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The effect of high doses of the slimming agent 2,4 dinitrophenol in the rat brain: A biochemical and histopathological study

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ABSTRACT

The uncoupler of mitochondrial oxidative phosphorylation 2,4 dinitrophenol (2,4-DNP) is used as a weight reducing agent and there are reports of toxicity and deaths due to the agent. We aimed to examine the effects of high doses of 2,4-DNP on brain in rats. 2,4-DNP was intraperitoneally injected at doses of 10, 40, 80 and 160 mg/kg and rats euthanized 4h later. Markers of oxidative stress including lipid peroxidation (malondialdehyde), reduced glutathione and nitric oxide were measured. In addition, paraoxonase-1 (PON-1) and butyrylcholinesterase (BCHE) activities were determined. Histopathological changes were evaluated using of haematoxylin and eosin staining. Periodic acid Schiff's (PAS) staining for mucopolyssacharides and immunostaining for cleaved casapase-3. Results showed that 2, 4-DNP caused inhibition of lipid per oxidation, nitric oxide and depletion of reduced glutathione. There were also inhibition of brain PON-1 and BCHE activities. The histopathological study revealed brain spongiform degeneration by 10 mg/kg 2,4-DNP while extensive neuronal necrosis and aggregates of focal gliosis occurred after the higher doses. There were also increased PAS reaction and cleaved caspase-3 immunostaining. Collectively, these results indicate deleterious effects for high doses of 2,4-DNP on brain tissue which is caused by uncoupling of mitochondrial oxidative phosphorylation and cellular energy depletion.

Keywords: 2,4-dinitrophenol, oxidative stress, mitochondrial uncoupling, apoptosis.

1. INTRODUCTION

The process of oxidative phosphorylation which occurs in the inner mitochondrial membrane involves the oxidation of NADH to produce energy that is used to phosphorylate adenosine di-phosphate (ADP) to adenosine triphosphate (ATP). 2,4-dinitrophenol (2,4-DNP) is a mitochondrial

uncoupling agent i.e., uncouples the electron transport chain from oxidative phosphorylation with the net result that no ATP being produced. The free energy is dissipated in the form of heat, thereby, increasing the basal metabolic rate (Terada, 1990). For this reason, 2,4-DNP has been used in the past (1930s) as a slimming agent, but banned later in 1938 by USA Food and Drug Administration (FDA) because of severe toxicity and fatalities (Grundlingh et al., 2011). There is renewed interest in using 2,4-DNP as a slimming agent since 2001 and this is especially common among body builders, with purchasing being through the internet (Grundlingh et al., 2011; Yen et al., 2012; Kamour et al., 2015; Bartlett et al., 2010). This coincided with several reports of toxicity and deaths despite a warning from the UK Food Standard Agency in 2003 that 2,4-DNP is 'not fit for human consumption' (Grundlingh et al., 2011). Toxicity is associated with uncontrollable hyperthermia, tachyarrhythmia, pulmonary oedema, muscular rigidity, metabolic acidosis, acute renal failure, ultimately leading to death (Bartlett et al., 2010; Hoch et al., 1973; Miranda et al., 2006; Tewari et al., 2009; Holborow et al., 2016). Unfortunately, there is no antidote for 2,4-DNP and management is that of supportive measures and controlling hyperthermia, seizures, agitation, arrhythmia and cardiovascular collapse (Yen et al., 2012). The aim of this study was to investigate the effect of high doses of 2,4-DNP on brain in rats using biochemical, histopathological, histochemical techniques.

2. MATERIALS AND METHODS

Animals

This study was conducted on female Sprague-Dawley rats weighing 120-130 g. Rats were group housed under temperature and light controlled conditions and allowed free access to standard laboratory rodent chow and tap water ad libitum. Eight rats were used per group. Animal procedures followed the guidelines of the Institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the US National Institutes of Health (Publication No. 85-23, revised 1996).

Chemicals and reagents

2,4 dinitrophenol (2,4-DNP) was purchased from Sigma (St. Louis, USA) and suspended in 1 w/v % methylcellulose solution. Other chemicals and reagents were of analytical grade (Sigma, St. Louis, USA).

