ToxEHS

Oxidative Stress Status, Caspase-3, Stromal Enzymes and Mitochondrial Respiration and Swelling of *Paramecium caudatum* in Responding to the Toxicity of Fe_3O_4 Nanoparticles

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Abstract

The number of industrial and consumer products which contain engineered nanomaterials (ENMs, materials with at least one dimension 1-100 nm) are increasing exponentially and there is a concern regarding their occupational and environmental safety. 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 toxicity 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 a unicellular protozoa *Paramecium* and the effect on mitochondrial swelling and respiration were assessed. Fe_3O_4 resulted in increase of toxicity markers, lipid peroxidation, protein and ROS formation. Mitochondrial enzymes and swelling were elevated with decreased respiration level. Caspase 3 activity was also increased.
Finally, our study suggested that the mitochondrial disease and dysfunction with elevated oxidative stress in Paramecia treated with 200 and 300 ppm during 15 days is the original of toxicity and maybe the original cause of many environmental pathologies.

Keywords: Fe_3O_4, *Paramecium*, Mitochondrial swelling, Oxidative stress, Respiration, Caspase-3

Introduction

Nanotechnology and engineered nanoparticles may become a real environmental contaminant in near future. this is shown by the great increase of their production and application in various area of life[1]. Iron oxide nanoparticles are very interesting; they are used in cell labeling[2], drug targeting[3], gene delivery[4], biosensors, hyperthermia therapy and as contrast agents in magnetic resonance imaging[5-7]. However, their biological reactivity can be enhanced and can lead to potential toxic interactions. Thus, Zhu *et al*. (2010)[8] were 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[9,10]. Remyaa *et al*. (2014)[11] have 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 and many other organs.

Usage of non-target organisms in environmental toxicology is needed to understand the wide range of toxic effects caused by the xenobiotics on different organisms, the use of undifferentiated cells and also of single-cell organisms in toxicological investigations has the advantage of enabling general statements about the reactivity and the influence of pollutant on cell metabolism to be made[12]. Fish and other aquatic biota that

were commonly used as bio-indicators of persistent organic pollutants[13], by regulatory agencies to determine the effects of potentially toxic materials and monitor environmental contamination may be time-consuming, labour-intensive, and costly. In order to develop alternate tests that are more rapid, less labour-intensive, and inexpensive, ciliated protistes have proven to be ideal alternatives for currently used regulatory assays[14]. Protozoa, algae and bacteria from broad base of food chains and protozoan cells are often used as boindicators of chemical pollution[15] especially in aqueous environment[16-19]. Among protozoan, *Paramecium sp* is ubiquitous element of aquatic ecosystems. The short life cycle of 8 h in axenic cultures allows easy cultivation under laboratory conditions. Toxicity test are sensitive, easy and rapidly performed. In addition, the cilia of ciliate protozoa exhibit comparable characteristics with human respiratory epithelia-cells[20]. These protozoa facilitate the study of biochemical and biological processes and effects on locomotor behavior by the microtubular system and mitochondria[21]. It was able to accumulate high amounts of Anthracene without any transformation[22]. Also it stores and secretes hormone-like materials known at higher level of phylogeny. These hormones are insulin, adrenocorticotrophic hormone, relaxin[23], endorphin[24]. Therefore, the aim of this work was to investigate the potential toxic effect of Fe_3O_4-NPs on Paramecia and to show the link between molecular mechanisms in terms of oxidative stress and mitochondrial dysfunction with of course possible repercussion on ecosystems.

Results

Assessment of GSH and MDA

Treatment of paramecium with 200 and 300 ppm of the Fe_3O_4-NPs during 15 days, results a highly significant ($P \leq 0.01$) decrease of the glutathione content in the paramecium mitochondria (5.31 ± 0.0001) $*10^{-6}$ and (3.33 ± 0.0001) $*10^{-6}$ µM/mg) compared to the control group ((6.36 ± 0.002) $*10^{-6}$ µ/mg) (Figure 1).

