treated with a specially fabricated cholesterol-rich diet composed of standard diet with an additional 1% cholesterol (Merc103672, Germany) and tapwater for 12 weeks. The experimental group-II (EG-II) was treated with the cholesterol-rich diet for four weeks and then treated orally with TT extract, once a day for 8 weeks in addition to the cholesterol-rich diet. The dose of 5 mg/kg/day of TT extract dissolved in physiological saline solution was administered to EG-II as recommended in the previous studies (Gauthaman et al., 2002).

Transcardiac perfusion was performed with Ringer's solution (NaCl, 111.87 mM; KCl, 2.47 mM; CaCl$_2$, 1.08 mM; NaHCO$_3$, 2.38 mM, pH 7.2) for each rabbit (Demir

et al., 2005). Brain tissues from the temporal lobe were removed and followed for both light and electron microscopy. Tissue samples were embedded in paraffin after fixation in 10% formalin for the light microscopy. In addition serum cholesterol levels were measured.

Biochemical analysis

Blood samples were collected at day 0, and weeks 4 and 12 from all groups and TC, HDL-C, and LDL-C and TG levels in serum were measured on Beckman Coulter Synchron LX20 PRO (Beckman-Coulter Inc., Fullerton, CA) using Syncron system reagents.

Immunohistochemistry

The details about the procedures used were described in our recent study (Sati et al., 2007). Briefly, serial paraffin sections were collected on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO), dewaxed, dehydrated, and placed in citrate buffer. To unmask antigens, an antigen-retrieval procedure was performed by treating the samples twice in a microwave oven at 750W for 5 min each time. After cooling for 20 min at room temperature, the sections were washed in phosphate buffered saline (PBS) and then kept in 3% H_2O_2 for 15 min to remove endogenous peroxidase activity, followed by three washes with PBS. After blocking with Ultra V blocking reagent (Lab Vision, Fremont, CA) for 10 min at room temperature to reduce non-specific binding, sections were incubated with 1:200 dilution of goat polyclonal anti-collagen type I (Chemicon, Temecula, CA), and 1:300 dilution of goat polyclonal anti-vimentin (Innovex Biosciences) antibodies and incubated overnight at 4°C. The antibodies were detected with LSAB2 Streptavidin–biotin horseradish peroxidase kit (Dako, Glostrup, Denmark) for 30 min at room temperature. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (Lab Vision) prepared as 1-2 drops (40-100µl) of DAB chromogen with each 1ml of DAB substrate. Sections were counterstained with Mayer's hematoxylin (Dako, Denmark) for 10 seconds, dehydrated, mounted, and examined with an Axioplan microscope (Zeiss, Oberkochen, Germany). Additionally, tissue samples were stained with CD34 (Santa Cruz, CA), an endothelial marker, in order to show the surface lining of the blood vessels. Negative control stainings were performed by replacing the primary antibodies with normal goat IgG serum (Dako, Denmark) at the same concentration.

The immunoreactivity was evaluated according to our recent paper (Sati et al., 2007). Briefly, staining intensity of target cells was denoted as 0 (undetectable), 1+ (weak), 2+ (distinct), 3+ (intense). For vimentin immunostaining, an HSCORE value was calculated for each slide by summing up the percentages of cells grouped in one intensity category and multiplying this

number with the weighted intensity of the staining, using the formula [HSCORE=Pi (i+1)], where i represents the intensity scores and Pi is the corresponding percentage of the cells. Two observers blinded to the tissues performed the HSCORE evaluations at different times. Since the collagen type-I is a fibrillar structure no HSCORE evaluation was made.

Ultrastructural analysis

Following cardiac puncture, aorta was catheterized and the brain was fixed with 2.5% glutaraldehyde in 0.08M phosphate buffer, pH 7.4. Additionally, the glutaraldehyde-osmium tetroxide double fixation method was applied in order to examine the samples by transmission electron microscopy (TEM). The tissue samples were post-fixed in 1% osmium tetroxide solution in isotonic phosphate buffer, pH 7.4 solution at 4°C for one hour as described previously (Demir, 1980; Acar et al., 2004). Following dehydration steps samples were embedded in Araldite kit (Electron Microscopy Sciences, Fort Washington, PA, USA). The semithin and thin sections were cut using an LKB Nova ultramicrotome. Thin sections were contrasted with uranyl acetate (5 g uranyl acetate in 100 ml methanol) and Reynolds's lead citrate solution (1.76 g sodium citrate, 1.33 g lead nitrate, 50 ml distilled water and 8 ml NaOH) (Demir et al., 2005). The thin sections were examined under Leo 906 Electron microscope.