Study design

Rats were randomly assigned into five different groups (8 rats each). Group 1 was intraperitoneally (IP) treated with vehicle and served as control. Groups 2,3 and 4 were treated IP, once with 2,4-DNP at different doses of 10, 40, 80 and 160 mg/kg and euthanized 4h later.

Tissue processing

Rats were quickly euthanized by decapitation, their brains moved. One half of the brain, the right was kept on icecold glass plate, washed with ice cold phosphate buffered saline at pH 7.4, weighed and stored at -80 ^{o}C until the biochemical assays. Tissue homogenization was done with 0.1 M phosphate buffered saline (pH 7.4) to give a final concentration of 10% weight/volume. The other half of the brains from all dissected animals were kept in 10% for mol saline for histopathological processing.

Biochemical analyses

Determination of lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation was determined in brain tissue homogenates according to the method of (Ruiz-Larrea et al., 1994). In this assay thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid to form TBA-MDA adduct which can be measured using spectrophotometer at 532 nm.

Determination of nitric oxide

Nitric oxide was determined in brain tissue homogenates using Griess reagent. In this assay nitrate is converted to nitrite via nitrate reductase. Griess reagent then acts to convert nitrite to a deep purple azo compound that can be determined using spectrophotometer at 540 nm (Moshage et al., 1995).

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Determination of reduced glutathione

Reduced glutathione (GSH) was determined in tissue homogenates using the procedure of (Ellman, 1959). The assay is based on the reduction of Ellman´s reagent (DTNB; 5, 5′-dithiobis (2-nitrobenzoic acid)) by the free sulfhydryl group on GSH to form yellow colored 5-thio-2-nitrobenzoic acid which can be determined using spectrophotometer at 412 nm.

Determination of paraoxonase-1

The aryl esterase activity of PON-1 was determined by a colorimetric method in which phenyl acetate used as a substrate. In this assay, PON-1 catalyzes the cleavage of phenyl acetate resulting in the formation of phenol. The rate of phenol formation was measured via monitoring the increase in the absorbance at 270 nm at 25°C. One unit of arylesterase activity is equal to 1 μmole of phenol formed per minute. The PON1 activity is expressed in KU/L, based on the extinction coefficient of phenol of 1310 $M^{-1}cm^{-1}$ (Haagen et al., 1992).

Cholinesterase activity

Butyrylcholinesterase (BCHE) activity was measured in brain tissue supernatants using commercially available kit (Biodiagnostics, Egypt). BCHE catalyzes the hydrolysis of butyrylthiocholine as a substrate into butyrate and thiocholine. The latter reacts with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) producing a yellow chromophore which then could be quantified using spectrophotometer at 412 nm (Ellman et al., 1961).

Histopathological studies

Haematoxylin and Eosin staining

Five μm thick paraffin sections were stained with Hematoxylin & Eosin (HX&E).

Periodic acid Schiff's staining

Periodic acid Schiff's (PAS) was used for staining mucopolysaccharides contents in brain.

Immunohistochemistry for cleaved caspase-3

Mouse monoclonal caspase-3 antibodies were used for detection of the caspase cleavage. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with anticaspase overnight at 4°C. After washing with PBS, followed by incubation with biotinylated goat antirabbitimmunoglobulin G secondary antibodies (1:200 dilution: Dako Corp.) and streptavidin/alkaline phosphates complex (1:200 dilution: Dako) for 30 min at room temperature, the binding sites of antibody were visualized with DAB (Sigma, USA). After washing with PBS, the samples were counterstained with H & E for 2–3 min, dehydrated using graded alcohols and xylene and mounted on slides. The immunostaining intensity and cellular localization of cleaved caspase-3 were analyzed by light microscope.

Quantitative measurements

Quantitative measurements of mucopolysaccharide content and cleaved caspase-3 immunostaining were performed using computerized image analyzer (Leica Qwin 500 image) in the Image Analyzer Unit, Pathology Department, National Research Centre.

Statistical analysis

Data in the study were presented as mean ± standard error of the mean (SEM). Statistical significance was assessed using one way ANOVA with Duncan's multiple range test post hoc test. Graphpad Prism software, version 6 (GraphPad Prism Software Inc., San Diego, CA, USA) was used. A probability value of less than 0.05 was considered as statistically significant.

