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Ameliorating effect of selenium nanoparticles on cyclophosphamide induced hippocampal neurotoxicity in male rats: light, electron microscopic and immunohistochemical study

Short running title: Hippocampus, neurotoxicity, nanoselenium

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Abstract

Background: Cyclophosphamide (CPH) is a widely used chemotherapeutic drug that can affect the hippocampal neurocytes with a subsequent effect on memory and cognitive functions. Nanomedicine has the potential to overcome the current chemotherapeutic side effects, because of the unique nanoscale size and distinctive bioeffects of nanomaterials. So, the present study aims to investigate the potential ameliorative effect of the biologically synthesized nano-selenium (nano Se) on CPH induced hippocampal neurotoxicity.

Materials and methods: Twenty four rats were randomly classified into four groups of 6 rats each: control group, nano Se group (dose of 0.5 mg biological nano Se /kg daily via oral gavage), CPH group (dose of 20 mg CPH/kg daily intraperitoneally), and CPH plus Nano Se group. After 4 weeks, the rats were sacrificed and the hippocampus was excised and processed. Sections were stained with H&E stain and immunohistochemically stained for caspase 3 (apoptosis marker) and GFAB

(astrocytic activity marker). Morphometric analysis and transmission electron microscopic (TEM) examination were also done.

Results: Control and nano Se groups revealed no structural changes. By light microscopy, CPH group showed degeneration and necrosis of hippocampal neurocytes, significantly reduced thickness of the neurocyte cell layers, increased expression of GFAB and caspase 3 immunostains and significantly elevated apoptotic index. Moreover, neurocytes damage, mitochondrial cristolysis, mild dilation of rough endoplasmic reticulum (Er), and disrupted neurolemmal sheaths of nerve fibers were also demonstrated by TEM. Nano Se cotreatment in the fourth group reversed all the aforementioned deleterious changes that induced by CPH in the hippocampal neurocytes.

Conclusions: Treatment with CPH caused damage to hippocampal neurocytes that can be reversed by biological nano Se co-treatment.

Key words: cyclophosphamide, hippocampus, neurotoxicity, nano selenium

INTRODUCTION

Selenium (Se) is an indispensable trace element, needed for the maintenance of growth and health [47]. It plays a role in illness prevention and fertility [9,10]. Recently, with the progress of the nanotechnology field, nano selenium (nano Se) has gained widespread attention because the nanoparticles exhibit innovative properties such as a powerful adsorbing capacity, high surface activity, high catalytic ability, and less toxicity [46] Some studies have also pointed out the possibility of use of nano Se in the field of medical treatment as antimicrobial, anti-biofilm [45] and anticancer [57].

Nano Se can be synthesized by chemical and biological methods. In chemical methods, the chemical stabilizers used may lead to environmental pollution [15]. On the contrary, biological synthesis is preferred as it is a nontoxic, clean, cost-effective, and more accessible approach. Moreover, nanoparticles produced by the biological methods have more novel properties [36,19]. There are relatively few reports on the

biologically synthesized nano Se and it is still a big challenge for researchers to elucidate the potential health benefits of nano Se [24].

Cyclophosphamide (CPH) is an anticancer agent, which is ordinarily used to treat numerous types of cancer [37]. Administration of CPH triggers oxidative stress in the brain [25] and massive cellular degeneration [39], consequently provoking apoptosis [32], and damage to cancer and healthy cells [49]. CPH uptake into healthy cells is higher than in cancer cells, rendering healthy cells more susceptible to damage [8]. The antineoplastic effects of CPH are associated with the production of acrolein which is the cause of the toxic side effects of CPH [28]. Acrolein diminishes cellular defense against oxidative stress, which can corrupt the blood-brain barrier [3,50]. Moreover, CPH can alkylate DNA, inhibits the duplication of the genome in dividing cells, and arrests the cell cycle particularly at the S-phase [6,51].

Treatment with CPH induces cognitive and psychological impairments especially the hippocampal-dependent memory task due to the negative impact of CPH on hippocampal neurogenesis [23,43]. Neurogenesis continues in the hippocampus throughout adulthood [23]. The neurotoxic effects of CPH include not only neuronal damage but also disruption of the cholinergic pathways [2]. The aforementioned reports point out that there is a necessity to explore the possible therapeutic approaches to mitigate the neurotoxicity induced by cyclophosphamide on hippocampal neurons. Therefore, the present study was conducted to investigate the potential ameliorative effect of the biologically synthesized nano Se on CPH induced hippocampal neurotoxicity.

MATERIALS AND METHODS

Drugs

Cyclophosphamide (CPH)

Cyclophosphamide monohydrate, trade name Endoxan, was manufactured by Baxter Oncology GmbH (Kantstrasse, Germany). It was available in the form of vials. Each vial contained 1 gm cyclophosphamide (dry powder). CPH was dissolved in saline to be intraperitoneally injected in rats.

Selenium nano-particles (nano Se):

Biologically synthesized nano Se was prepared using yoghurt culture (*Lactobacillus delbrueckii subsp. bulgaricus* (*L. bulgaricus*, NCAIM B 02206) and *Streptococcus thermophilus* (*S. thermophiles*, CNCM I-1670) cultivated in MRS medium as described by Prokisch and Zommara, 2010 [41]. Nano Se size ranged from 55-238 nm with an average of 122.6 ± 34.6 (SD) or 122.6 ± 8.6 (SE). Scanning electron microscopic (JSM-IT100, JEOL Co. Japan) photos of purified nano Se were used for nano Se size determination according to Nagy *et al.* 2016 (Fig.1) [35].

Animals

24 adult male albino rats with an average weight of 200-250 gm were used in the current experimental study. The animals were treated according to the guidelines of the CARE (Committee of Animal Research Ethics) of the faculty of medicine, Ain-Shams University. The animals were housed in a convenient environment; six rats per cage, food, and water were freely allowed. All rats were exposed to 12 hours light-dark cycle, good ventilation, and suitable temperature 22°-25° C.

Experimental design

Animals were divided into four groups:

- Group I (control group): 6 rats received 0.1 ml of a saline intraperitoneal injection daily for 4 weeks.
- Group II (Nano Se group): 6 rats received nano Se (0.5 mg/kg body weight) by oral gavage daily for 4 weeks [16].
- Group III (CPH group): 6 rats received a CPH (20 mg/kg body weight) intraperitoneal injection daily for 4 weeks [34].
- Group IV (CPH plus nano Se group): 6 rats received a CPH (20 mg/kg body weight) intraperitoneal injection daily and nano Se (0.5 mg/kg body weight) by oral gavage daily for 4 weeks.

