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Physical & mental activities enhance the neuroprotective effect of vinpocetine & coenzyme Q10 combination against Alzheimer & bone remodeling in rats

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ABSTRACT

Background: Alzheimer's disease is a neurodegenerative disorder characterized by a progressive decline of cognitive abilities as well as bone loss. Physical and mental activities maintain cognitive functions as well as increase bone mass by inhibiting bone resorption. VIN and CoQ10 are neuroprotective drugs that possess anti-inflammatory and antioxidant properties.

Aims: To study the effect of PH&M on enhancing the neuroprotective role of VIN and CoQ10 combination during induction of AD model in rats besides their role against bone mass loss associated with AD model.

Main methods: Six groups of rats were received saline, $AlCl_3$, and PH&M daily either alone or with a combination of VIN and CoQ10 for 4 weeks. Various biochemical analyses were performed to evaluate the extent of brain damage such as ACHE, β-secretase, chitinase, Aβ, tau protein, and monoamines besides the inflammatory and antioxidant parameters. Serum levels of minerals as well as 25-OHD, PTH, RANKL, and OPG levels were measured to detect the extent of bone impairment. Also, histopathological changes were evaluated in different brain regions and hind paw.

Key findings: VIN and CoQ10 combination together with PH&M significantly attenuated the neurodegeneration induced by $AlCl_3$ administration through the improvement of AD markers in brain tissue as well as oxidant and inflammatory markers. Bone resorption markers, serum minerals, and PTH levels were also normalized too.

Significance: Neuroprotective drugs together with PH&M have a more protective effect against AD and bone loss rather than PH&M alone.

1. Introduction

Alzheimer's disease (AD) known as senile dementia that considered one of the main neurodegenerative diseases that exerted a negative influence on family and society [1]. AD characterized by cognitive dysfunction and progressive memory loss in old age individuals [2]. Furthermore, recent reports showed that sensory and motor dysfunction might precede the cognitive symptoms of AD, since increasing frailty, lower aerobic capacity, weight loss, and motor inability were common in AD [3,4]. At the molecular level, AD characterized by senile plaques and neurofibrillary tangles that composed of beta-amyloid (Aβ) and tau phosphorylated protein subsequently [5]. This Aβ formed from the sequential cleavage of β-amyloid precursor protein (APP) by β- and γ–secretases [6]. Additionally, Aβ plaques have many pathological roles in AD incidence as potent synaptotoxins, proteasome inhibitors, suppress mitochondrial function, change intracellular Ca^{2+} levels, and enhance the neuronal inflammatory processes [7,8]. Earlier studies documented that Aβ is involved in AD induction due to reactive oxygen species (ROS) generation and oxidative stress resulting in damaged mitochondria and hence neurotoxicity [9,10].

Bone health is an important matter in individuals with AD because hip fractures due to bone fragility become a major public health issue in

Abbreviations: AD, Alzheimer's disease; PH&M, physical and mental activities; VIN, vinpocetine; CoQ10, coenzyme Q10; ACHE, acetylcholinesterase; Aβ, beta-amyloid; BDNF, brain-derived neurotrophic factor; ALP, alkaline phosphatase; DA, dopamine; NE, norepinephrine; 5-HT, serotonin; 25-OHD, 25-(OH) vitamin D; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor-κB; OPG, osteoprotegerin

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the elderly population as they are subjected to poor recovery and high mortality rates [11,12]. On the other hand, some studies suggest that low bone mineral density (BMD) is related to the increased risk of AD [13,14] and cognitive decline [15]. Also, reduced physical activity and vitamin D (25OHD) deficiency are major risk factors for low BMD occurrence in AD individuals [16].

Aluminum (Al) is a highly toxic metal that causes bone disease, microcytic anemia, and encephalopathy in renal failure patients [17–19]. Chronic administration of $AlCl_3$ accumulates effectively in specific regions in the central nervous system (CNS) inducing degenerative changes [20]. Al induces overexpression of APP which mediates the pathogenesis of AD due to activation of different signaling pathways involved in inflammation and neurodegeneration [21]. Moreover, Al exposure causes aggregation of proteins akin to neurofibrillary tangles in CNS that induces neurological abnormalities [22]. Additionally, $AlCl_3$ administration adversely affects bone formation resulting in bone loss and impairment mediated by oxidative stress as well as osteoclast cell activation [23,24].