3. RESULTS

Biochemical results

Results are presented in Table 1 & Figures 1 & 2. Compared with the control group, the administration of 2,4-DNP resulted in significant decrease in malondialdehyde, nitric oxide and reduced glutathione contents in brain tissue. There was also significant inhibition of brain PON-1 and BCHE activities. These effects of 2,4-DNP were dose dependent.

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Table 1 Effect of different doses of 2,4 dinitrophenol (2,4-DNP) on brain malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO), paraoxonae-1 (PON-1) and butyrylcholinesterase (BCHE)

Groups / Parameters	Control	2,4-DNP 10 mg/kg	2,4-DNP 40 mg/kg	2,4-DNP 80 mg/kg	2,4-DNP 160 mg/kg
MDA (nmol/g. tissue)	10.8±1.9	8.86 ± 0.09* (-18.0%)	6.04± 0.1* (-44.1%)	5.69 ± 0.08*+ (-47.3%)	4.25±0.08*+ (60.6%)
GSH (µmol /g.tissue)	5.43±0.11	4.55 ± 0.07* (-16.2%)	4.47 ± 0.09* (-17.7%)	4.13 ± 0.10* (-23.9%)	3.86 ± 0.05*+ (-28.9%)
NO (µmol/g.tissue)	9.1 ± 0.54	8.0 ±0.66 (-12.1-%)	6.5 ±0.48* (-28.6-%)	3.77 ±0.21*+ (-58.6-%)	3.6±0.18*+ (-60.4-%)
PON-1 (kU/l)	14.0±0.16	9.95 ±0.44* (-28.9-%)	9.45±0.48* (-32.5-%)	7.95±0.7*+ (-43.2%)	4.9±0.27*+ (-65.0%)
BCHE (U/l)	128 ±4.9	118.5 ±3.8 (-7.4-%)	101.2 ±5.4* (-21.0-%)	87.7± 1.8*+ (-31.5-%)	73.1 ±2.9*+ (-42.9-%)

Data are expressed as mean ± SE. The percent change from the control value is shown in parenthesis. Statistical analysis was carried out by one-way ANOVA. *: P<0.05 vs. Control. +: P<0.05 vs. 2,4-DNP 10 mg/kg.