Light microscopic studies

At the end of the 4 weeks experimental period, rats were anesthetized by ether, and then sacrificed also with a fatal dose of ether. For each animal, the brain was

extracted. The cerebral hemispheres were separated into right and left halves. Then, the posterior portion of the brain containing the hippocampus was cut. The right hippocampus was put in 10% formal saline (5 days). Specimens were then dehydrated, cleared, and embedded in paraffin. Sagittal sections (5- μ m) were cut and stained with H&E [4], and immunohistochemical staining for caspase 3 (apoptotic marker) and GFAP (glial fibrillary acidic protein) were also performed. GFAP was utilized to investigate the astrocytes distribution and their response to neurocyte damage [31]

Immunohistochemical staining

Tissue sections were deparaffinized, rehydrated and 10% hydrogen peroxide was added for endogenous peroxidase inhibition. Then, boiling with citrate buffer pH 6.0, For antigen retrieval was done. Sections were also incubated with the primary antibodies: anti-caspase 3 (BIOCYC GmbH, Germany), and anti-GFAP (Goat Polyclonal IgG, anti-rat antibody; Dako Cytomation, USA). Subsequently, incubation with the secondary antibody, a biotinylated goat anti-rabbit immunoglobulin and a streptavidin–biotin complex was done. The site of the reaction was visualized by adding diaminobenzidine HCl, which was converted into a brown precipitate by peroxidase. Finally, hematoxylin was utilized for counterstaining [5].

Transmission electron microscopy

The left hippocampal formation was extracted immediately after sacrificing the animals; samples were cut into small pieces (1 \times 1 \times 1 mm). The pieces were thereafter fixed in formal glutaraldehyde 2.5% for 2 days. Then, postfixation with a 1% osmium tetroxide solution was performed. Washing four times with PBS and dehydration through graded ethanol was done followed by clearing in propylene. Finally, the specimens were embedded in epoxy resin. Semithin sections were cut (1 μ m) and stained with toluidine blue and inspected by light microscope. Then, cutting the ultrathin sections (60nm) and staining with uranyl acetate and lead citrate was done. The sections were examined using a transmission electron microscope (Jeol 1200Ex, Japan) at the Electron Microscopy Center, Faculty of Science Ain Shams University, Cairo, Egypt.[42]

Morphometric and statistical analysis

The following data were measured:

- The thickness of the neurocyte cell layer i.e. the pyramidal cell layer in CA1 and CA3 zones and the granular cell layer in dentate (DG) gyrus. Vertical lines were drawn, by the software (Image Pro- Plus), from the uppermost pyramidal cell (or granule cell) to the lower most one detected. The lines were then measured.
- The area percentage of GFAP reaction, and the number of GFAP positive cells in CA1 in all groups. Briefly, The GFAP positive cells were marked by the software and the area percentage and the number of cells were counted at the high-power field ($\times 400$ magnification).
- The area percentage of caspase 3 reaction in CA1 in all groups. The caspase 3 positive cells were marked by the software and the area percentage was counted at the high-power field ($\times 400$ magnification). The number of caspase 3 positive cells (apoptotic cells) was expressed frequently in the previous studies by the apoptotic index (Ap index). [55]

Five different captures in non-overlapping high-power fields ($\times 400$) were taken in the slides of each rat in all groups (the H&E stained slides and the immunostained slides). Then, the aforementioned parameters were measured by software. The Ap index represented the percentage of apoptotic cells in 1000 cells. Meanwhile, the Ap index was determined by counting at least 1000 cells per slide, subdivided into 10 fields chosen randomly. The counting process was done at $400 \times$ magnification. $\text{Ap index} = (\text{number of caspase 3 positive cells} / \text{total number of calculated cells}) \times 100$. Measurements were performed by an independent observer blinded to the specimens' details to achieve an unbiased evaluation. The morphometric analysis was performed on digital light microscopic images that had been captured by a digital camera Olympus (DP 20) on Olympus BX51 light microscope (Olympus, Hamburg, Germany). Image analysis procedures were carried out using the Image Pro-plus Program, Media cybernetics. Inc. (version 4.5.1.27).

All data were expressed as mean \pm SD and statistically analyzed by IBM SPSS Statistics version 20 using one-way ANOVA analysis of variance, followed by Tukey test to compare statistical differences between all groups at $P < 0.05$.

RESULTS

Light microscopic results

Group (I&II) (Control and nano Se groups). The microscopic sections of the control and nano Se groups were nearly similar. In both groups, examination of H&E stained sections of the hippocampus revealed that it was formed of the hippocampal proper (proprius) and the dentate (DG) gyrus. The hippocampus proper was composed of four zones (CA1, CA2, CA3, and CA4) of cornu ammonis. CA1 and CA2 were located dorsally while CA3 made the descending arch that ended at the beginning of the hilus of the DG gyrus. CA4 is situated inside the hilus of the DG gyrus. The DG gyrus appeared as a dark V-shaped structure, with its open part encircling the CA4 area of the hippocampus. Each area was formed of three layers: polymorphic, pyramidal, and molecular (Fig. 2A). The molecular and polymorphic layers contained few neurocytes (nerve cells), whereas the pyramidal layer was formed of numerous neurocytes (pyramidal cells) containing large vesicular nuclei with prominent nucleoli and pale basophilic cytoplasm. Glial cell interneurons were seen in all layers of the hippocampus. They had dark basophilic cytoplasm and deeply stained small rounded nuclei. The middle layer in the DG gyrus is called the granule cell layer instead of the pyramidal cell layer (Fig. 2B-D). The cellular structure of the various zones of the hippocampus proper is similar so figures of CA1 and CA3 were only included in the present study as examples to the different layers and to avoid repetition of unnecessary similar figures.

Group (III) (CPH group). Hippocampus proper: The thickness of the pyramidal cell layer was apparently decreased. The pyramidal cells were disorganized and appeared either degenerated or necrotic. The cytoplasm of the degenerated cells was vacuolated with loss of cellular characteristics. The necrotic cells had shrunken deeply stained nuclei i.e. pyknotic nuclei. Small perineural spaces were seen surrounding the necrotic pyramidal cells. Additionally, some cells had swollen

karyolytic pale stained nuclei with pale faint cytoplasm giving the ghost-like appearance. Few cells were still healthy with vesicular nuclei in between the damaged cells (Figs. 3A and B).

DG gyrus: The granule cell layer was diminished in thickness and the majority of its cells had deeply stained shrunken pyknotic nuclei or ghost-like nuclei (Fig. 3C).

Morphometrically, the thickness of the pyramidal cell layer and the granule cell layer (neurocyte cell layers) was significantly decreased as compared to the control group ($p < 0.05$). So, the morphometric data were in harmony with the morphological observations (table 1 & Fig. 4).