Quantitative analysis

In order to evaluate the fine structure of cerebral cortex cells, transmission electron micrographs were standardized at the same magnification (x2,500). Forty-five electron micrographs (15 from each group) were randomly selected and evaluated for: (a) total number of perikarya (23 pyramidal neurons for each group; total n=69) and their subcellular components, (b) lipofuscin pigment granules, (c) damaged mitochondria versus total mitochondria, (d) non-myelinated axon sections, (e) damaged myelin sheath, (f) vacuoles in the neuropil and (g) synapses in the neuropil. The quantification method was applied as described previously (Demir et al., 2002). Following the quantification, the electron micrographs were arranged according to experimental groups.

Statistical analysis

Data analysis was carried out by Sigma-Stat 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA). Differences among groups and the versus comparisons of the morphometric parameters were compared with One-Way ANOVA analysis on normally distributed data and the Kruskal-Wallis One-Way ANOVA on ranks on data that were not normally distributed. Following ANOVA, the Holm-Sidak all pair-wise multiple comparison method was also performed. All data are presented as mean ± SD.

Results

Immunohistochemical analysis

In all experimental groups (EG) morphology of the perikaryon and neuronal processes in the cortical layers of motor cortex were histologically normal. Even though there was a weak vimentin immunoreactivity in the different areas of cerebral cortex, a strong immunopositivity was observed around the vascular structures (Fig. 1A,D,G,M) as observed for collagen type-I immunoreactivity (Fig. 1B,E,H). The intensity of
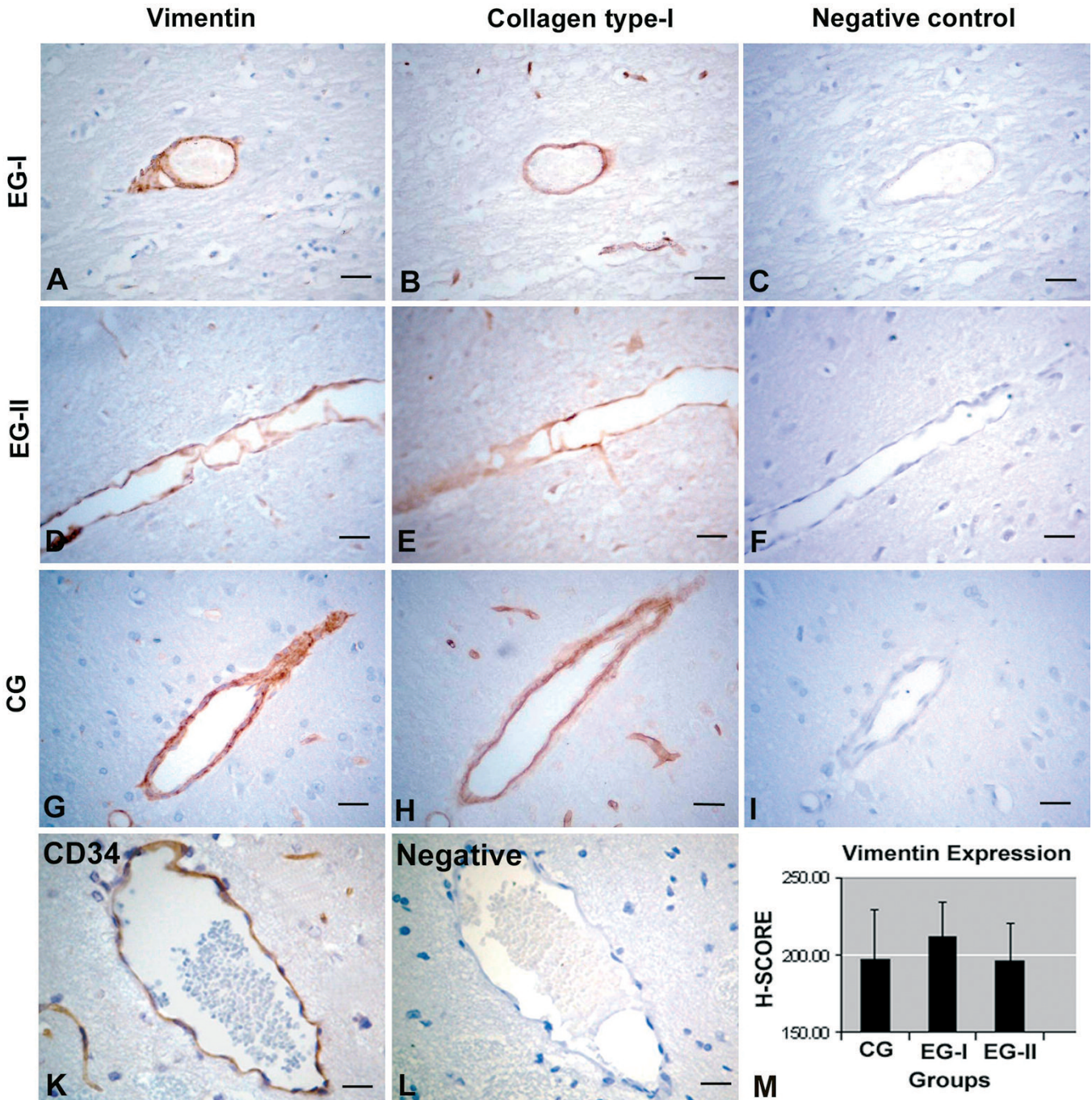
Fig. 1. Immunohistochemical staining of vimentin **(A, D, G)** and collagen type-I **(B, E, H)** around the microvascular structures in the brain tissues. Negative control slides showed no staining **(C, F, I)**. CD34 immunostained vascular structures **(K)** and the negative control **(L)** are presented. The immunohistochemical distribution of vimentin immunostaining by means of HSCORE **(M)**. Scale bars: 200 μm.

