ToxEHS

Toxicity of Fe_3O_4 Nanoparticles on Oxidative Stress Status, Stromal Enzymes and Mitochondrial Respiration and Swelling of *Oryctolagus cuniculus* Brain Cortex

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Abstract

Fe_3O_4 nanoparticles are the most widely used metal oxide nanoparticles especially, in biomedical applications. Although, nanoparticles can enter to the different organs, little is known so far on the neurotoxic potential and oxidative stress of Fe_3O_4. Here the understanding of the effect of Fe_3O_4 nanoparticles on the general Redox state of rabbit brain and the effect on mitochondrial swelling and respiration were assessed. Fe_3O_4 resulted in increase of brain markers, lipid peroxidation, protein and ROS formation. Mitochondrial enzymes and swelling were elevated with decreased respiration level. Caspase 3 activity and TNF-α level were also increased. Finally, our study suggested that the mitochondrial disease and dysfunction with elevated oxidative stress in rabbit brain treated with 200 and 300 µg/kg *per Os* is the original of neurotoxicity and maybe the original cause of neurodegenerative disease.

Keywords: Fe_3O_4, *Oryctolagus cuniculus*, Neurotoxicity, Mitochondrial swelling, Oxidative stress, Respiration, Nervous system, TNF-α, Caspase-3

Introduction

Iron oxide nanoparticles are very interesting; they are used in cell labeling[1], drug targeting[2], gene delivery[3], biosensors, hyperthermia therapy and as contrast agents in magnetic resonance imaging[4-6]. However, their biological reactivity can be enhanced and can lead to potential toxic interactions. Thus, (Zhu *et al*., 2010)[7] demonstrated that superparamagnetic iron oxide nanoparticles (SPION) generate injuries in endothelial cells and might play a key role in downstream cardiovascular diseases such as atherosclerosis, hypertension and myocardial infarction. Owing to their ultra-fine sizes, ION can elicit a spectrum of tissue responses including cell activation, generation of reactive oxygen species (ROS), and cell death[8,9]. Remyaa *et al*. (2014)[10] found that iron oxide NPs affected hematology and iono-regulation and gill K^+/Na^+ ATPase activity; all this leads us to think about the effect of nanoparticles on nervous system as a possible target to their toxicity.

Neurodegenerative diseases are very dominant illnesses; it is linked directly to the environment quality. The most common form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space (IMS)[11]. There is strong evidence that mitochondrial dysfunction and oxidative stress play a causal role in neurodegenerative disease pathogenesis, including in four of the more well-known diseases Alzheimer's, Parkinson's, Huntington's, and Amyotrophic lateral sclerosis[12].

Based on these finding, we hypothesized that Fe_3O_4-NPs induced neurotoxicity. Therefore, the aim of this work was to investigate the potential toxicity effect of Fe_3O_4-NPs in rabbit and to show the link between molecular mechanisms in terms of oxidative stress and apoptotic mediators.

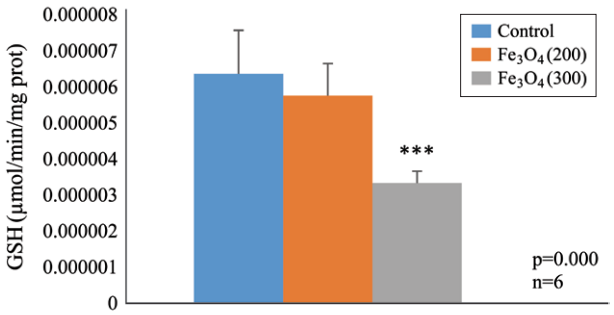

Figure 1. Variation of stromal GSH (µM/mg prot.) in the control and treated rabbits brain cortex after 14 days of treatment ($p<0.01$).

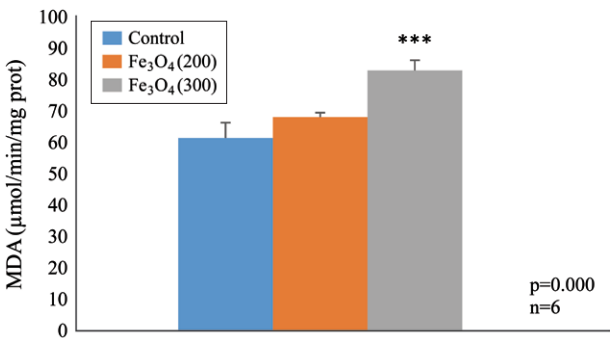

Figure 2. Variation of stromal MDA (µM/mg) level in brain cortex after 14 days of treatment with 200 and 300 µg/kg of Fe_3O_4-NPs ($p<0.001$).