Vinpocetine (VIN), a synthetic derivative of the alkaloid vincamine that is used to improve the cognitive function in cerebrovascular diseases. VIN has a neuroprotective effect due to its antioxidant and anti-inflammatory properties [25]. Also, VIN possesses an important effect on the brain via increasing cerebral circulation and metabolism [26].

Coenzyme Q10 (CoQ10) is a pseudovitamin that acts as a component of the electron transport chain and generates ATP in the mitochondria [27]. CoQ10 possesses an antioxidant and free radical scavenger activity. It helps in preventing neurodegeneration against mitochondrial deficiency and oxidative stress and thus, improvement of cognitive disorders [28]. It appeared that CoQ10 is decreased in patients susceptible to AD, so its administration is a promising approach in alleviating neurodegenerative disease [29].

Physical and mental activities (PH&M) improve cognition and memory by increasing blood flow to the brain as well as enhancing neurogenesis in the hippocampus [30–32]. Thus, this study designed to detect the effect of PH&M in addition to VIN and CoQ10 combination therapy in reducing AD and the associated low bone density.

2. Materials and methods

2.1. Drugs and chemicals

Aluminum chloride - hydrated ($AlCl_3 \cdot 6H_2O$), VIN, and CoQ10 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of the highest grade-commercially available. $AlCl_3$ was freshly dissolved in distilled water. VIN was suspended in saline with the help of tween 80 and daily administered to rats by gavage (p.o.) at a dose of 20 mg/kg [33]. CoQ10 was suspended in saline with the help of tween 80 and daily administered to rats by gavage (p.o.) at a dose of 200 mg/kg [34].

2.2. Animals

The animal experiments were carried out according to the guidelines of the Ethical Committee of Al-Azhar University in Egypt for animal care and use. Male Sprague Dawley rats weighing 250–280 g were used in all experiments of this study. Rats were obtained from the Nile Co. for pharmaceuticals and chemical industries, Cairo, Egypt. Rats were housed in faculty of pharmacy, Al-Azhar University in Egypt animal house under standard housing conditions (temperature of 25 ± 1 °C) with 12-h light and dark cycles. They were kept for four weeks on the water with ad-libitum and standard diet pellets that composed of protein (20%), fiber (5%), fat (3.5%), ash (6.5%) and vitamin mixture (El-Nasr, Abu Zaabal, Cairo, Egypt) [35].

2.3. Experimental design

Rats were randomly divided into six groups; each group contains eight numbers of rats. During these four weeks of the experiment, the control groups of rats were given saline daily and the other two groups received saline together with PH&M (Swimming and Y-maze tests) either alone or with a combination of VIN (20 mg/kg, p.o.) and CoQ10 orally (200 mg/kg, p.o.) suspended in tween 80. The other three groups served as an AD model group that received $AlCl_3$ (70 mg/kg, i.p.) daily [36]. The $AlCl_3$ groups are either untreated or exposed to PH&M alone or combine with VIN and CoQ10.

2.4. Physical and mental activities (PH&M)

Both swimming and Y-maze test are the physical and mental activities subsequently used during this experimental study. Rats were exposed to PH&M once per week for four weeks [37] to illustrate the protective effect of these activities on $AlCl_3$ treated groups (AD model).

The swimming test was carried out according to other described and modified methods [38,39]. The test was done in a glass tank apparatus that filled to its half with water of adjusted temperature (26 °C to 27 °C). The ramp was placed at one side of the glass tank and swimming was started from the other side of the tank. Rats were placed individually at the starting point and take 3 min till reaching the ramp with forepaws.

On the other hand, Y-maze test was used according to the previously described method [40] to enhance the activity of different parts of the brain [41]. The apparatus is a 3-arm maze shaped like a capital Y, each arm spaced at an angle of 120°. During the test, each rat placed at the center of the maze then the sequence of entries into the three arms was recorded over a period of 8 min [42].