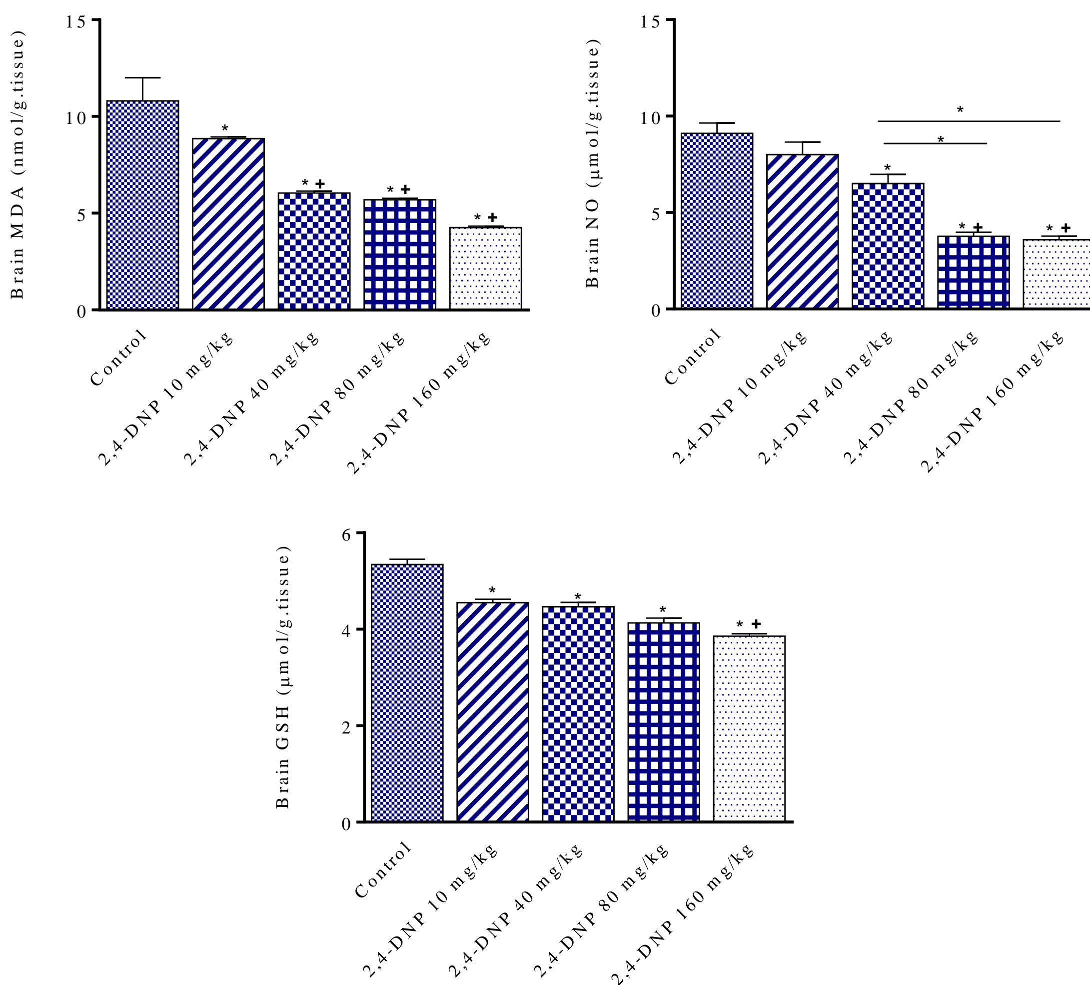

Figure 1 Effect of different doses of 2,4 dinitrophenol (2,4-DNP) on brain malondialdehyde (MDA), Reduced glutathione (GSH) and nitric oxide (NO). *: P<0.05 vs. Control and between different groups as indicated in the graph. +: P<0.05 vs. 2,4-DNP 10 mg/kg.

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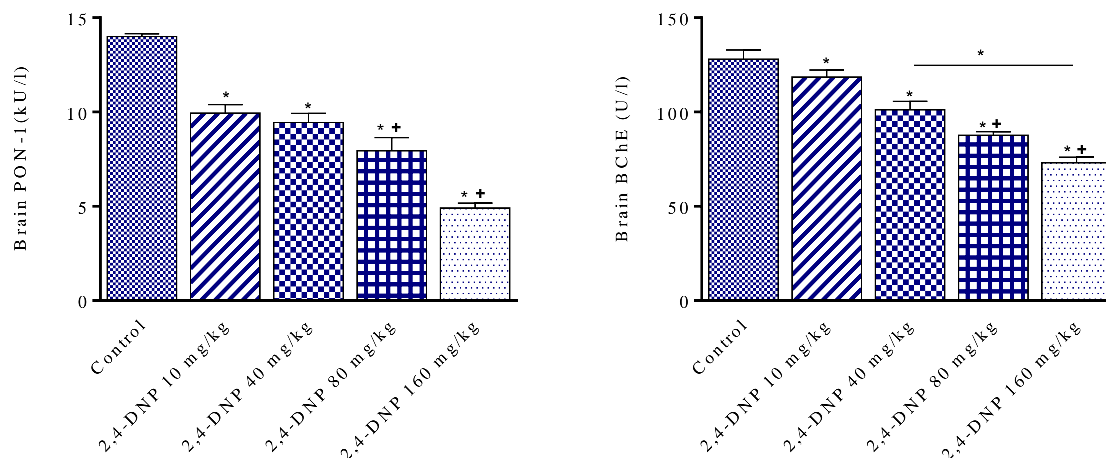

Figure 2 Effect of different doses of 2,4 dinitrophenol (2,4-DNP) on brain paraoxonase1 (PON-1) and butyrylcholinesterase (BCHE). *: P<0.05 vs. Control and between different groups as indicated in the graph. +: P<0.05 vs. 2,4-DNP 10 mg/kg.