Group (IV) (CPH plus Nano Se group). Examination of H&E-stained sections in the hippocampus of rats treated with CPH plus Nano Se revealed that most of the pyramidal cells and granule cells appeared healthy with large rounded or oval vesicular nuclei. Few cells with shrunken pyknotic nuclei with dark cytoplasm could be also seen in between the healthy cells (Fig. 3D, E, and F). The thickness of the pyramidal cell layer and the granule cell layer was significantly elevated as compared to CPH group by morphometric analysis (table 1 & Fig. 4).

Immunohistochemical results

Immunohistochemistry for GFAP. The control group and the Nano Se group (I&II groups) showed few positive brownish star-shaped GFAP immunoreactive cells. The immunoreactive cells were large branched cells dispersed among various cell layers of the hippocampus. They were most probably activated astrocytes (Fig. 5A). The CPH group (group III) showed an apparent increase in the number and size of the star-shaped GFAP immunoreactive cells (Fig. 5B). The CPH plus nano Se group (group IV) showed less number and size of the GFAP immunoreactive cells compared to the group of CPH treated Rats (Fig. 5C). Morphometrically, the area percentage of the GFAB reaction and the mean number of immunoreactive cells were significantly increased in the CPH group as compared to the control group. Meanwhile, these measured parameters were significantly reduced in CPH plus nano Se group as compared to the CPH group (Table 2 & Fig, 7A).

Immunohistochemistry for Caspase 3. Caspase 3 was rarely expressed in the neurocytes of the control and Nano Se groups (group I&II) (Fig. 6A). Enhanced staining of caspase 3 was observed in the neurocytes of the CPH group (group III) compared with the control group, indicating increased apoptosis (Fig. 6B). The CPH plus Nano Se group (group IV) showed decreased staining in comparison with the CPH group (Fig. 6C). Morphometrically, the area percentage of caspase 3 reaction, and the apoptotic index were significantly increased in the CPH group compared to the control group. Meanwhile, these measured parameters were significantly decreased in CPH plus Nano Se group as compared to the CPH group. (Table 2 & Fig. 7B).

Transmission electron microscopic (TEM) results

Group (I&II) (Control and nano Se groups). The Neurocytes (nerve cells) of the control group showed large oval or rounded euchromatic nucleus with dispersed chromatin and a regular nuclear membrane. The cytoplasm contained multiple cisternae of rough endoplasmic reticulum (Er), several free ribosomes, and intact mitochondria with prominent cristae (Fig. 8).

Group (III) (CPH group). The nuclei of many cells had irregular outlines and condensed chromatin and some of them appeared shrunken and deeply stained i.e. pyknotic (Fig. 9 A and C). The nuclei of the degenerated cells appeared electro-lucent but their cytoplasm showed large vacuolation with remnants of the degenerated organelles (Fig. 9 B). The mitochondrial cristae appeared damaged i.e. cristolysis and the cisternae the rough Er were slightly dilated (Fig 9 D). Some neurolemmal sheaths of the nerve fibers were disrupted (Fig. 9A and C).

Group (IV) (CPH plus Nano SE group). The majority of cells had electro-lucent nuclei with nearly regular outlines and dispersed chromatin and their cytoplasm showed healthy mitochondria and a nearly normal rough Er. The neurolemmal sheaths of the nerve fibers appeared intact (Fig. 10 A and B). Some cells had normal cytoplasmic organelles and electro-lucent chromatin, but their nuclei still had irregular outlines (Fig. 10 C). While most of the cells appeared healthy, few cells still had deeply stained nuclei and/or cytoplasmic vacuolation (Fig. 10 D).

DISCUSSION

Chemotherapeutic drugs as cyclophosphamide have been a cornerstone of cancer therapy. Extensive effort was done on the synthesis of more efficient and less toxic chemotherapeutic drugs, but less attention has been paid to factors that may decrease the harmful side effects created by the antineoplastic agents. Moreover, the clinical outcome of treatment with these agents is critically restricted, frequently due to their toxicity to normal tissues. Consequently, there was a need to develop adjuvant treatment to enhance the effectiveness and/or diminish the associated side effects. Nanoparticles have the potential to overcome the obstacles in the treatment of cancer by the traditional antineoplastic agents, due to the unique nano size and novel characteristics of nanomaterials [27,59].

In the current study, H&E stained sections of the group of rats exposed to CPH showed either degeneration or necrosis of the neurocytes of the hippocampus i.e. the pyramidal cells of the hippocampus proper and granule cells of the dentate gyrus. The thickness of the pyramidal and granule cell layers was also significantly reduced. The results of the current study were similar to the observations of Shaibah et.al.,2016 who postulated that CPH caused highly significant dystrophic and apoptotic alterations in the neurocytes of the hippocampus with a marked reduction in the neurocyte cell layer thickness [44]. On the contrary, Lyons et.al.,2011 pointed out that CPH did not affect the survival of the cells of the hippocampus in rats after a short-term treatment and it is less neurotoxic than other antineoplastic agents. [30] Consequently, it is concluded that CPH could provoke neuronal damage in the hippocampus due to the long term and not in short term treatment.

The H&E stained sections of the rats treated with CPH plus nano SE, in the present study, showed that most of the neurocytes were healthy in the hippocampus apart from few cells exhibited dystrophic changes. Additionally, the thickness of the pyramidal cell layer and granule cell layer was significantly increased as compared to the CPH group. These ameliorating results induced by nano Se were in harmony with the previous studies insuring the ameliorating effect of nano Se on the different tissues against the tissue deleterious effect of cyclophosphamide and the other injurious factors. In this context, Bhattacharjee et. Al.,2014 reported that nano Se alleviated degenerative changes in the liver induced by CPH. [7] Moreover, Hamza et. al.,2020 reported that nano Se could protect also against the hepatocellular damage

induced by acrylamide [17]. Additionally, Dkhil et.al.,2016 stated that nano Se prevented and minimized diabetes-induced spermatogenic cell loss in diabetic rats. [13]. Similarly, Abdel Hakeem EA et. al.,2020 reported that nano Se could mitigate the degeneration, necrosis, and inflammation of pancreatic acini and Langerhans cells caused by acute pancreatitis [1].

The protective effect of nano Se on the hippocampus and the other tissues may be due to its antioxidant power against the oxidant damaging effect of CPH as stated by the previous studies. Akomolafe et. Al., 2020 Postulated that CPH has a pro-oxidant character and its administration is associated with induction of oxidative stress by the generation of free radicals [2]. Moreover, Valko et. al.,2006 reported that higher levels of malondialdehyde (MDA) were noted in the brain of CPH treated mice. MDA is an outcome of lipid peroxidation and has been recorded to cause neuronal damage [52]. On the other hand, nano Se significantly inhibited CPH induced free radicals formation which may be due to its antioxidant property [7]. Nano Se played important roles in the enhancement of the antioxidant defense system and scavenging free radicals by increasing activities of both and glutathione S-transferase and Glutathione peroxidase [20,40,58]

In the present study, the thickness of the neurocyte cell layers (pyramidal and granule layers) was significantly reduced in the group treated with CPH. This reduction occurred due to the decrease in the neurocyte population owing to the cellular damage (necrosis and/or apoptosis). Additionally, the reduction in the population of cells may be also due to the inhibition of hippocampal neurogenesis by CPH that was mentioned recently in numerous studies. The reduction in hippocampal neurogenesis by CPH could affect the hippocampal related cognitive functions and memory [12,56]. Nano Se, in the present study, prevented the decrease in the thickness of neurocyte cell layers in the group treated with CPH plus nano Se. This may be due to the prevention of cellular degeneration, necrosis, and apoptosis by nano Se. The positive impact of nano Se on neurogenesis may be also assumed which could be an additional cause of increased neurocyte cell layers thickness.