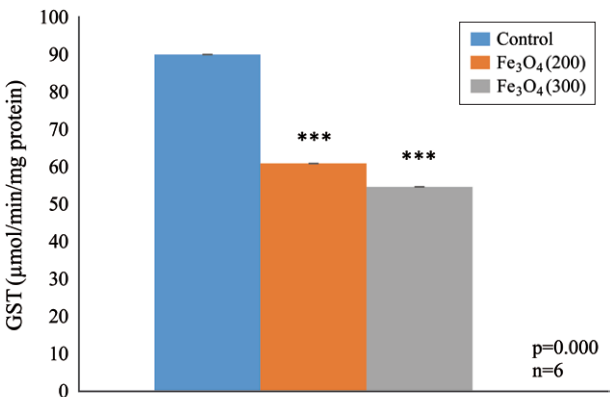

Figure 3. Effect of Fe_3O_4-NPs on the variation of stromal GST (µM/mg) after 14 days of per os administration ($p<0.01$).

Results

Assessment of GSH and MDA

Treatment of rabbits with 200 and 300 µg/kg/day of the Fe_3O_4-NPs during 14 days, results a highly significant ($p\leq0.01$) decrease of the glutathione content in the brain cortex mitochondria (0.055 ± 0.0001 and 0.035 ± 0.0001 µM/mg) compared to the control group (0.09 ± 0.002 µM/mg) (Figure 1).

The Figure 2 shows the impact of Fe_3O_4-NPs in two doses 200 and 300 µg/kg/day on the lipidic peroxidation in brain cortex cells by the estimation of the MDA level. These two doses cause a very significant ($p<0.001$) increase in MDA level (83.05 ± 0.9 µM/mg) and (68.11 ± 0.5 µM/mg) against (61.49 ± 0.9 µM/mg) in controls.

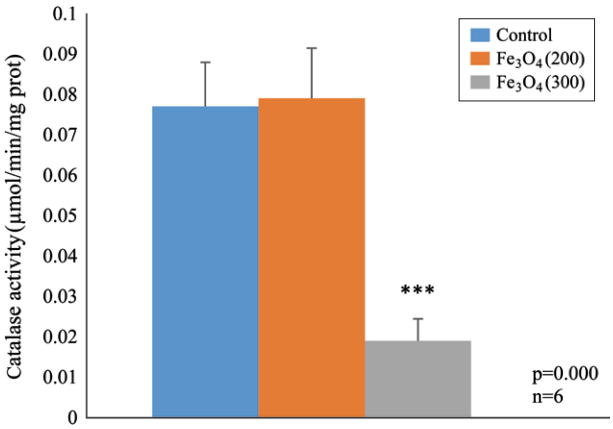

Figure 4. Effect of Fe_3O_4-NPs on the variation of stromal CAT (µM/mg) after 14 days of per os administration ($p<0.001$).

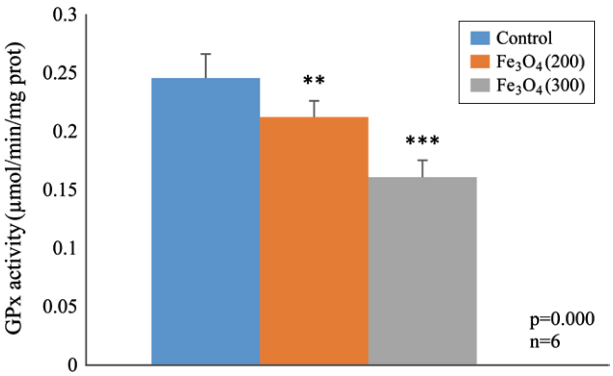

Figure 5. Effect of Fe_3O_4-NPs treatments on the variation of stromal GPx (µM/mg) after 14 days of per os administration ($p<0.001$).

Assessment of Stromal Oxidative Stress Enzymes

Treatment by 200 and 300 µg/kg/day of Fe_3O_4-NPs causes a high significant decrease in enzymatic activity of GST, CAT and GPx and SOD respectively, according to the group control (Figures 3, 4, 5 and 6).