2.5. Tissue sampling and preparation

At the end of the fourth week, rats were sacrificed then brain and hind paw tissues were dissected and thoroughly washed with isotonic saline. Bone tissues were stored for histopathological examination in 10% buffered formalin, while the whole brain tissues were divided into two parts. These parts were one for histopathological examination and another part was immediately homogenized to give 10% (*w/v*) homogenate in ice-cold medium containing 50 mM Tris-HCl (PH 7.4) and 300 mM sucrose [43]. The homogenate was centrifuged at 1800 × *g* for 10 min. at 4 °C. The supernatant (10%) was used for the assessment of β-secretase, chitinase, and acetylcholine esterase (ACHE) activity. Moreover, β-amyloid (Aβ), brain-derived neurotrophic factor (BDNF), alkaline phosphatase (ALP), tau protein, and brain monoamines [Dopamine (DA), Norepinephrine (NE), and Serotonin (5-HT)] content were measured in this brain tissue homogenate. In addition, oxidative stress markers as malondialdehyde (MDA), total antioxidant capacity (TAC), and superoxide dismutase (SOD) activity. Furthermore, inflammatory mediators as tumor necrosis factor-alpha (TNF-α) and Interleukin1β (IL-1β) were measured in this homogenate too.

2.6. Sera sampling and preparation

Serum samples were collected from the six groups and centrifugated at 3000 rpm for 10 min to determine the bone measured parameters. Furthermore, the sera were used for measuring calcium ions (Ca^{2+}), magnesium ions (Mg^{2+}), 25-(OH) vitamin D (25OHD), parathyroid hormone, receptor activator of NF-kB ligand (RANKL), and osteoprotegerin (OPG) content. These parameters were measured to evaluate the impact of PH&M when combined with VIN and CoQ10 on AD model and their protective role in bone remodeling.