Caspase-3 has been known as an essential therapeutic target in neurodegeneration owing to its involvement in neuroinflammation and apoptosis [21]. Immunohistochemical examination and morphometric analysis, in the current study, revealed that CPH injection significantly increased the area percentage of caspase 3 reaction and the apoptotic index which reflected the increase in the number of cells

showing positive reactions to caspase 3 (an apoptosis marker). This result was in line with the observations of Shaibah et.al.,2016 who reported that immunohistochemical section of rats injected with CPH showed a large number of apoptotic neurons with a positive reaction for p53 (an apoptosis marker) and a less positive reaction for Bcl2 (anti-apoptotic marker) [44].

The immunohistochemical results also revealed that nano Se reduced the area percentage of caspase 3 reaction and the apoptotic index in the group exposed to CPH plus nano Se. Our results were in harmony with that of Wang et.al.,2019 who studied the effect of nano Se on cultured pancreatic cells. They stated that nano Se completely inhibited the activation of caspase-3, 8, and 9 and consequently, suppressing apoptosis [53]. On the contrary Huang et. al., 2010 proved that nano-Se provoked initiation of apoptosis through destabilization of the membrane potential of mitochondria and raised the expression of many pro-apoptotic caspases [18]. These differences in results may due to the variations in doses and sizes of nano Se utilized. As for nano Se to produce apoptosis, it should reside within the cell at high concentrations. Moreover, the diversity in cell type upon which nano Se acts, the variations in cellular redox status, and different durations of administration may be other causes [22]

GFAP is a cellular protein that is found in various cell types of the brain particularly astrocytes [48]. GFAP is known to be essential for changing astrocyte shape and mobility by increasing the structural stability of the processes of astrocytes [29]. GFAP is also known as a specific marker for the maturity of astrocytes [14]. It was found that injury of the brain, whether because of chemical agents, diseases, or trauma led to astrogliosis. In astrogliosis, rapid synthesis of GFAP occurs and is detected by immunostaining with GFAP antibodies [26]. In the current study, CPH triggered the activation of astrocytes that was manifested in the form of increased expression of GFAP. This might be a compensatory mechanism for neuronal damage. Moreover, nano Se significantly reduced the expression of GFAB which indicated that nano Se could inhibit CPH induced neuronal damage and its concomitant astrogliosis.

So far, there was very limited data in the previous studies about the effect CPH on the ultrastructure of the hippocampus in adult rats. By TEM, the results of the present study revealed that CPH produced mild dilation of rough ER., mitochondrial cristolysis, disrupted neurolemmal sheaths of nerve fibers, neuronal degeneration,

and necrosis which was greatly reversed by nano Se co-treatment. Our observations were in harmony with the developmental study of Xiao et.al.,2007 who reported that CPH decreased the growth and viability of neurons, destroyed nuclear DNA, and provoked apoptotic morphological alterations in rat embryos. [54]. CPH may induce neuroinflammation and neurodegeneration due to enhancing NO synthesis and increasing arginase activity. Experimental studies have revealed that elevated arginase activity and changes in NO levels may contribute to neurodegeneration [11,38]. Additionally, Mohammed and Safwat, 2013 reported that the administration of nano Se significantly decreased the NO concentration in brain tissue [33]. So, the reduction in the NO level caused by nano Se may be one of the causes of its neuroprotective effects.

CONCLUSIONS

Cyclophosphamide injection in rats triggered cellular degeneration, necrosis, and apoptosis of hippocampal neurocytes. Moreover, it caused mild dilation of rough endoplasmic reticulum, mitochondrial cristolysis, and disrupted neurolemmal sheaths of nerve fibers at the ultrastructural level. Nano selenium co-treatment mitigated these deleterious changes induced by cyclophosphamide. So, nano selenium Supplementation to the diet of patients treated with cyclophosphamide may be useful. Further studies are required to elucidate the mechanism of action nano selenium as a protective factor against chemotherapeutic drugs as cyclophosphamide.

Ethics approval and consent to participate

All the procedures of the experiment were carried out according to the guide rules of the Committee of the Animal Research Ethics (CARE), Faculty of Medicine, Ain Shams University.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the

corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Thickness of the pyramidal cell layer in CA1 and CA3 and the granular cell layer in dentate gyrus of the hippocampus in different groups (in μ M)

	Control	Nano Se	CPH	CPH+ Nano Se
CA1	84.28 \pm 7.41	87.48 \pm 7.75	40.26 \pm 4.11 ^a	76.94 \pm 7.18 ^b
CA3	112.34 \pm 5.50	116.56 \pm 6.27	57.97 \pm 5.46 ^a	107.06 \pm 8.00 ^b
Dentate gyrus	69.61 \pm 4.67	71.92 \pm 4.06	40.31 \pm 3.70 ^a	63.91 \pm 6.95 ^b

Values are expressed as mean \pm standard deviation. N=6 for each group. One-way ANOVA. a= significant versus the control group; b= significant versus the CPH group; P<0.05 was considered significant.

Table 2. The mean GFAP area %, number of GFAB positive cells, caspase 3 area %, and the apoptotic index in different groups

Groups	GFAP area %	number of GFAB positive cells	caspase 3 area %	apoptotic index
Control	2.99±1.57	2.73±1.44	1.44±0.85	0.43±0.25
Nano Se	2.24±1.53	1.73±1.23	1.24±0.92	0.32±0.15
CPH	18.56±10.25 ^a	19.23±6.60 ^a	15.06±6.12 ^a	33.66±5.14 ^a
CPH+ Nano Se	7.07±2.96 ^b	5.47±2.11 ^b	5.73±1.61 ^b	10.54±2.58 ^b

Values are expressed as mean ± standard deviation. N=6 for each group. One-way ANOVA. a= significant versus the control group; b= significant the CPH group; P<0.05 was considered significant.