Histopathological results

Microscopic examination of H&E-stained sections from control rats showed normal histological structure of the cerebral cortex with normally arranged cortical layers, outer and inner granular layer and outer, inner pyramidal layer. These layers showing acidophilic neuropile and rounded open face nuclei with prominent nucleoli of the neurons, cerebral blood vessel (Figure 3). Rats treated with 2,4-DNP at 10 mg/kg exhibited spongiform degeneration as numerous vacuolated areas in the cortex and microgliosis adjacent to inflammatory mononuclear perivascular cuff (Figure 3B). Multiple focal homogenous deeply eosinophilic plaques with various sizes were seen (Figure 3C). Rats treated with 2,4 DNP at 40 mg/kg showed spongiform degeneration and neuronal cell degeneration, pyknotic black neurons with condensed nuclei. Some neurons appeared apoptotic and others exhibited basophilic neuronal necrosis. Dilated and congested blood vessels in cerebral cortex were observed (Figures 3D & E). Severe chromatolysis in neurocytes and pyknotic pyramidal cells, eosinophilic plaques and well delineated dense aggregates of cotton wool plaques were clearly seen in the group treated with 2,4 DNP at 80 mg.kg (Figure 3F). Moreover, treatment with 2,4 DNP at 160 mg/kg showed neuronal degeneration with pyknotic black neurons and condensed nuclei (hyperchromatic nuclei). Aggregates of focal gliosis and congested cerebral blood vessel were seen (Figures 3G & H).

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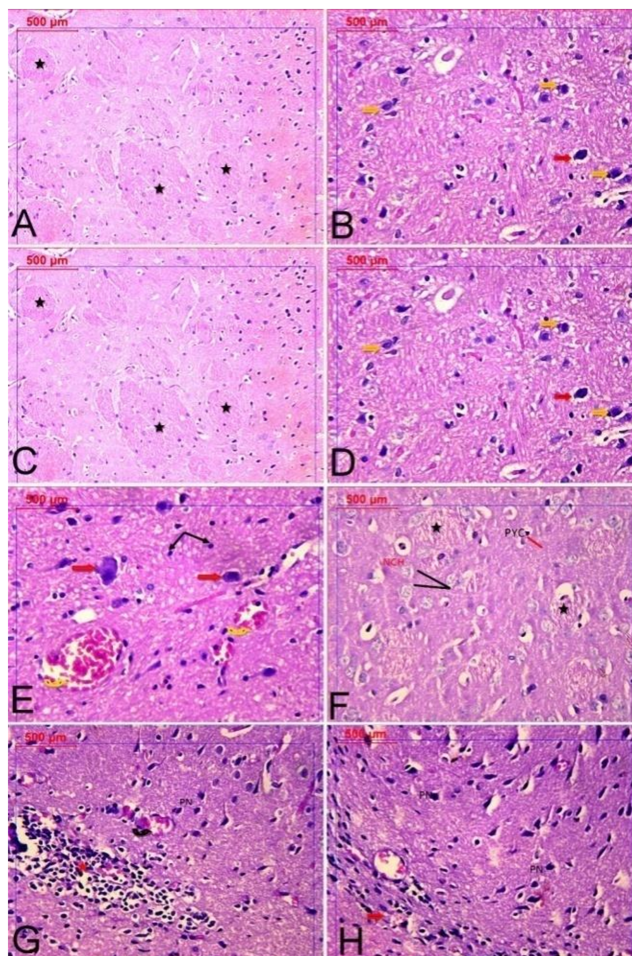

Figure 3 Photomicrographs of sections of the cerebral cortex after treatment with: (A) Vehicle control: Normally arranged cortical layers. (B) 2,4-DNP 10 mg/kg: spongiform degeneration, microgliosis, inflammatory mononuclear perivascular cuff (arrow). (C) 2,4-DNP 10 mg/kg: Deeply eosinophilic plaques. (D) 2,4-DNP 40 mg/kg: Spongiform degeneration and neuronal cell degeneration, pyknotic black neurons (orange arrow) with condensed nucleus. Some neurons appeared apoptotic (red arrow). (E) 2,4-DNP 40 mg/kg: Basophilic neuronal necrosis (red arrow), congested blood vessel (orange arrow), glial cells (black arrow). (F) 2,4-DNP 80 mg/kg: Severe chromatinolysis in neurocytes (NCH), pyknotic pyramidal cells (PYC). Eosinophilic plaque (star), cotton wool plaques. (G) 2,4-DNP 160 mg/kg: Neuronal degeneration (perikaryons with pyknotic nuclei) (PN), focal gliosis (red star), congested blood vessel (black curved arrow). (H) 2,4-DNP 160 mg/kg: Perikaryons with pyknotic nuclei (PN), focal gliosis (star), congested blood vessel (black arrow) (HX & EX 400).