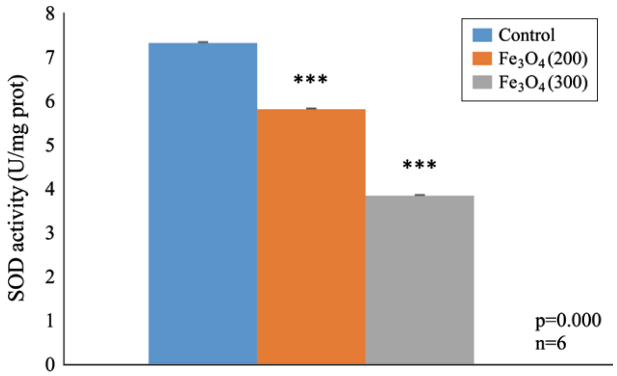

Figure 6. Effect of Fe_3O_4 at 200 and 300 µg/kg on stromal superoxide dismutase (SOD) activity (U/mg prot) after 14 days of per os administration ($p<0.001$).

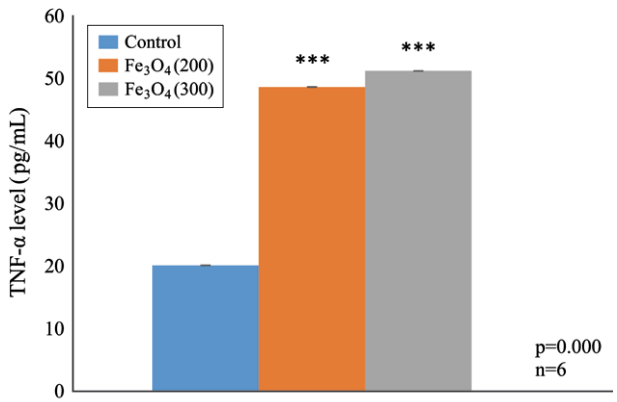

Figure 7. Effect of Fe_3O_4 at 200 and 300 µg/kg on brain cortical TNF-α level (pg/mL) after 14 days of per os administration ($p<0.001$).

Effect of Fe_3O_4-NPs on TNF-α, Caspase-3 of Brain Cortex

Impact of Fe_3O_4-NPs on TNF-α and caspase-3 of brain cortex homogenates is shown in the Figures 7 and 8, it is clear that the two doses of iron nanoparticle increase the level of these biomarkers significantly ($p<0.01$).

Mitochondrial Assays

Effects of Fe_3O_4-NPs on brain cortex mitochondrial swelling, permeability and respiration are shown in the Figures 9, 10 and 11 it is clear that the two doses of iron nanoparticle increase the mitochondrial swelling by the increase of its permeability significantly ($p<0.01$).

Mitochondrial Respiration was also influenced in dose-dependence manner; it shows a clear decrease of this parameter (Figure 11).

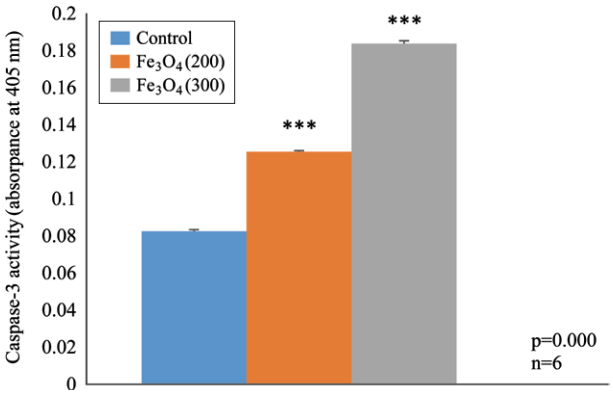

Figure 8. Effect of Fe_3O_4 at 200 and 300 µg/kg on brain cortical caspase-3 activity (OD at 405 nm) after 14 days of *per os* administration ($p<0.001$).

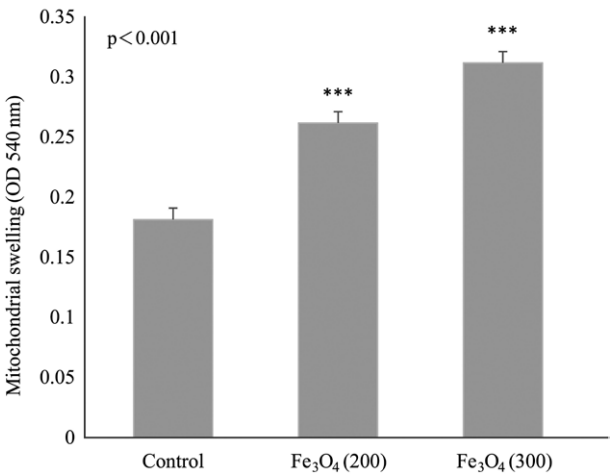

Figure 9. Effect of Fe_3O_4 at 200 and 300 µg/kg on brain cortex mitochondria swelling at OD = 540 nm after 14 days of per os administration ($p<0.001$).