the immunolabeling for vimentin in EGs was not statistically significantly different from CG (Fig. 1M). However, the intensity of collagen type-I immuno-labelling in EG-I was stronger than EG-II and CG. Additionally, tissue samples were stained with CD34, regarded as a common diagnostic endothelial marker in order to determine vessels (Fig. 1K,L).

*Biochemistry: serum lipid profiles*

The biochemical analyses were submitted in our recent study (Tuncer et al., 2009). These results are summarized as follows: at the end of week 4 with the cholesterol-rich diet there was a significant increase in the lipid profiles of both EGs compared to CG (Table 1). Following TT extract treatment, TC, LDL-C, HDL-C and TG levels decreased significantly, 65%, 66%, 64% and 55 % respectively (p<0.001) (Table 1) (Tuncer et al., 2009).

Ultrastructural analysis

Control group (CG)

The cerebral cortex showed no significant ultrastructural degeneration in the perikarya or neuropili,
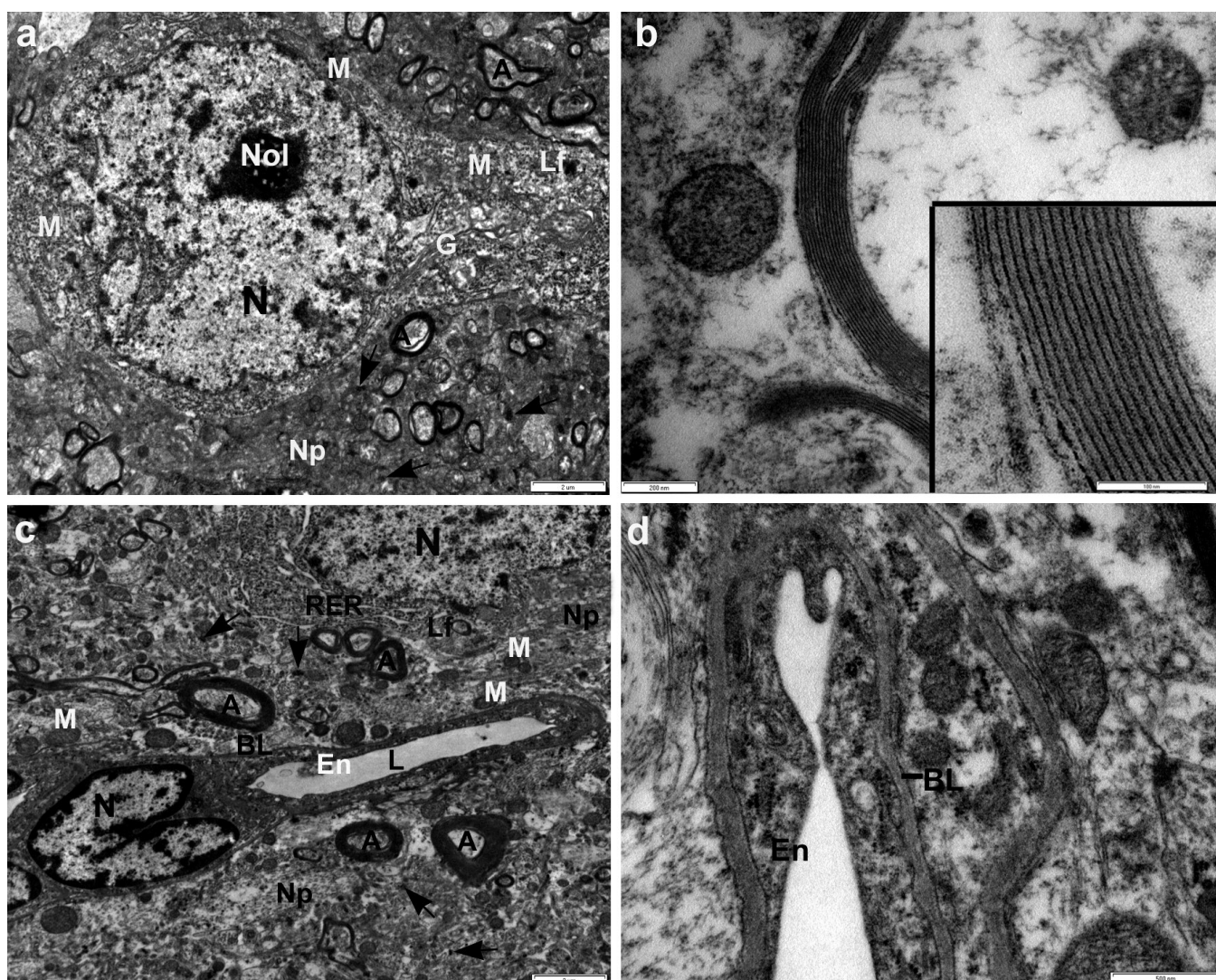