The Figure 2 shows the impact of Fe_3O_4-NPs in two doses 200 and 300 ppm on the lipidic peroxidation in paramecium cells by the estimation of the MDA level. These two doses cause a very significant ($p < .001$) increase in MDA level (83.03 ± 0.9 µM/mg) and (68.51 ± 0.5 µM/mg) against (60.69 ± 0.9 µM/mg) in controls.

Assessment of Stromal Oxidative Stress Enzymes

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

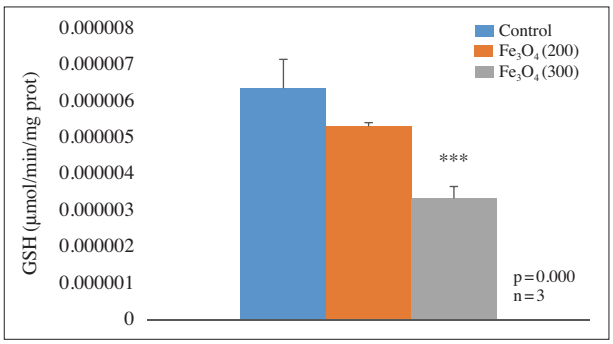

Figure 1. Variation of stromal GSH (µM/mg prot.) in the control and treated paramecia after 15 days of treatment ($p < 0.01$).

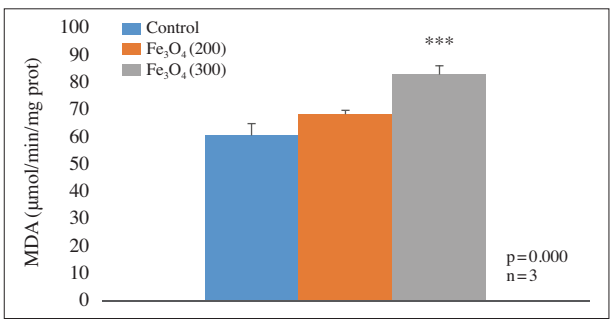

Figure 2. Variation of MDA (µM/mg) level in *Paramecium caudatum* after 15 days of treatment with 200 and 300 ppm of Fe_3O_4-NPs ($p < 0.001$).

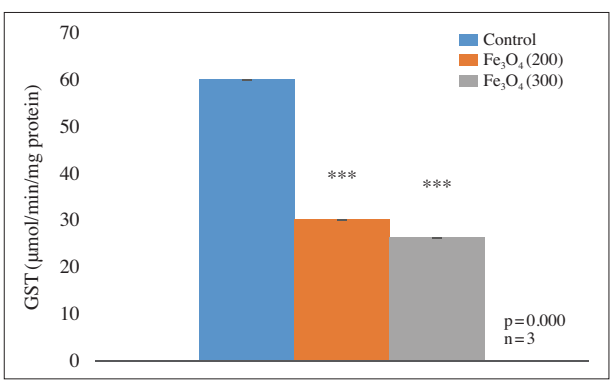

Figure 3. Effect of Fe_3O_4-NPs on the variation of stromal GST (µM/mg) after 15 days of treatment ($p < 0.01$).

Effect of Fe_3O_4-NPs on Caspase-3

Impact of Fe_3O_4-NPs on caspase-3 of paramecium cells homogenate is shown in the Figure 7, it is clear that the two doses of iron nanoparticle increase the

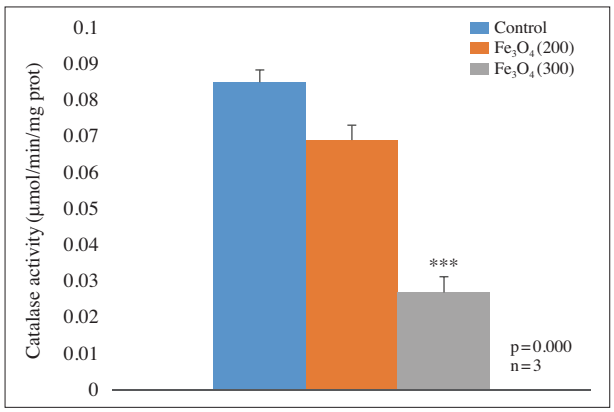

Figure 4. Effect of Fe_3O_4-NPs on the variation of stromal CAT (µM/mg) after 15 days ($p < 0.001$).