Periodic acid Schiff's staining

The cerebral cortex of control rats showed normal distribution of PAS reaction in the cytoplasm of the granular cells (Figure 4A).The glycogen particles showed slight increase in rats treated with 2,4-DNP at 10 or 40 mg/kg (Figures 4B & C), moderate increase in rats treated with 2,4-DNP at 80 mg/kg and high increase after treatment with 160 mg/kg 2,4-DNP. The increased glycogen particles more apparent within degenerated neurons (Figures 4D & E).

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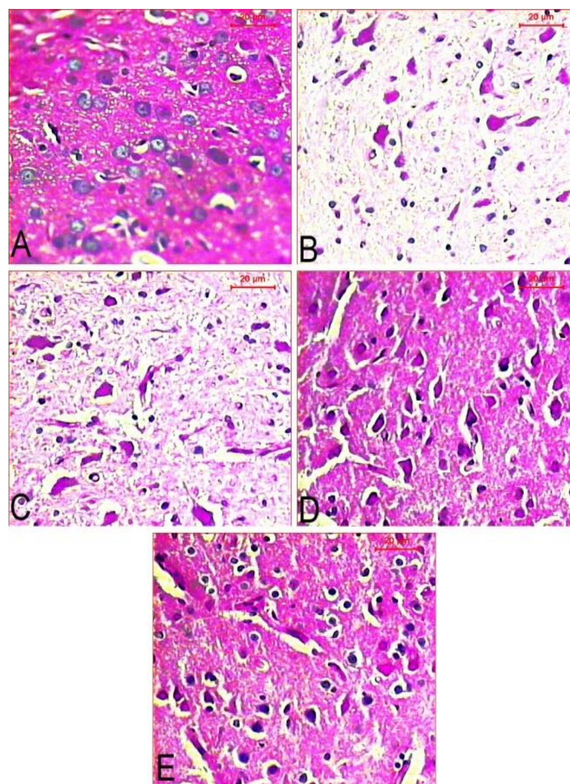

Figure 4 Photomicrographs of sections of the cerebral cortex stained with Periodic acid Schiff's (PAS). (A) Control (B & C) 2,4-DNP 10 and 40 mg/kg: Slight increase in PAS reaction. PAS inclusions were present in cerebral cortical neurons. (D) 2,4-DNP 80 mg/kg: Moderate increase in PAS stain. (E) 2,4-DNP 160 mg/kg: Highly increased mucopolysaccharide content in abnormal granular neuron (PAS reaction counterstained with HX & EX400).

Caspase-3 immunoreactivity

No caspase-3 immunoreactivity was detectable in the brain of control rats (Figure 5A). Rats treated with 2,4-DNP 10 and 40 mg/kg exhibited mild increase in caspase-3 immmunostaining (Figures 5B & C). The expression of caspase 3 revealed moderate increase after 2,4-DNP 80 mg/kg (Figure 5D) and was highly increased after 2,4-DNP 160 mg/kg (Figure 5E). The results of morphometric measurements of PAS area % and caspase-3 area% in different treated groups are shown in Figure 6.