Fig. 2. The ultrastructure of brain tissue with some cellular components in control group (CG). Perikaryon and neuropil (Np) with subcellular elements in normal appearance **(a)** and normal myelin sheaths with many regular lamellae and axoplasm (**b**, inset picture), and numerous synaptic points (**a, c**; with single arrows) are seen. The blood-brain barrier (BBB) unit with healthy main structural components are presented **(c, d)**. N: nucleus, Nol: nucleoli, M: mitochondrion, G: Golgi complex, Lf: lipofuscin body, En: endothelium, BL: basal lamina, L: lumen, A: axon, RER: rough endoplasmic reticulum. Scale bars: a, c, 2 µm; b, 200 nm, inset, 100 nm; d, 500 nm.

including myelinated and non-myelinated axons (Fig. 2a). The subcellular components and synaptic connections also showed normal morphology throughout the cortical layers. The pyramidal neurons were clearly observed with euchromatic nucleus, including very active nucleoli and subcellular organelles (Fig. 2a). Myelin sheaths with axoplasm appeared in healthy condition (Fig. 2b; inset).

The ultrastructure of the blood-brain-barrier (BBB) unit was observed and the endothelium with basal lamina and glial cell endings around the vascular structures were also in normal condition (Fig. 2c,d).

Experimental group-I (EG-I)