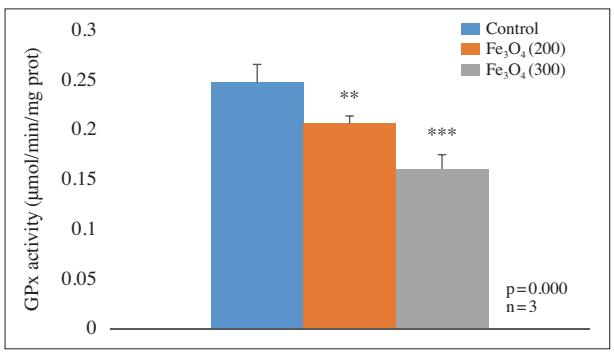

Figure 5. Effect of Fe_3O_4-NPs treatments on the variation of stromal GPx (µM/mg) after 15 days ($p < 0.001$).

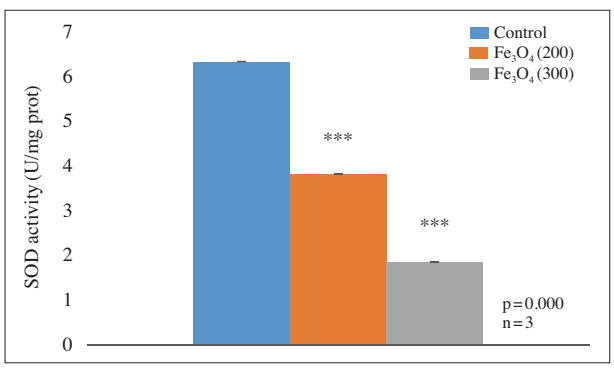

Figure 6. Effect of Fe_3O_4 at 200 and 300 ppm on stromal superoxide dismutase (SOD) activity (U/mg prot) after 15 days of exposure ($p < 0.001$).

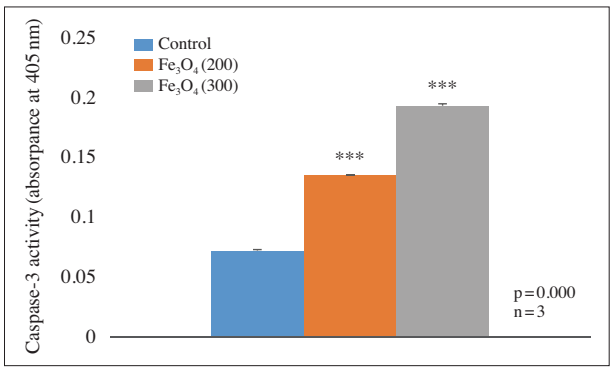

Figure 7. Effect of Fe_3O_4 at 200 and 300 ppm on caspase-3 activity (OD at 405 nm) after 15 days of treatment ($p < 0.001$).

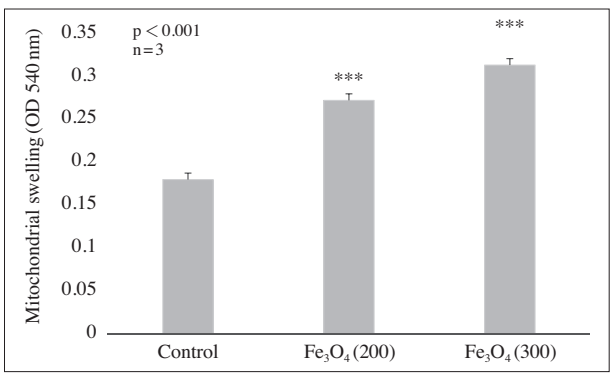

Figure 8. Effect of Fe_3O_4 at 200 and 300 ppm on paramecium mitochondrial swelling at OD_{540} nm after 15 days ($p < 0.001$).