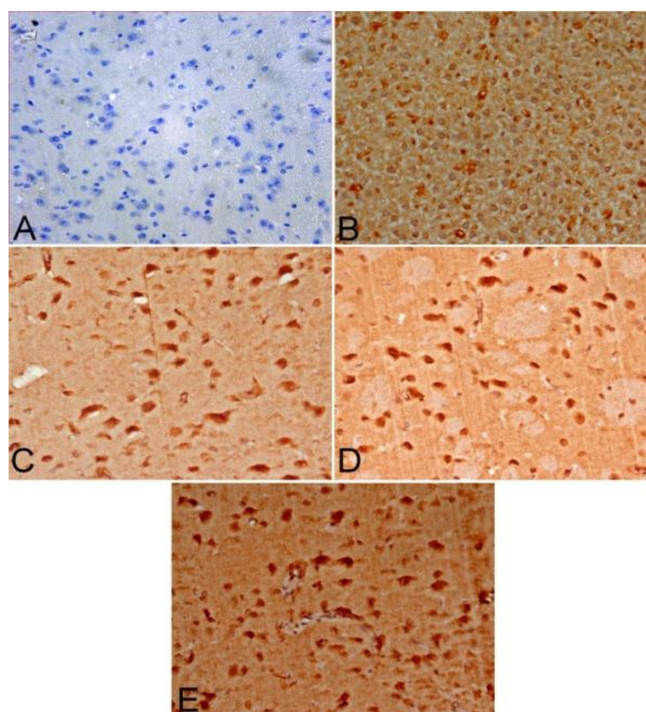

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Figure 5 Photomicrographs of sections of the cerebral cortex immunostained by caspase-3 antibody showing: (A) No expression of cleaved caspase-3 in control group. (B & C) Mild increase in expression of cleaved caspase3 in the nerve cell bodies of rats treated with 2,4-DNP at 10 and 40 mg/kg. (D) Moderate positive cleaved caspase-3 immunoreactivity after 2,4-DNP 80 mg/kg. (E) Highly increased cleaved caspase-3 immunostatining in rats treated with 2,4-DNP 160 mg/kg (X200).

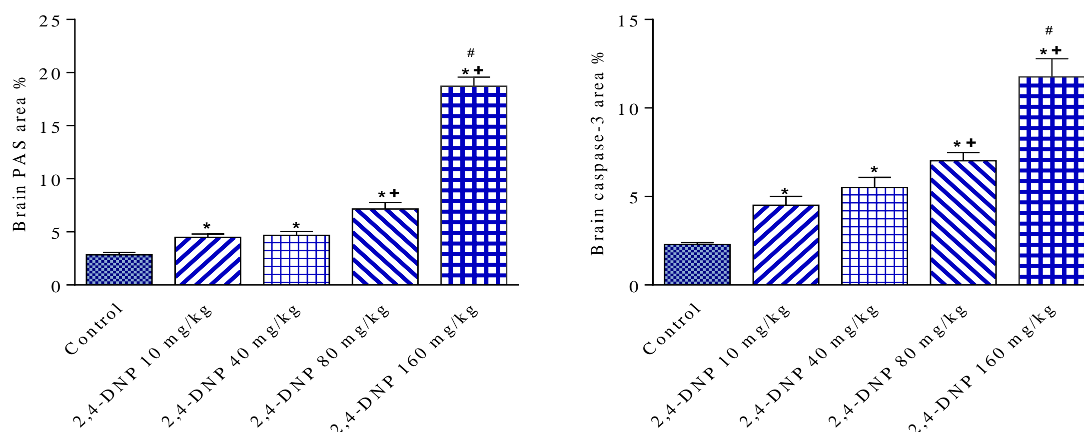

Figure 6 Results of morphometric measurements of PAS area% and caspase-3 area % in different groups. Data are expressed as mean ± SE. Statistical analysis was carried out by oneway ANOVA. *: P<0.05 vs. Control. +: P<0.05 vs. 2,4-DNP 10 mg/kg. #: P<0.05 vs. 2,4-DNP 40 mg/kg.

4. DISCUSSION

The results of this study clearly indicate deleterious effects for 2,4-DNP on several organs which explains the toxicity associated with this slimming agent. 2,4-DNP did not increase oxidative stress. In contrast, inhibition of malondialdehyde, an end product of lipid peroxidation (Gutteridge, 1995) in brain tissue was demonstrated. The major source for reactive oxygen metabolites in the cell is the mitochondria where the superoxide radical ($O_2^{-\bullet}$) is generated as a byproduct of electron transfer through respiratory chain complexes. The superoxide anion can then result in the formation of more reactive oxygen and nitrogen species eg., hydroxyl radical, peroxynitrite and lipid peroxides (Figueira et al., 2013). 2,4-DNP uncouples the electron transport chain from oxidative phosphorylation with the result that no ATP is produced Terada, (1990) which explains the decrease in nitric oxide levels presumably from the decrease in nitric oxide synthase activity. In support of the above notion is the observed decline in the activity of both PON-1 and BCHE enzymes in brain of 2,4-DNP-reated rats. We also found depletion of the antioxidant and free radical scavenging reduced glutathione following 2,4-DNP, suggesting decreased synthesis. Taken together, the observed biochemical alterations in the tissues of 2,4-DNP-treated rats could be the result of failure of ATP synthesis.