In contrast to the control group, there was evidence of subcellular degeneration in the perikarya. Some lipofuscin pigment bodies, often called aging pigments,
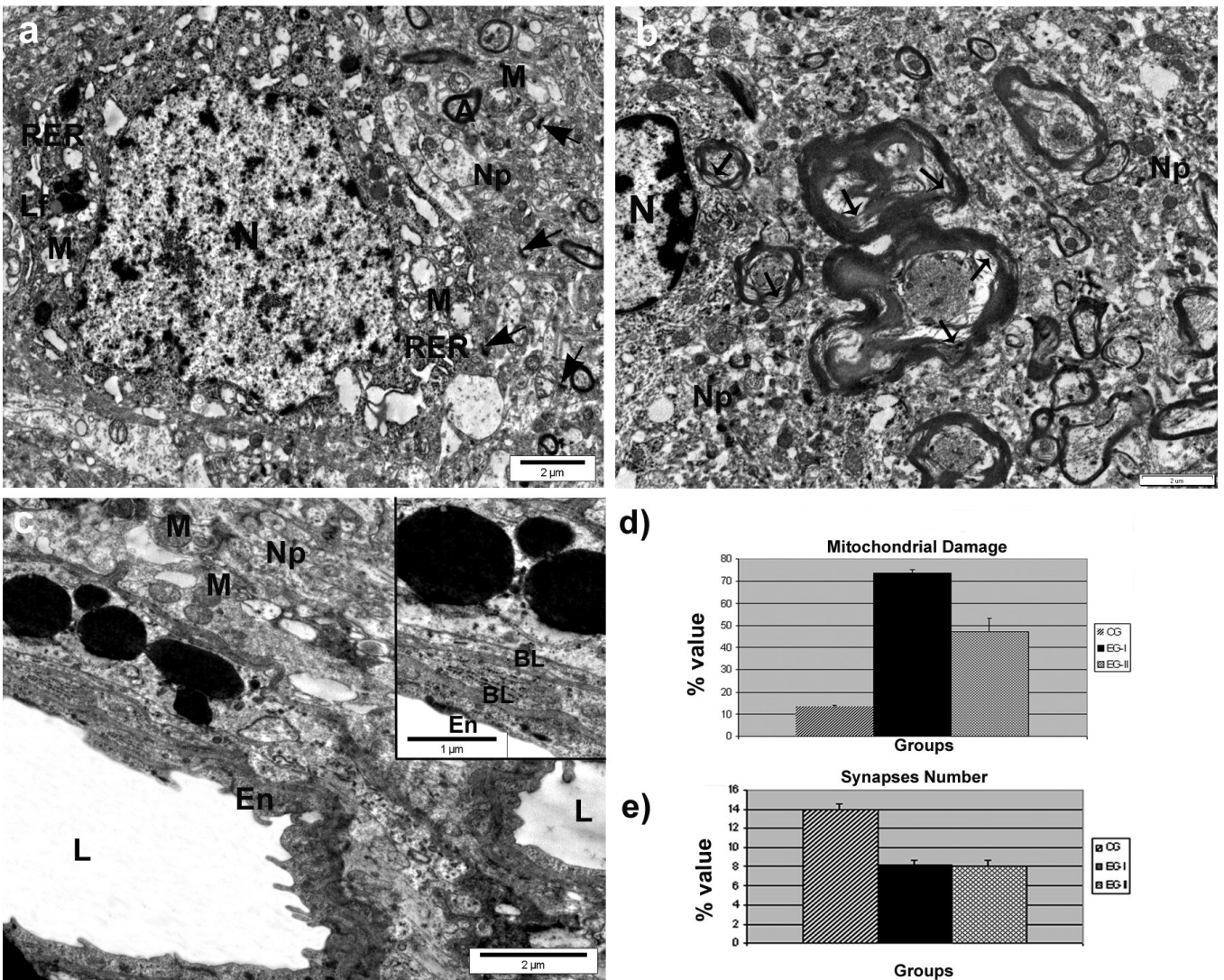
Fig. 3. The structural alterations in cerebral cortex of EG-I. A pyramidal neuron with subcellular components and neuropili (Np) were observed. The cytoplasm of the perikaryon with many organells and amount of inclusions and heterochromatic nucleus (N) are seen (a). Marked inner membrane forming cristae break down and matrix loss in some mitochondria (M) **(a)**, rough endoplasmic reticulum cisternae (RER) with numerous ribosomes and polysomes (Nisll bodies), a lot of lipofuscin pigment bodies (Lf) are seen **(a)**. The value of mitochondrial damage in all groups are compared. The mitochondrial damage was increased in EG-I compared to EG-II and CG (P<0.001) **(d)**. Myelin sheath degenerations **(b)** and separations with shrinkage of axoplasma (with fine arrows) with slightly increased cytoskeletal components of axonal organization. Interestingly, some very dense electron bodies (**c**, inset) stored in blood-brain barrier unit that are enveloped by cellular processes and extracellular matrix (inset). Synapse points are seen (with thick arrows; a), and there weren't any significant differences between EGs regarding to the number of synapses **(e)**. However, significant differences were observed between EGs and CG. En: endothelium, BL: basal lamina, L: lumen, A: axon, Np: neuropil, RER: rough endoplasmic reticulum. Scale bars: a, b, c, 2 µm; inset, 1 µm.

were observed with different sizes and shapes. Lipofuscin bodies were the most abundant pigments observed in both EGs compared to the CG (Figs. 3a, 4a,b). In addition, dilated endoplasmic reticulum and small vacuoles were present in the cytoplasm. Although obvious mitochondrial damage was observed in both EG-I and EG-II, cristae degeneration and matrix loss were more severe in EG-I compared to EG-II (Figs. 3a, 4a). However, there was an apparent decrease in the degree of mitochondrial damage in EG-II followed by TT treatment (Fig. 3d).