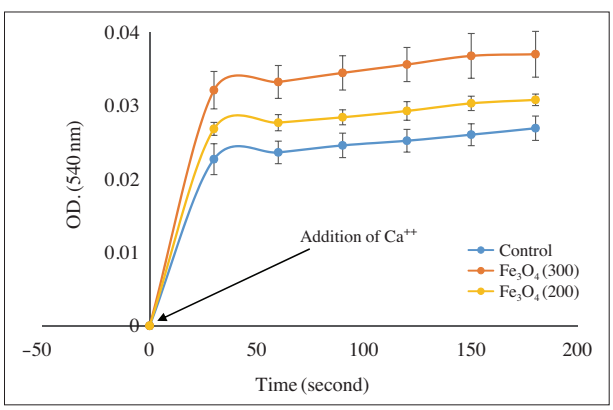

Figure 9. Effect of Fe_3O_4 at 200 and 300 ppm on mitochondrial permeability after 15 days ($p < 0.001$).

level of this biomarker significantly ($p < 0.01$).

Mitochondrial Assays

Effects of Fe_3O_4-NPs on paramecium mitochondrial swelling, permeability and respiration are shown in the Figures 8, 9 and 10, it is clear that the two doses of iron nanoparticle increase the mitochondrial swelling by the increase of its permeability significantly ($p < 0.01$).

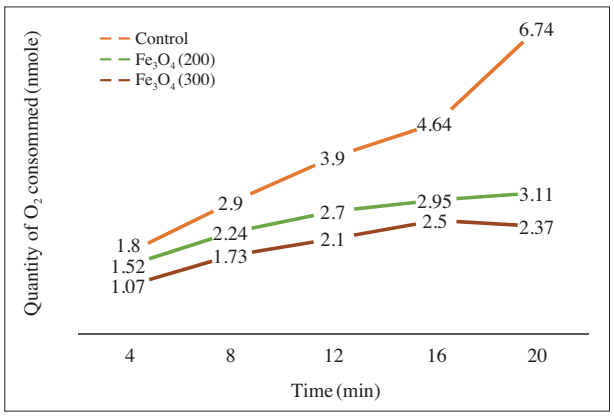

Figure 10. Effect of Fe_3O_4 at 200 and 300 ppm on mitochondrial respiration (nmoles) after 15 days of administration ($p < 0.001$).

Mitochondrial respiration was also influenced in dose-dependence manner; it shows a clear decrease of this parameter (Figure 10).

Mitochondrial Swelling

(See the Figure 8)

Mitochondrial Permeability

(See the Figure 9)

Mitochondrial Respiration

(See the Figure 10)

Discussion and Conclusions

The use of metal-based nanoparticles (NPs) is increasing which leads to their release in water *via* various waste streams, and thus warrants risk assessment. Consistent biological-effect data of NPs for environmentally relevant test species, which are accompanied by thorough characterization of NPs are indispensable for understanding the risks of these new xenobiotics.

Nanoparticles are used in various medical and industrial process; in cell labeling[2], drug targeting[3], gene delivery[4], biosensors, hyperthermia therapy and as contrast agents in magnetic resonance imaging[5-7]. However, their biological reactivity can be enhanced and can lead to potential toxic interactions. In this study, the exposure to two doses (200 and 300 ppm) of Fe_3O_4-NPs of *Paramecium caudatum* cells caused very large perturbation on enzymatic, redox state and mitochondrial parameters.