Our study showed that 2,4-DNP in high doses causes spongiform degeneration, microgliosis, eosinophilic plaques, foci of gliosis and neuronal cell degeneration. Other researchers demonstrated neurodegeneration in the rat brain after treatment with 2,4-dinitroanisole (80 mg/kg/day for 90 days) (Lent, 2016). There were variable degenerated neurons and lesions in cerebellar peduncle, consisting of focal loss or attenuation of the neuropil, shrunken Purkinje cells increase in glial cell numbers, occasional hemorrhage. It should be noted, however, that low concentration of DNP protects neurons against the toxicity of the amyloid-β peptide and the pesticide rotenone (De Felice et al., 2006; Abdel-Salam et al., 2017). The useful actions of DNP at low concentrations including neuroprotection against different types of insults, blockade of amyloid aggregation, stimulation of neurite outgrowth and neuronal differentiation were reported using DNP doses between 1-5 mg/kg (De Felice et al., 2006) which is not the case in the present study. Some of these effects appear to be due to mild mitochondrial uncoupling and prevention of oxidative stress, whereas other actions are related to activation of additional intracellular signaling pathways (Korde et al., 2005).

We used Periodic acid Schiff (PAS) for delineating the effect of 2,4-DNP on the cell's glycogen content in brain. The PAS reaction has been used clinically to identify progressive neurodegeneration in Alzheimer's disease (Naderali et al., 2010), presenile dementia (Kobayashi et al., 1992) and Parkinson's disease (Forno et al., 1993). Also, Bennett et al., (1995) demonstrated that the distribution of PAS positive material in brain tissue varied according to gross morphological alteration accompanying neurodegeneration. Caspases (cysteine aspartic proteases) are a family of cysteine proteases that play an essential role in apoptosis or "programmed cell

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death" (Steller, 1995; Greenhalgh, 1998). Caspase-3 activation plays a key role in triggering apoptosis in neuronal cells (Stefanis et al., 1999; Jin et al., 2004). Here we showed that cleaved caspase-3 immunostaining increases in brain after toxic doses of 2,4-DNP. Our results are clearly in contrast to the effect of 2,4-DNP at doses of 1 and 3 mg/kg (Abdel-Salam et al., 2017). These doses are considerably much lower than those used in the current study and fell within the dose range reported to exert neuroprotective effects (De Felice et al., 2006; Jin et al., 2004; Maragos et al., 2004; Ferreira et al., 2007). In contrast to detrimental effects of high doses of 2,4-DNP that depleted cellular energy, mild uncoupling of oxidative phosphorylation with small doses (in range of 1-3 mg/kg) reduces the production of oxygen reactive metabolites especially superoxide by the mitochondria which acts to protect the mitochondria from damage by free radical (Maragos et al., 2004).

4. CONCLUSION

In summary, the present study indicates that the administration of high doses of 2,4-DNP was associated with histologic brain damage and apoptosis. 2,4-DNP resulted in inhibition of lipid peroxidation, nitric oxide, depletion of reduced glutathione levels as well as depressed brain activities of PON-1 and BCHE enzymes. These deleterious effects of 2,4-DNP are accounted for by uncoupling of oxidative phosphorylation and the consequent energy failure.

Author contribution

OMEAS and AAS designed and conducted the research. DM performed the biochemical studies. FAM, MES and NNY performed the histopathological studies and its interpretation. OMEAS prepared the manuscript. OMEAS, AAS, FAM, MES, NNY and DM approved the final version of the manuscript.

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There are none.

Ethical approval

Animal procedures followed the guidelines of the Institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the US National Institutes of Health (Publication No. 85-23, revised 1996).

Conflict of Interest:

The authors declare that there are no conflicts of interests.

Data and materials availability:

All data associated with this study are present in the paper.

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