Marked myelin sheath degeneration, shrinkage in the axoplasm with damaged subcellular elements were observed in all cholesterol-rich fed samples (Fig. 3b). These degenerative changes were frequently dispersed in every cortical layer, but not homogenously distributed because some myelin sheaths were observed to be more affected than the others.

In addition, the quantity of neurotubules and density of the neurofilaments in the axoplasm were increased in both EGs, particularly in EG-I (Fig. 3b). Some large electron dense bodies with different sizes and irregular shapes were seen under the basal lamina of the vascular endothelium (Fig. 3c, inset). A strong envelope of
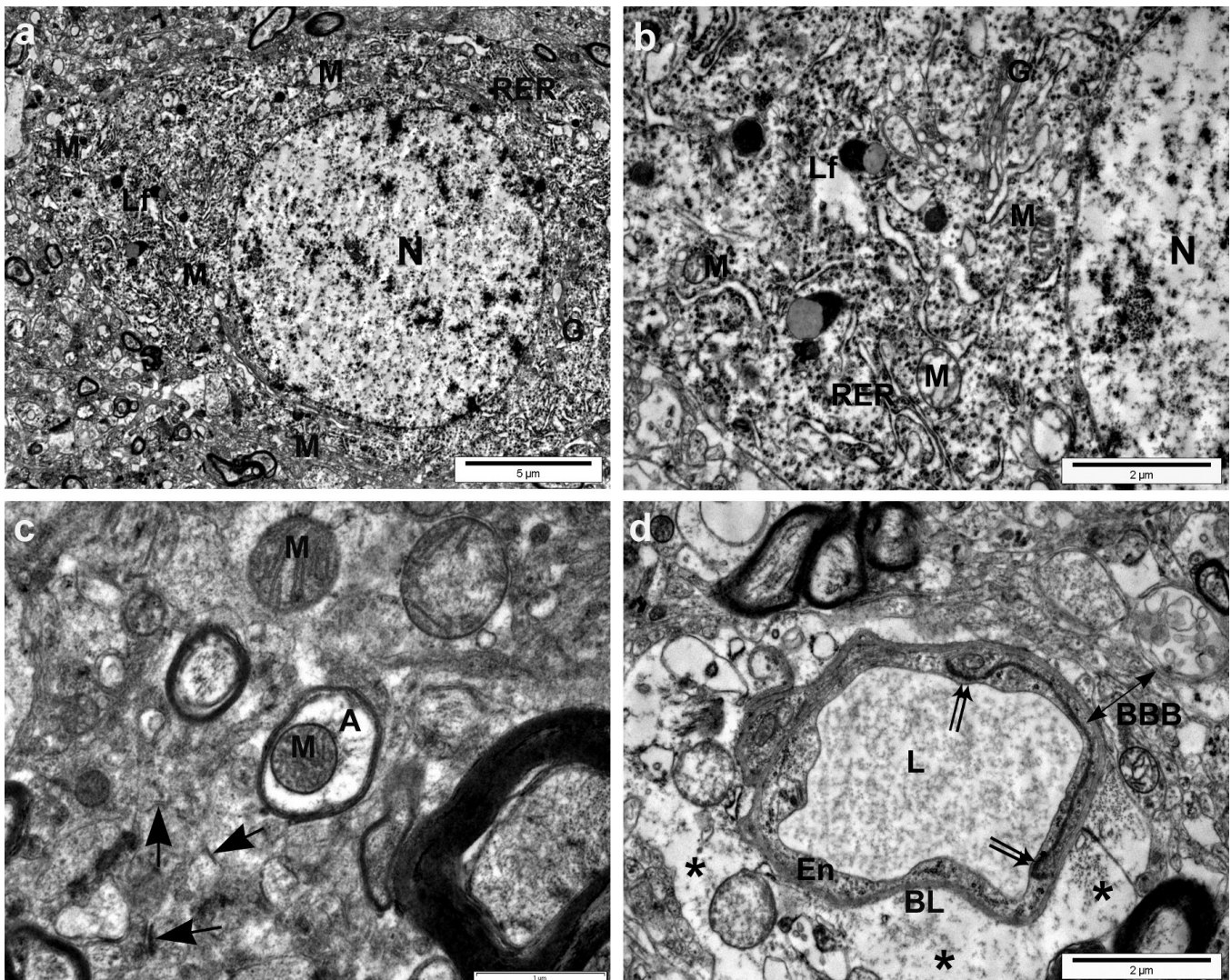
Fig. 4. The structural alterations in cerebral cortex of EG-II. Well protected perikaryon with prominent nucleus (N), rough endoplasmic reticulum (RER) and mitochondrion (M) are presented **(a, b)**. Damaged mitochondria and electron dense lipofuscin bodies (Lf) were decreased in the perikarya and neuropil (Np) with subcellular components **(a, b)**. Preserved myelin sheaths and axoplasma with definitive mitochondria and neuropilic arrangement with many synapses (thick arrows) are observed compared to EG-I **(c)**. The structure of endothelium (En) with definitive connecting complexes (double arrows) and its basal laminae (BL) are in normal condition, but very large oedematous areas (asterix) are present around the vascular structures forming neurovascular unit **(d)**. A: axon, BBB: blood-brain barrier. Scale bars: a, 5 μm; b, d, 2 μm; c, 1 μm.