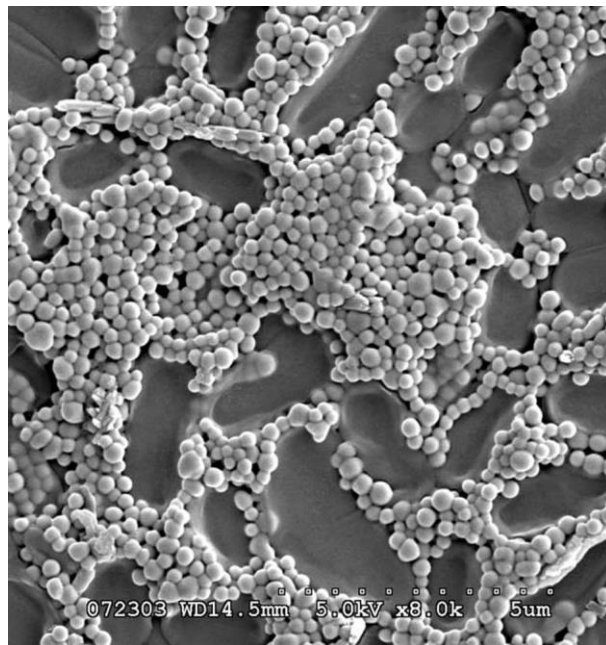


Figure 1. Scanning electron microscopic photograph of a yoghurt culture-nano Se suspension.

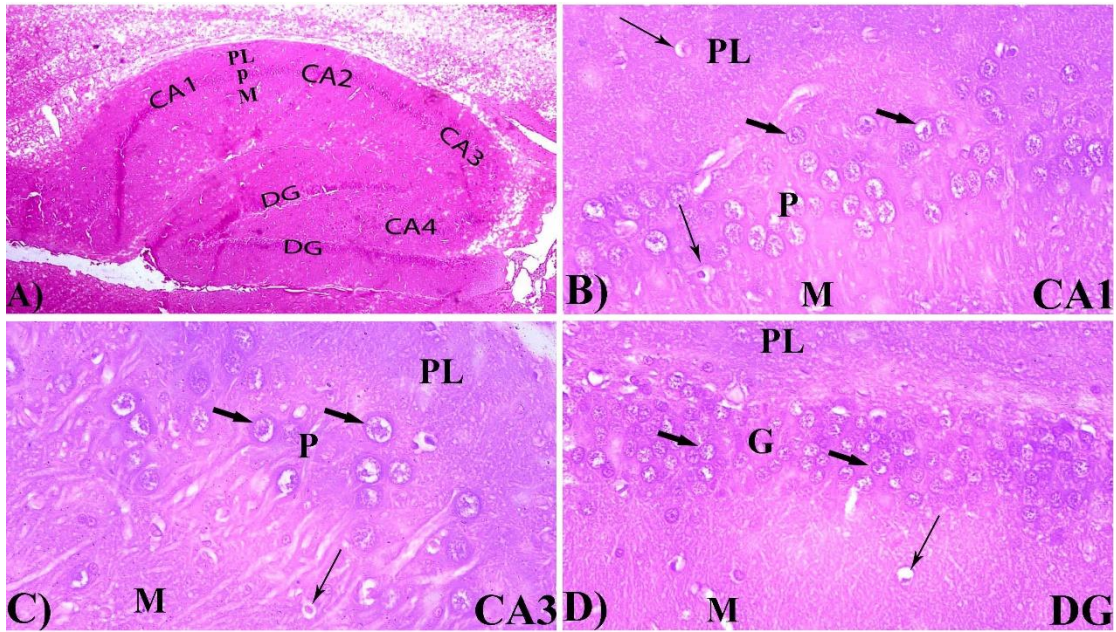


Figure 2. A) hippocampus of control rat showing the DG gyrus and the four regions of hippocampus CA1, CA2, CA3, and CA4. B, C, and D) showing that CA1, CA3 and DG gyrus are formed of 3 layers: PL=polymorphic layer, p=pyramidal cell layer, and M=molecular cell layer. The pyramidal cell layer is replaced by granule cell layer (G) in DG gyrus. The thick arrows point to the pyramidal and granule cells and the thin arrows for the interneurons. (H&E stain A X40 B, C, and D X400)

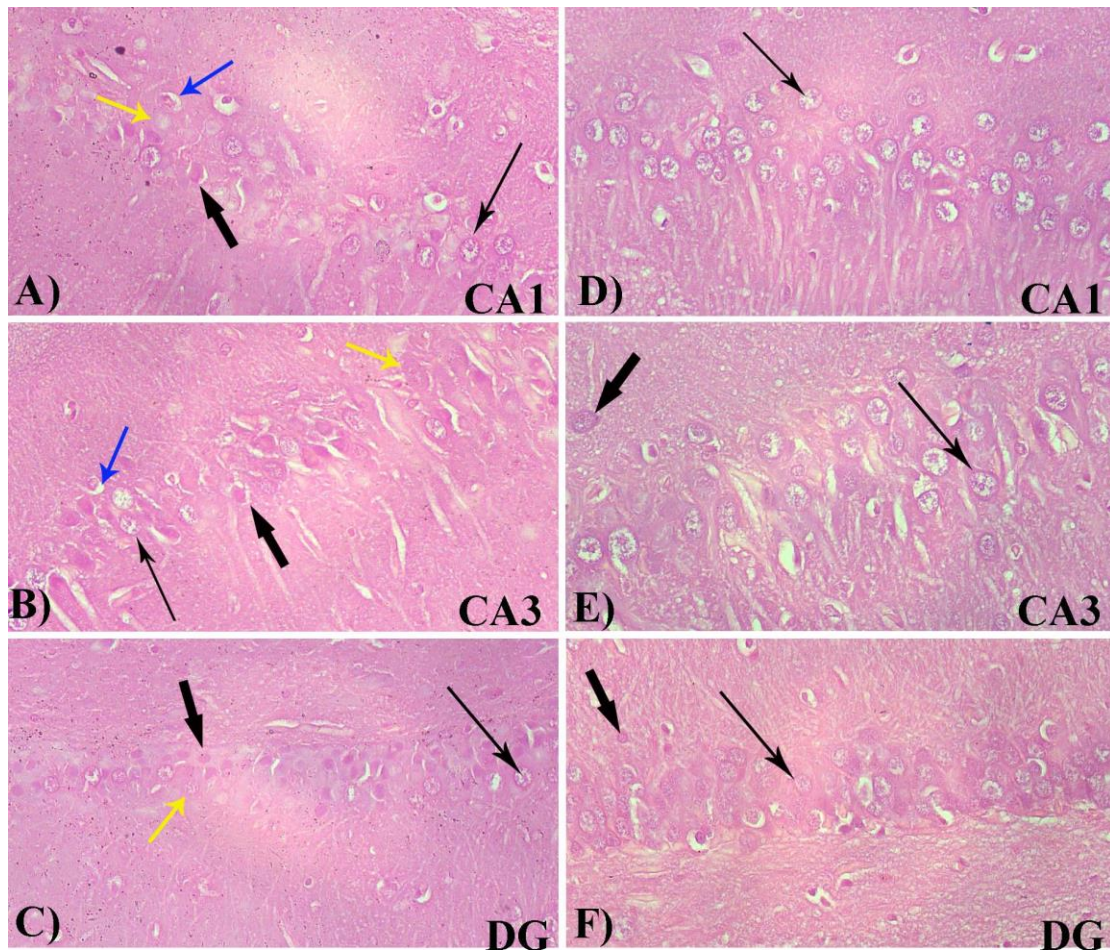


Figure 3. A,B, and C) Sections from CPH group showing the necrotic and degenerated neurocytes in all zones separated by few healthy cells. D,E, and F) sections in CPH plus Nano SE group showing that most of the neurocytes are healthy. Thick arrow= necrotic cell, thin arrow= healthy cell, yellow arrow= ghost like cell, blue arrow = cytoplasmic vacuolation in a degenerated cell. (H&E stain X400)