2.7. Histopathological examination of brain tissue and hind paw

Brain and hind paw tissue samples were fixed in 10% formalin for

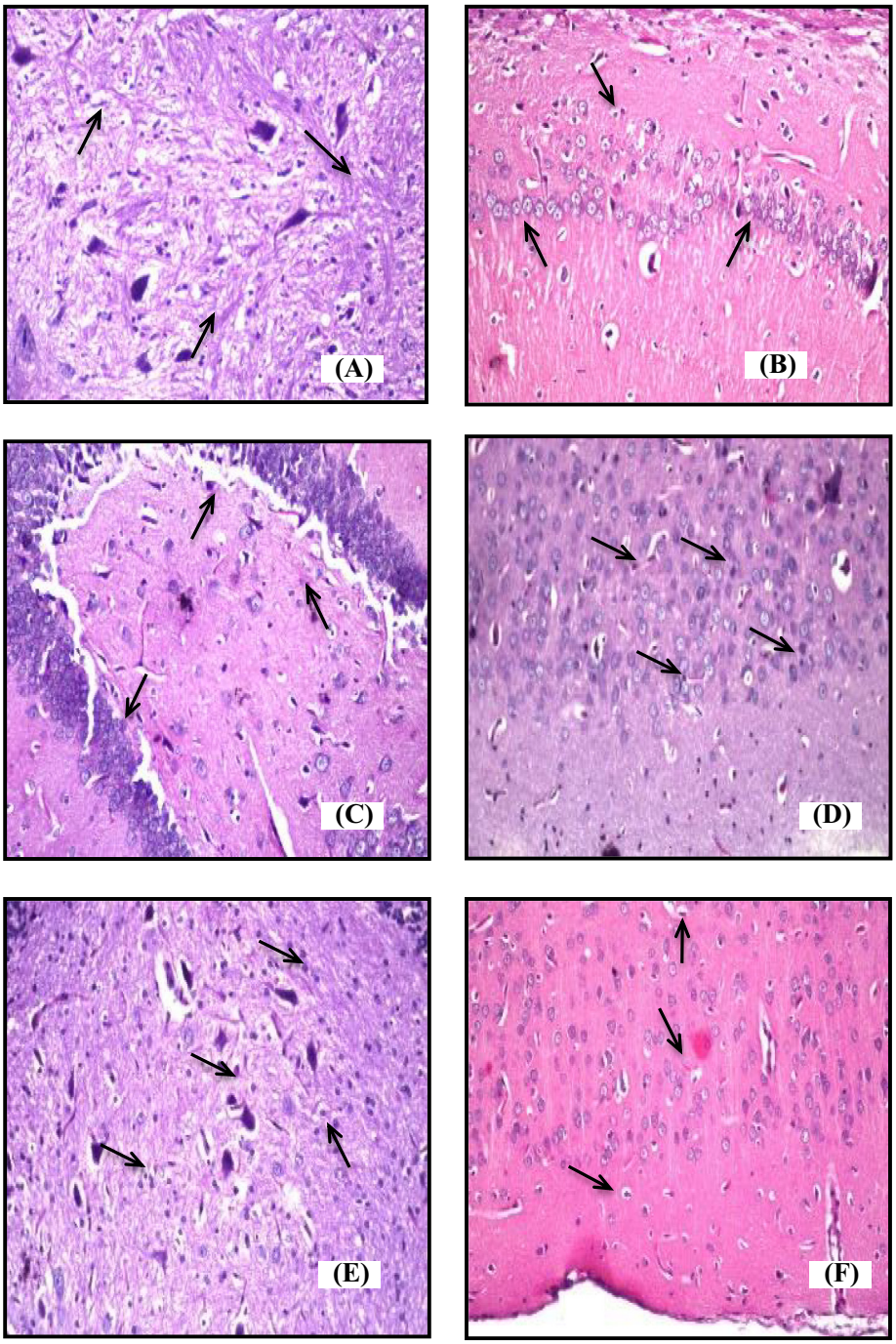

Fig. 1. Representative H&E staining images to detect the effect of physical and mental activities when combined with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) co-administration during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) on histopathological examination of different brain regions in rats.

24 h subsequently washed with water and serial dilution of alcohol for dehydration. Specimens were embedded in paraffin and sectioned by microtome to 4 μm thickness. Afterward, the obtained tissue sections were collected on glass slides for deparaffinized, then stained with hematoxylin and eosin stain for the routine histological examination using light microscopy [44].

2.8. Biochemical measurements

2.8.1. Determination of acetylcholinesterase (ACHE), β-secretase, and Chitinase content in brain tissue

ACHE, β-secretase, and chitinase content were detected in brain tissue homogenate according to the previously described methods respectively [45–47]. The ELISA kit numbers (MAK119, CS0010, and CS1030) were used respectively for this detection and supplied by Sigma-Aldrich Co. (St. Louis, MO, USA).

2.8.2. Determination of beta-amyloid (Aβ) and tau protein content in brain tissue

Aβ and Tau protein contents were measured in brain tissue homogenate according to the kit manufacturer's instructions. The ELISA kits, supplied by My BioSource, Inc., San Diego, USA, number (MBS702915) and (MBS725098) were used for Aβ and Tau protein detection respectively.