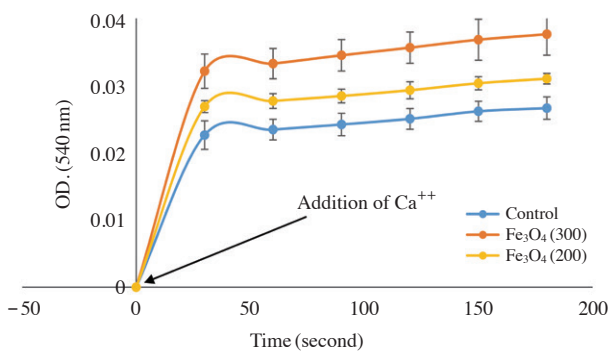

Figure 10. Effect of Fe_3O_4 at 200 and 300 µg/kg on brain cortex mitochondria permeability after 14 days of per os administration ($p<0.001$).

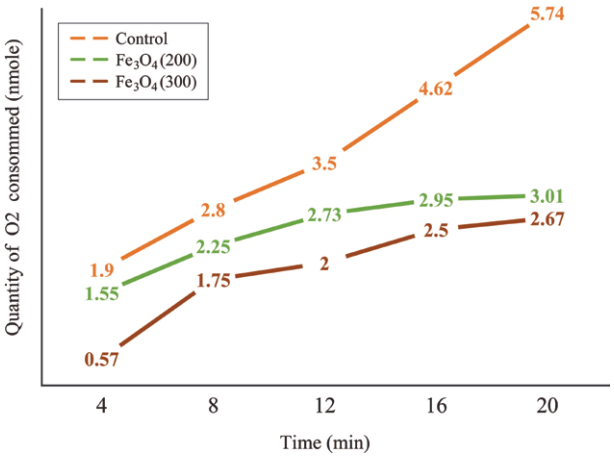

Figure 11. Effect of Fe_3O_4 at 200 and 300 µg/kg on brain cortex mitochondria respiration (nmoles) after 14 days of *per os* administration ($p < 0.001$).

Mitochondrial Swelling

See the Figure 9.

Mitochondrial Permeability

See the Figure 10.

Mitochondrial Respiration

See the Figure 11.

Discussion and Conclusions

Iron oxide nanoparticles are very interesting; they are used in cell labeling[1], drug targeting[2], gene delivery[3], biosensors, hyperthermia therapy and as contrast agents in magnetic resonance imaging[4-6]. However, their biological reactivity can be enhanced and can lead to potential toxic interactions. In this study, the administration *per-os* of two doses (200 and 300 µg/kg) of Fe_3O_4-NPs to the male rabbits caused very large perturbation on enzymatic, redox state and mitochondrial parameters.

Treatment by these two doses decreased significantly the amount of stromal GSH concordant to the results of[13,14] and increased the MDA level according to the controls, this due by the increased utilization of GSH to scavenge the ROS and by consequence increase the oxidative stress and increase the MDA level by the enhancement of lipid degradation, these results are in concord with the results of[15] Rouabhi *et al.* (2015). Garcia *et al.* (2011)[16] found that the Iron oxide nanoparticles inhibit the development of some laboratory test models by the influence on general metabolism including the β-oxidation. In this work, stromal GST, CAT, GPx and SOD enzymes activities were significantly decreased in the rabbits treated by the two doses of Fe_3O_4-NPs, which induced a total paralyze in the defensive state resulting an increased amount of ROS and these results was in discordance with the results of Rouabhi *et al.* (2008)[17] and Rouabhi *et al.* (2015)[15]. A gradual increasing of the ROS amount can induce a mitochondrial disturbance translated by the increase of the swelling and permeability. Lakroun *et al.* (2014)[18] found that the END induced a mitochondrial swelling and an increased oxidative stress state.

Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9. It is encoded by the *CASP3* gene. *CASP3* orthologs have been identified in numerous mammals for which complete genome data are available. The CASP3 protein is a member of the cysteine-aspartic acid protease (caspase) family[19,20]. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6 and 7; and the protein itself is processed and activated by caspases 8, 9, and 10. It is the predominant caspase involved in the cleavage of amyloid-beta 4A precursor protein, which is associated with neuronal death in Alzheimer's disease. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways[21,22]. The zymogen feature of caspase-3 is necessary because if unregulated, caspase activity would kill cells indiscriminately[23].