Table 1. The lipid profiles of EG-I, EG-II and CG measured in the different periods of the experiment (mg/dL).

	Day 0	After 4 weeks	After 12 weeks
EG-I			
TC	32.3±13.3	869.1±167.2	849.1±165.1
LDL-C	11.4±6.4	414.5±237.3	433.7±268.6
HDL-C	31.3±14.3	287.7±14.3	294.8±17.2
TG	61.6±38.6	159.4±43.4	179.3±72.3
EG-II			
TC	31.1±12.1	787.9±285.1	278.3±119.2
LDL-C	10.2±3.6	418.7±253.0	144.4±82.9
HDL-C	29.4±9.3	316.1±56.6	113.9±50.6
TG	42.7±29.1	117±28.9	52.9±34.9
CG			
TC	30.3±12.3	32.2±14.1	33.1±13.1
LDL-C	12.2±3.9	10.2±2.1	14.1±10.0
HDL-C	28.5±11.4	32.4±15.2	30.5±15.3
TG	56.6±29.4	53.4±17.2	57.5±23.4

TC: total cholesterol, LDL-C: low density lipoprotein-cholesterol, HDL-C: high density lipoprotein-cholesterol, TG: triglyceride (Tuncer et al., 2009).

cellular processes and amorphous substances were observed around these electron-dense bodies. In contrast, the vascular structures participating in the BBB, including endothelium and basal lamina, were structurally normal (Fig. 3c, inset). However, some perivascular areas had very slight dense substances between basal lamina and neuropilic elements. Although there was a pronounced decrease in the number of synapses in EGs compared to CG (p=0.002), the difference between EGs was not statistically significant (Fig. 3e).

Experimental group-II (EG-II)

In general, the structural damage in the cerebral cortex was less prominent than that of EG-I. The perikaryon and its subcellular components seem to be repaired to various degrees compared to EG-I. In some perikarya, the mitochondrial destruction was even more severe and the matrix was almost completely lost in EG-I (Fig. 3a,b) but the mitochondrial damage was decreased in EG-II compared to EG-I (Figs. 3a,d, 4a). Lipofuscin bodies were the most abundant pigment granules observed in the EG-I and EG-II compared to the CG, although this feature was not frequent and the number of lipofuscin granules were increased in some perikarya as well. The myelin sheath was structurally normal and lamellar organization was clearly seen. There was no obvious axonal shrinkage or myelin damage compared to EG-I (Fig. 4c). In contrast to preservation of normal structure in endothelium and its basal lamina, very large oedematous areas were observed around the vascular structures (Fig. 4d). These large oedematous areas had some slightly condensed substances between basal lamina and neuropilic

elements. In both EGs, the number of synaptic points were not significantly different in the neuropili, but there was a significant difference between CG and EGs (Fig. 3e). Some necrotic perikarya were observed with an irregular shape and very dense cytoplasm containing many vacuoles, and a definitive pycnotic nucleus with nucleoli. They were compressed and enveloped by the neuropilic tissues.

Discussion

The present study was designed to investigate the effects of long-term TT exposure following a cholesterol-rich diet in rabbit cerebral cortex by immunohistochemistry and electron microscopy. According to light microscopy, there seems to be no major abnormality due to high serum cholesterol levels and TT extract treatment. From immunohistochemical aspects, collagen type-I intensity was very strong around the vascular structures in EG-I. This feature was not frequent in all sections, suggesting a non-specific reaction or, less probably, an increased expression of collagen due to cholesterol-rich diet.