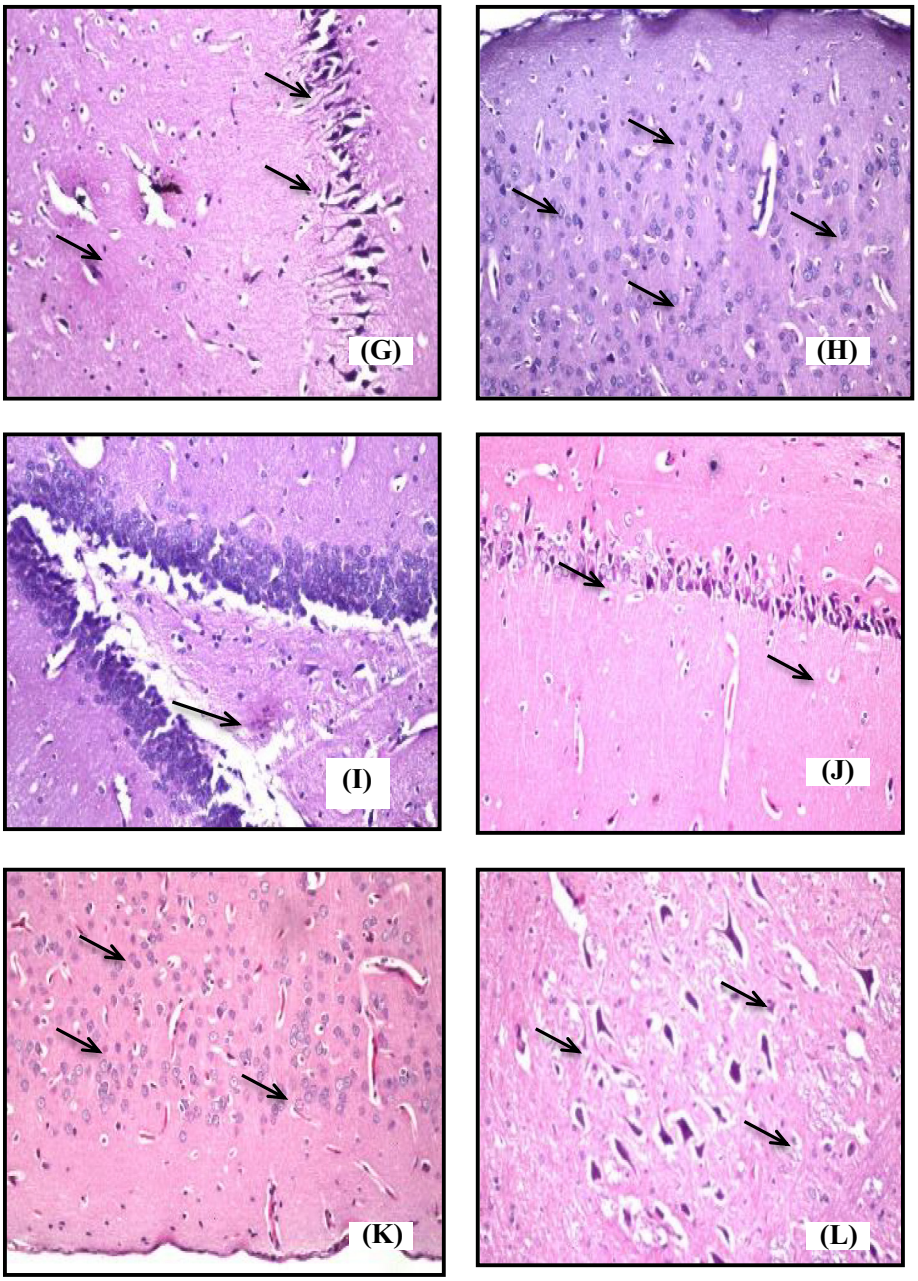

Fig. 1. (*continued*)

2.8.3. Determination of alkaline phosphatase (ALP) and brain-derived neurotrophic factor (BDNF) in brain tissue

Content of ALP in brain tissue homogenate was assayed by ELISA method using assay kit purchased from My BioSource, Inc., San Diego, USA. Product Number MBS011598 was used in such detection. Moreover, the same manufactured company supplied a Product Number MBS494147 for the ELISA method used in BDNF determination. The method used in the detection of BDNF in brain tissue was described by Kovalchuk et al. [48].

2.8.4. Determination of brain monoamine parameters (DA, NE, and 5-HT)

Immediately after sacrificing the rats, concentrations of brain monoamines were detected because changes in the level of brain monoamines may occur within a few minutes. Fluorometric assay of DA, NE and 5-HT were determined according to the previously described method [49].

2.8.5. Determination of oxidative stress markers (MDA, SOD, and TAC) in brain tissue

Brain lipid peroxidation was measured in terms of malondialdehyde (MDA) using thiobarbituric acid (Chemie Gmbh, Steinheim, Germany) [50]. SOD enzyme activity was determined based on its inhibitory effect to reduce nitro blue tetrazolium dye [51]. Finally, the antioxidants reactions with exogenously provide H_2O_2 was used for TAC assessment. The residual H_2O_2 was determined colorimetrically [52].

2.8.6. Determination of brain inflammatory mediators (IL-1β and TNF-α)

Using ELISA Kit supplied by Ray Biotech did determination of both IL-1β and TNF-α in brain tissue homogenate. Product Number (RTA00, SRTA00, PRTA00) and (ELR-IL1b) were used respectively for this determination according to the manufacturer's instructions.