Increasing level of ROS induces a significant elevation of caspase-3 level which induced an apoptotic state in neurons, this results is confirmed by the elevated amount of TNF-α that stimulate the activation of caspase-3 and the apoptotic pathway, this is confirmed by the work of Zhao *et al.* (2001)[24] and Utaisincharoen *et al.* (2000)[25].

TNF-α with TNFR stimulate and activate NF-κB by the inhibition of a protein, IκBα, that normally binds to NF-κB and inhibits its translocation, is phosphorylated by IKK and subsequently degraded, releasing NF-κB. NF-κB is a heterodimeric transcription factor that translocate to the nucleus and mediates the transcription of a vast array of proteins involved in inflammatory response and pro-apoptotic. Our results confirmed that the treatment by 200 and 300 µg/kg of Fe_3O_4-NPs induce an elevated oxidative stress state followed by an apoptosis confirmed by a high mitochondrial swelling and permeability, that make us thought about the exo-diffusion of the cytochrome B to the neuronal cytoplasm because of the high permeability, following by the ex-

ecution of the apoptotic phenomenon, these results are in concord with the results of Yang *et al.* (2006)[26] and Zhang *et al.* (2011)[27]. This inflammation and cell death conducted to the neurodegenerative disease like Alzheimer.

Fe_3O_4 with two doses and 14 days of treatment caused a decrease in the mitochondrial respiration probably by the direct effect on the mitochondria that confirm our results about the swelling and the permeability increase. The dysfunction of mitochondria results a decrease amount of oxygen a substrate of monooxygenases and influence directly on the activity of oxidative stress enzymes this finding is in concordance with those of Baratli *et al.* (2014)[28].

In conclusion: IONPs are very used in biomedical field, this work showed a toxic effect of these nanoparticles on mitochondrial enzymes of brain cortex and induced many effects conducting to the death of cells (neurons) and cause a neurodegenerative disease as Alzheimer.

Materials and Methods

Chemicals

Fe_3O_4-NPs nanopowder (< 50 nm average particle size, characterized by TEM), bovine serum albumin (BSA), Bradford reagent, collagenase type I, Dulbecco's modified eagle's medium (DMEM) and Fetal bovine sera (FBS), 1-Chloro-2,4-dinitrobenzene (CDNB), dimethyl sulphoxide (DMSO), 5,50-dithiobis-(2-nitrobenzoic acid) [DTNB, (Ellman's reagent)], ethylene diamine tetra acetic acid (EDTA), 20,70-dichlorofluorescein diacetate, glacial acetic acid, trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), N-ethylmaleimide (NEM), nicotinamide adenine dinucleotide reduced disodium salt (NADH), nitro blue tetrazolium chloride (NBT), phenazine 116 methosulphate (PMT), potassium dihydrogen phosphate 117 (KH_2PO_4), reduced glutathione (GSH) and thiobarbituric acid (TBA), were provided from toxicology laboratory, Tebessa University, Algeria.

Animals

Male rabbits strain *Oryctolagus cuniculus* body weight 1.5-1.9 kg was used in this study. They have been housed individually in stainless metal cages during an adaptation period of 14 days in a controlled temperature/humidity/photoperiod room ($22 \pm 2°C$; 50%; 12 h dark/light cycle). The feeding of rabbits was based on specific artificial diet for rabbits, and they accessed water freely.

Treatment Protocol

Rabbits were divided in 03 lots of 06 individuals each: Controls (no treatments), treated with Fe_3O_4-NPs at 200 μg/kg/day, treated with Fe_3O_4-NPs at 300 μg/kg/day. All treatments are per os (p. o.) way, for 14 days and carried out with conformity to the international guidelines for the care and use of laboratory animals.

After 14 days of treatment, the rabbits were sacrificed and the brains were picked up, (liver and kidney were recovered, weighed and preserved for other assays) and mitochondrial extraction were proceeding. Some biochemical metabolites (proteins, carbohydrates and fats), and some parameters of oxidative stress in the brain cortex mitochondria (GSH, GPx, CAT, MDA, GST, SOD) was investigated crowned by the estimation of swelling and respiration parameters.