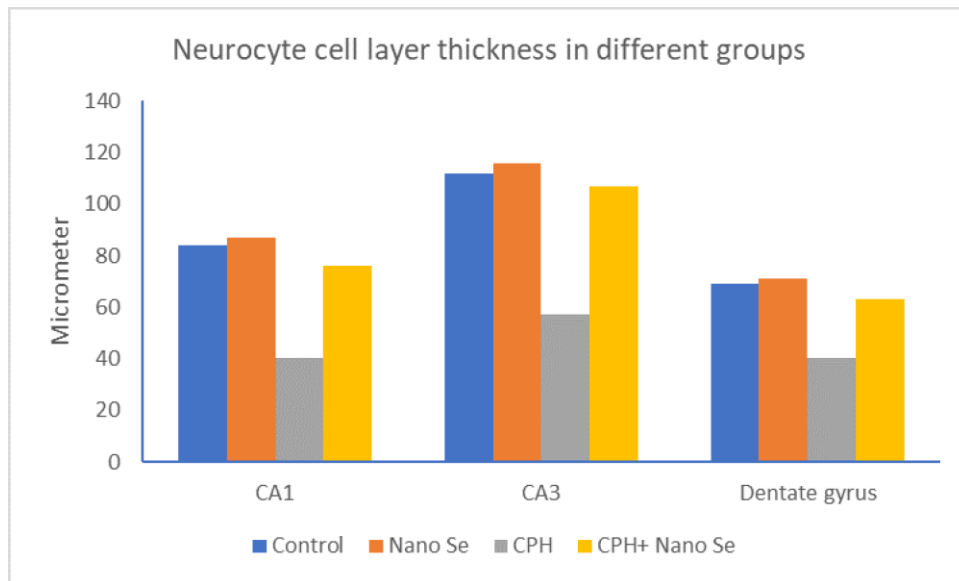


Figure 4. A histogram comparing the neurocyte cell layer thickness of the different groups.

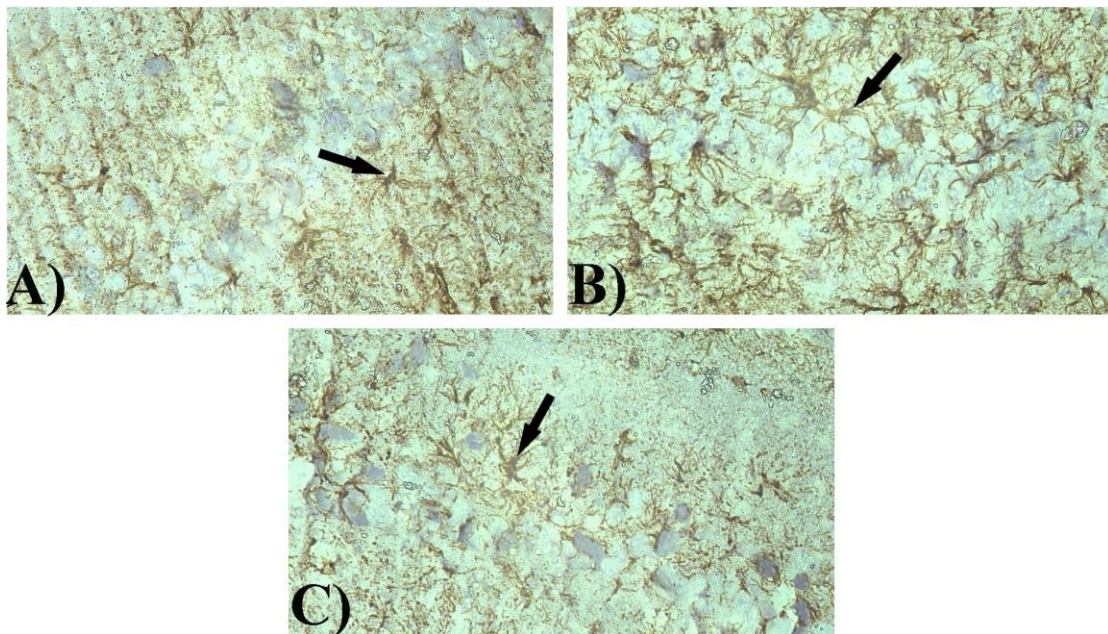


Figure 5. Sections in CA1 A) Few cells with positive reaction to GFAB in the control group. B) Many cells with positive reaction in CPH group. C) Few cells with positive reaction in CPH plus Nano Se group. (GFAB immune- stain X 400)