However, detailed ultrastructural analysis revealed a marked damage due to cholesterol-rich diet. In both experimental groups, cholesterol-rich diet led to some ultrastructural damages in the perivascular structures of BBB, including vascular endothelium with a definitive basal lamina and glial process endings in the vascular periphery. Additionally, prominent damage was evident in myelin sheath and axons, and elements of the cytoskeleton and mitochondria. 5 mg/kg/day of TT extract dissolved in physiological saline solution was administered to EG-II as recommended in the previous studies (Gauthaman et al., 2002). On the other hand, the cholesterol-rich diet caused mild oedema around the perivascular areas of BBB, as well as many lipofuscin granules. In addition to these dramatic changes, following TT extract treatment, the oedematous areas were found to be wider, although there was no damage in the vascular barrier, axons and myelin sheaths. Wide oedematous areas and the presence of electron dense bodies between neuropil and endothelial basal lamina may be a kind of protective reaction to prevent more cholesterol transportation into the neuron (Lutjohann and Von Bergmann, 2003; Vaya and Schipper, 2007). This protective barrier may play an important role in the preservation of the myelin sheath and neural processes and may be related to the action of the saponin component of the TT extract (Arcasoy et al., 1998).

Normal functioning of myelinated nerve fibers depends on the integrity of both the axon and myelin sheath. Myelin is an electrical insulator in neuronal signal transmission and its absence or damage leads to loss or reduced nerve conduction (Douglas et al., 1991). A cholesterol-rich diet caused signs of pronounced destruction in the myelin sheath and axonal shrinkage, but according to our experiments, with long-term TT extract administration, the normal lamellar arrangement

of myelin was preserved.

Microtubules and microfilaments are important determinants of cell architecture, with key roles in intracellular transport, mitotic divisions and the formation of functional core of cilia and flagella. The transport performed by neurotubules in axons is essential for axonal growth and maintenance. In our experiments, it is clearly demonstrated that high cholesterol diet leads to some structural damage to neurotubules and neurofilaments, as well as a marked increase in the density of other cytoskeleton components. These results are very interesting for brain, since it can produce its own cholesterol containing substances (Wang et al., 2007). Moreover, TT extract treatment may also protect cytoskeleton structures in the cortical axons of rabbit brain.

According to our results, there was significant mitochondrial degeneration in the experimental groups, suggesting a possible relationship between the cholesterol-rich diet and cell degeneration via mitochondria. Our results also showed that the severe mitochondrial damage caused by high cholesterol diet became less prominent after TT extract treatment. Although TT extract was protective for myelin damage, it has a limited benefit to prevent mitochondrial degeneration due to high cholesterol. On the other hand, since the inner membrane of the mitochondrion is the site for oxidative phosphorylation and ATP production, high cholesterol levels may have disturbed the electrical activity of neurons via mitochondrial damage as well. Previously, it was suggested that a high cholesterol diet might lead to dysfunction in ion transport and neurotransmitter release (Bekpinar et al., 1989). However, it is still obscure whether mitochondrial damage is involved in the reaction of the neurons against high cholesterol exposure.

Our study suggests that the results should be interpreted with an integrative approach, and thus a concept of dynamic interaction between cells belonging to the neurovascular unit should emerge. The functionality of this unit is altered by the complex series of interconnected pathophysiological processes that damage the brain tissue due to neurotoxicity. It was suggested that the new therapeutic strategies should target both the preservation of endothelium integrity and the deleterious effects induced by ionic imbalance, excitotoxicity and the generation of reactive oxygen species within the neurovascular unit (Curin et al., 2006).

In conclusion, cholesterol-rich diet causes mitochondrial degeneration, axonal and myelin sheath and also neurotubules, neurofilament damage in the axonal processes of neurons in rabbit cerebral cortex. Following TT treatment, some of this neuronal damage appeared to have decreased or been prevented. However, although TT extract has a consistent effect on cerebral cortex and may be protective in limited structures, such as myelin sheaths, axons and synaptic contact points, it also causes damage on both perivascular structures of the BBB and the perikaryon elements. We may speculate that TT extract is similar to a double-edged sword. Thus, one should pay attention to the time and dosage of TT extract recommended for patients who are supplemented by these herbs in traditional medicine. We believe that it is necessary to investigate the advantages and disadvantages of the TT extract treatment at the biochemical and pharmacological levels in order to support our results.

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