Assay of Antioxidant Enzymes

For this study, SOD activity was measured by following the method of Kakkar *et al.* (1984)[29]. Briefly, cell lysate containing 5 mg protein was mixed with 0.52 M sodium pyrophosphate buffer, PMS (186 lM), NBT (300 lM). The reaction was started by the addition of NADH. The absorbance of the chromogen formed was measured at 560 nm. The enzymatic activity of GPx was measured by the method of Flohe and Günzler (1984)[30], using H_2O_2 as substrate. The spectrophotometric assay of catalase (CAT) activity was performed according to the method of Cakmak and Horst (1991)[31]. The decrease of absorbance is recorded for three minutes by a spectrophotometer at a wavelength of 240 nm and an extinction coefficient $\varepsilon = 39400\ L \cdot \mu M^{-1} \cdot cm^{-1}$. The activity of glutathione S-transferase (GST) was determined according to the method of Habig *et al.* (1974)[32]. It is based on the conjugation reaction between GST and a substrate, the CDNB (1-Chloro2, 4 dinitrobenzene) as a cofactor of glutathione (GST), the conjugation results in the formation of a new molecule: 1-S-glutathionyl 2-4 Di nitrobenzene to measure the activity of GST.

Assay of Glutathione and Lipid Peroxidation

Glutathione (GSH) level was determined according to the method of Weckbeker and Cory (1988)[33]. This assay is based on measuring the absorbance of the 2-nitro-5-mercapturic. The latter results from the reduction of the acide5,5′-dithiobis-2-nitrobenzoic acid (reagent Elleman) by groups (-SH) of glutathione. Once prepared, must undergo homogenate deproteinization (by 0.25% sulfosalicylic acid) to protect the SH-groups of glutathione.

MDA can be detected by a colorimetric reaction with thiobarbituric acid (TBA). Detecting MDA after degradation of polyunsaturated fatty acids 3 or 4 Double per-

oxidized bonds. This is a highly sensitive method for determining lipid peroxidation *in vitro*. The assay of MDA is carried out according to the method of Esterbauer *et al*. (1992)[34].

TNF-α Level Quantification

Determination of Tumor necrosis factor-α (TNF-α) level in rabbit brain cortex homogenates was performed using commercial kit (Rabbit TNF-α (Tumor Necrosis Factor Alpha) ELISA Kit from MyBioSource. USA) This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rabbit TNF-alpha. Standards or samples are added to the appropriate micro ELISA plate wells and bound by the specific antibody. Then a biotinylated detection antibody specific for Rabbit TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rabbit TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rabbit TNF-α in pg/mL. You can calculate the concentration of Rabbit TNF-alpha in the samples by comparing the OD of the samples to the standard curve.

Assay of Caspase-3 Activity Level

Determination of caspases-3 activity in rabbit brain cortex was performed using commercial kit (Caspase 3 Assay Kit (Colorimetric) (ab39401)) from Abcam tech according to manufacturer guidelines.

Extraction of Mitochondria

All operations were carried on ice. A piece of brain tissue was placed into buffer A containing 50 mM tris, 1 mM EGTA, 70 mM Sucrose, 210 mM Mannitol, pH 7.40 at +4°C. Tissues were finely minced with scissors, placed in buffer A and homogenized with a Potter-Elvehjem. Then, the homogenate was centrifuged at 1300 g for 3 min, 4°C. The supernatant was centrifuged at 10,000 g for 10 min, 4°C to sediment mitochondria. Finally, the mitochondrial pellet was washed twice and then suspended in 50 mM Tris, 70 mM sucrose, 210 mM mannitol, pH 7.4 at +4°C. Protein content was routinely assayed with a bradford assay using bovine serum albumin as a standard[35]. Mitochondria were kept on ice and used within 4 h.

Mitochondrial Respiration and Swelling Assay

According to the method of Krystal *et al*. (1996)[36], we carried out the estimation of the mitochondrial permeability based on the rate of traverse of Ca^{++} followed by an increase in mitochondrial size detected at 520 nm wavelength for 3 minutes and each 30 second.

Respiration was estimated using an Oxygraph (Hansatech) according to the method described by Rouabhi *et al*. (2006, 2009)[19,37].

Statistical Analysis

The numerical and graphical results are presented as mean 6 standard error (SE). The significance of the Difference between two treatment groups was verified by the Student's t-test. The degree of statistical significance was set at a level of $p < 0.05$. Statistical calculations were carried out using Minitab 17.1 statistical package and the Excel 16.0 (Microsoft, Inc.).

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