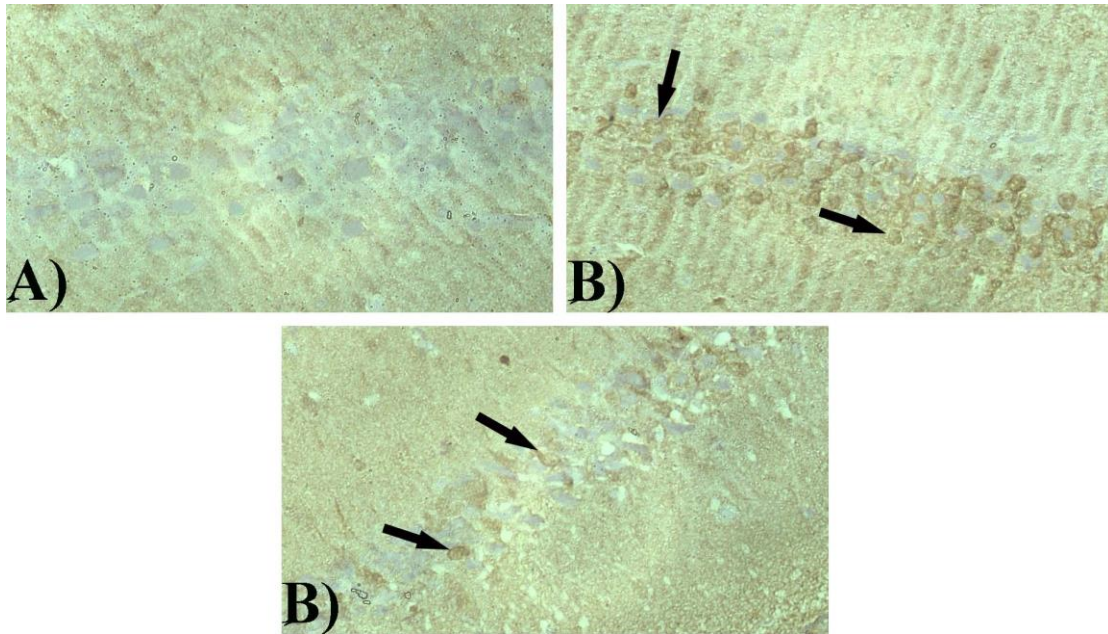


Figure 6. Sections in CA1 A) No positive reaction to caspase 3 in the control group. B) Many cells with positive reaction in CPH group. C) Few cells with positive reaction in CPH plus Nano Se group. (Caspase 3 immune- stain X 400)

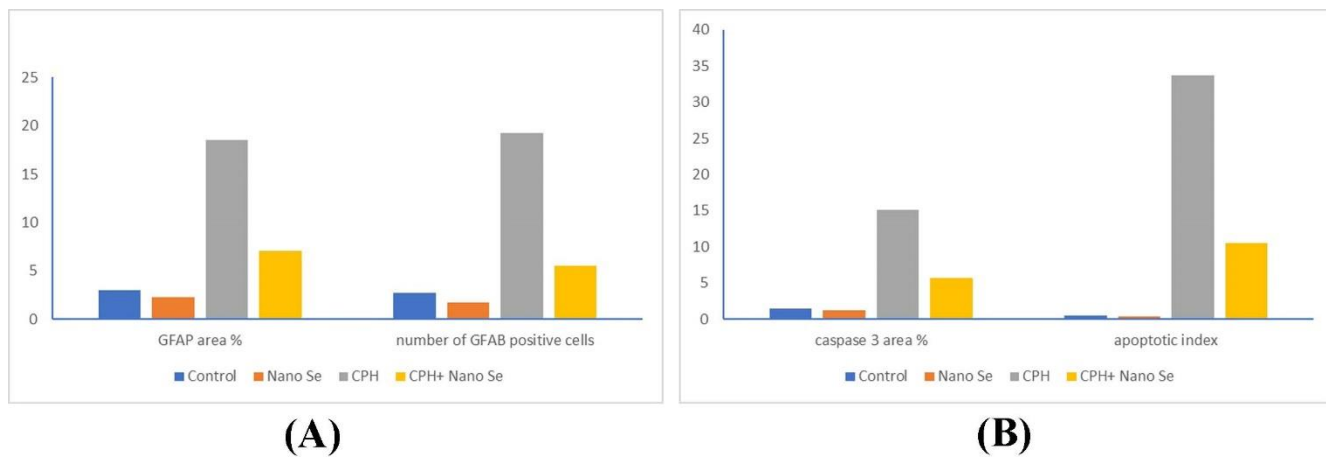


Figure 7. A histogram comparing; A) GFAP area percentage and number of GFAP positive cells in the different groups, B) Caspase 3 area percentage and apoptotic index in the different groups.

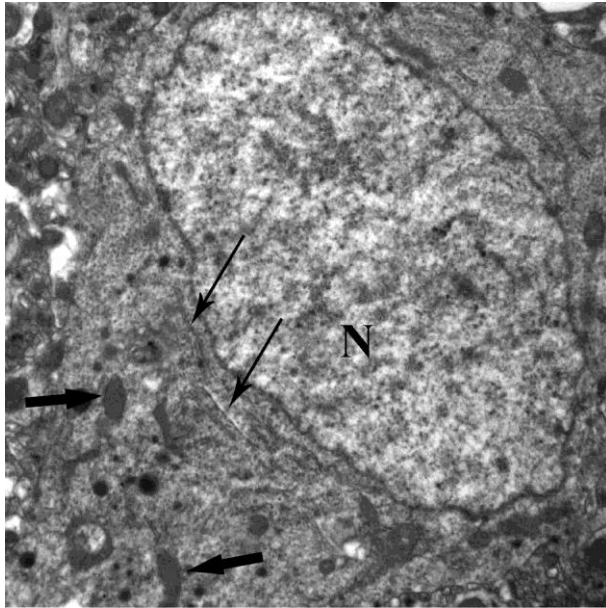


Figure 8. TEM micrograph of a control neurocyte showing a large oval euchromatic nucleus (N), cisternae of rough Er (thin arrow), and intact mitochondria (thick arrow) (X 2500)