Treatment by these two doses decreased significantly the amount of GSH concordant to the results of Ashley *et al.* (2013)[25] 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 Rouabhi *et al.* (2015)[26]. Garcia *et al.* (2011)[27] 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 paramecium mitochondria 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)[28], and Rouabhi *et al.* (2015)[26]. A gradual increasing of the ROS amount can induce a mitochondrial disturbance translated by the increase of the swelling and permeability. Aruoja *et al.* (2015)[29] found that the 12 NPs metals induced a disturbance in many parameters of algae protozoa and bacteria.

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[30]. 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. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways[31,32]. The zymogen feature of caspase-3 is necessary because if unregulated, caspase activity would kill cells indiscriminately[33].

Increasing level of ROS induces a significant elevation of caspase-3 level which induced an apoptotic state, this is confirmed by the work of (Zhao *et al.*, 2001)[34] and (Utaisincharoen *et al.*, 2000)[35]. Zou *et al.* (2013)[36] were found that exposure to ferroferric oxide NPs and arsenic decrease significantly the number of *T. pyriformis* cells which confirm our results on the apoptosis and cell death.

Our results confirmed that the treatment by 200 and 300 ppm 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 cyto-

chrome B to the cells cytoplasm because of the high permeability, following by the execution of the apoptotic phenomenon, these results are in concord with the results of (Yang *et al*., 2006)[37] and (Zhang *et al*., 2011)[38].

Fe_3O_4 with two doses and 15 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)[39].

In conclusion: IONPs are very used in biomedical field, this work showed a toxic effect of these nanoparticles on mitochondrial enzymes of *Paramecium caudatum* and induced many effects conducting to the disturbance of aquatic ecosystem organisms which may affect by consequence terrestrial organisms including human beings.

Materials and Methods

Chemicals

Fe_3O_4-NPs nanopowder (< 50 nm average particle size, characterized by TEM, synthetized by Spray and Microemulsion Methods), 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.

Cells Culturing And Treatments

Paramecium caudatum strain was used in the logarithmic phase of growth. The cells were grown at exponential phase in Proteose Peptone Yeast Medium (PPY), 2% proteose peptone and 5% yeast extract at pH 7.0-7.5, at 24 ± 2°C. The density of *Paramecium* cultures was adjusted in fresh PPY in order to obtain at least 10^4 cells per mL. Before the experiments on respiration metabolism, the cells were washed with fresh culture medium and were resuspended at the concentration of 5×10^4 cells mL^{-1} in 200 mL flask; we take 1 mL to test in oxygraph (each time we added the appropriate concentration of Fe_3O_4-NPs to the reactive chamber by microsyringe). The cells were not exposed to Iron oxide nanoparticles were used as control. For the evaluation of Fe_3O_4-NPs effect on paramecia's stress parameters, we added the xenobiotic in culture medium before the addition of paramecium cells, the used cells are starved for 96 h to become encysted. After 15 days of treatment mitochondrial extraction were proceeded. Some biochemical metabolites (proteins, carbohydrates and fats), and some parameters of oxidative stress, Caspase-3, mitochondrial (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)[40]. 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)[41], using H_2O_2 as substrate. The spectrophotometric assay of catalase (CAT) activity was performed according to the method of Cakmak and Horst (1991)[42]. 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)[43]. 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)[44]. 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 peroxidized 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)[45].

Assay of Caspase-3 Activity Level

Determination of caspases-3 activity in paramecium cells 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. cells were placed into buffer A containing 50 mM tris, 1 mM EGTA, 70 mM Sucrose, 210 mM Mannitol, pH 7.40 at +4°C. 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[46]. Mitochondria were kept on ice and used within 4 h.

Mitochondrial Respiration and Swelling Assay

According to the method of Kristal *et al*. (1996)[47], 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 540 nm wavelength for 3 minutes and each 30 seconds.

Respiration was estimated using an Oxygraph (Hansatech) according to the method described by Rouabhi *et al*. (2006, 2009)[17,18,48].

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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