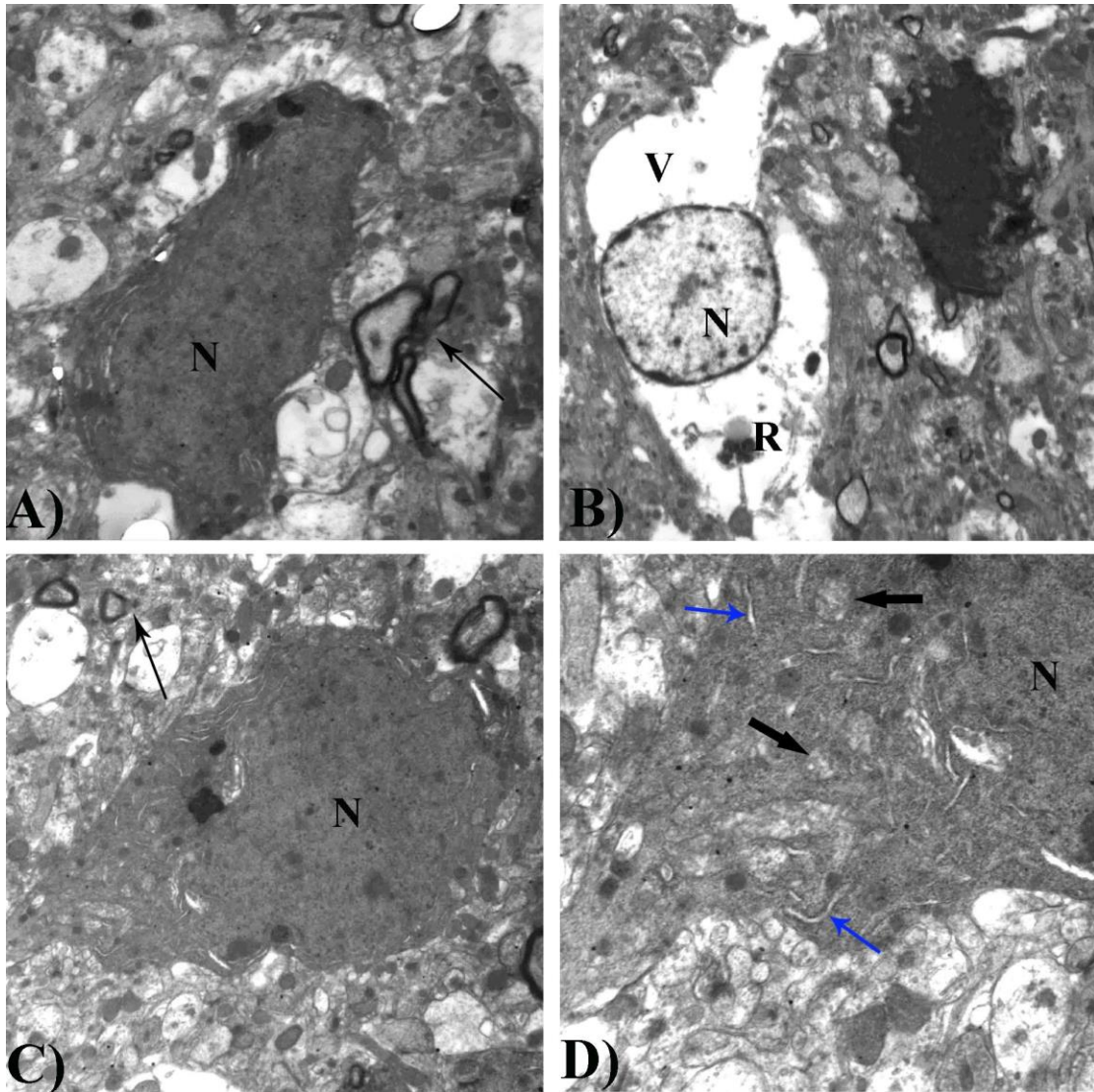


Figure 9. TEM micrographs of neurocytes of the CPH group A) showing a nucleus (N) with irregular outline and condensed chromatin. B) showing a degenerated cell with electron lucent nucleus (N), vacuolated cytoplasm (V), and remnants of the degenerated organelles (R). C) showing a necrotic cell with a pyknotic nucleus (N). D) a higher magnification of C showing the cristolysis of mitochondria (thick arrow) and the slightly dilated cisternae the rough Er (blue arrow). N.B.: The disrupted neurolemmal sheaths (thin arrow) are seen in A and C. (A&C X2500, B X1500, D X6000)

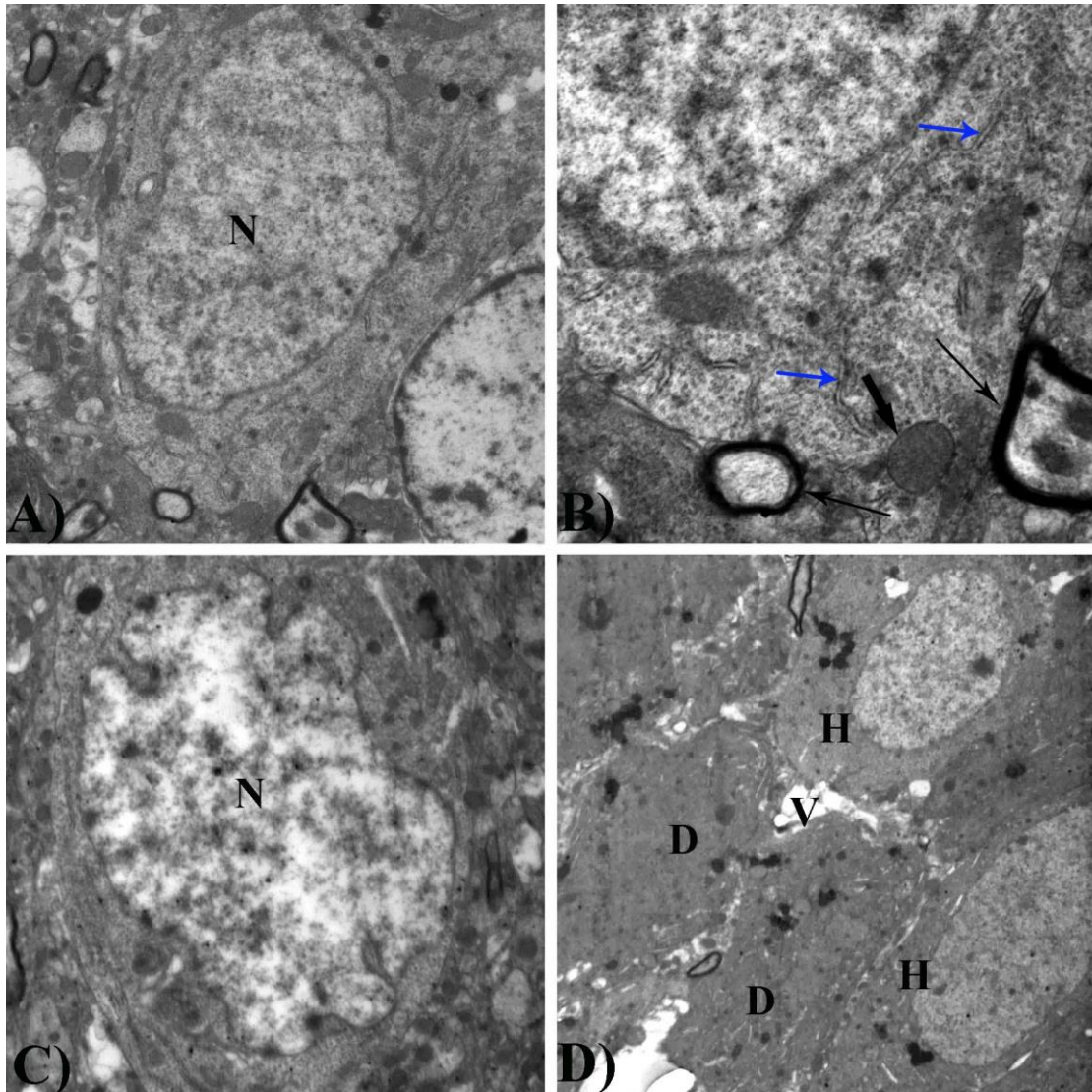


Figure 10. TEM micrographs of neurocytes of the CPH plus nano Se group A) showing a cell with electro-lucent nucleus (N) with a nearly regular outline and dispersed chromatin. B) a higher magnification of A showing healthy mitochondria (thick arrow), nearly normal rough E (blue arrow), and intact neurolemmal sheaths (thin arrow). C) showing a cell with electro-lucent nucleus(N) with irregular outline. D) showing healthy cells (H) and damaged cells (D) with deeply stained nuclei and cytoplasmic vacuolation (V). (A&C X2500, B X6000, D X1500)