2.8.7. Determination of calcium, magnesium, and phosphate ions in serum

Calcium Assay Kit (DICA-500) supplied by Quanti Chrom™ was used

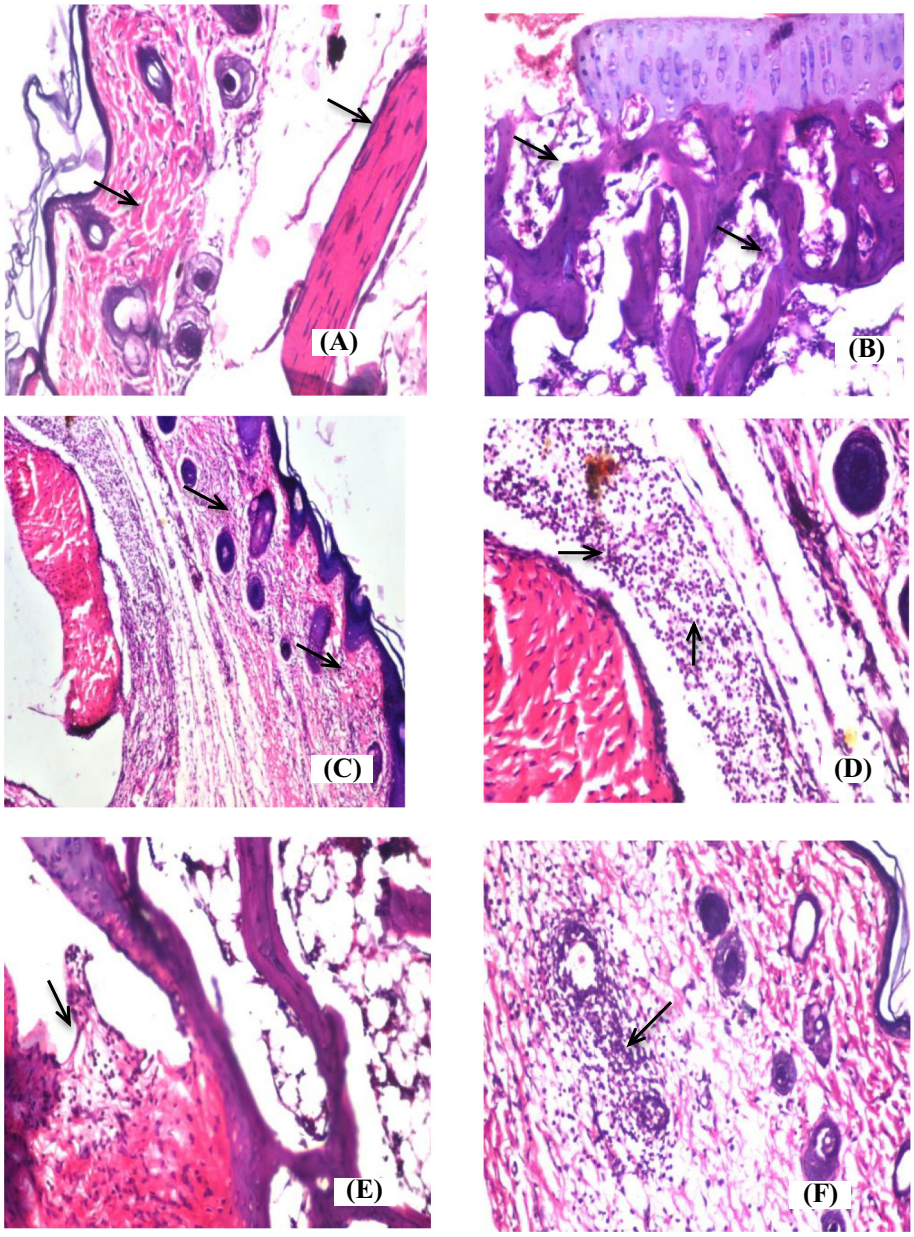

Fig. 2. Representative H&E staining images to detect the effect of physical and mental activities when combined with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) on hind paw sections in rats.

to determine quantitatively the calcium level. The determination method was mentioned previously [53]. Magnesium Assay Kit (DIMG-250) supplied by Quanti Chrom™ was used to determine quantitatively at 500 nm the magnesium level [54]. Moreover, phosphate Assay Kit (DIPI-500) supplied by Quanti Chrom™ was used to determine quantitatively the phosphate level. The manufacturer's instructions were the reference used for the assay methods of calcium, magnesium, and phosphate ions.

2.8.8. Determination of 25-(OH) vitamin D (25-OHD) and parathyroid hormone (PTH) levels in serum

Using ELISA Kit supplied by My BioSource (Inc., San Diego) and Sigma-Aldrich (Inc., St. Louis, MO, USA) correspondingly did determine both 25OHD and PTH in serum. Product numbers (MBS728692) and (SE120107) were used respectively for this determination according to the manufacturer's instructions.

2.8.9. Determination of receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) in serum

ELISA Kit (My BioSource, Inc., San Diego, MBS029351) was used for determining RANKL level in serum. Additionally, OPG was assessed in serum by using ELISA Kit supplied by Sigma-Aldrich (Inc., St. Louis, MO, USA), according to the previously described method [55].

2.9. Statistical analysis

Data are expressed as mean ± SD and multiple comparisons were performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. All statistical analysis and graphs were performed using GraphPad Prism (ISI®, USA) software (version 5).

3. Results

Effect of physical and mental activities when combined with VIN (20 mg/kg, p.o.) and CoQ10 (200 mg/kg, p.o.) co-administration during

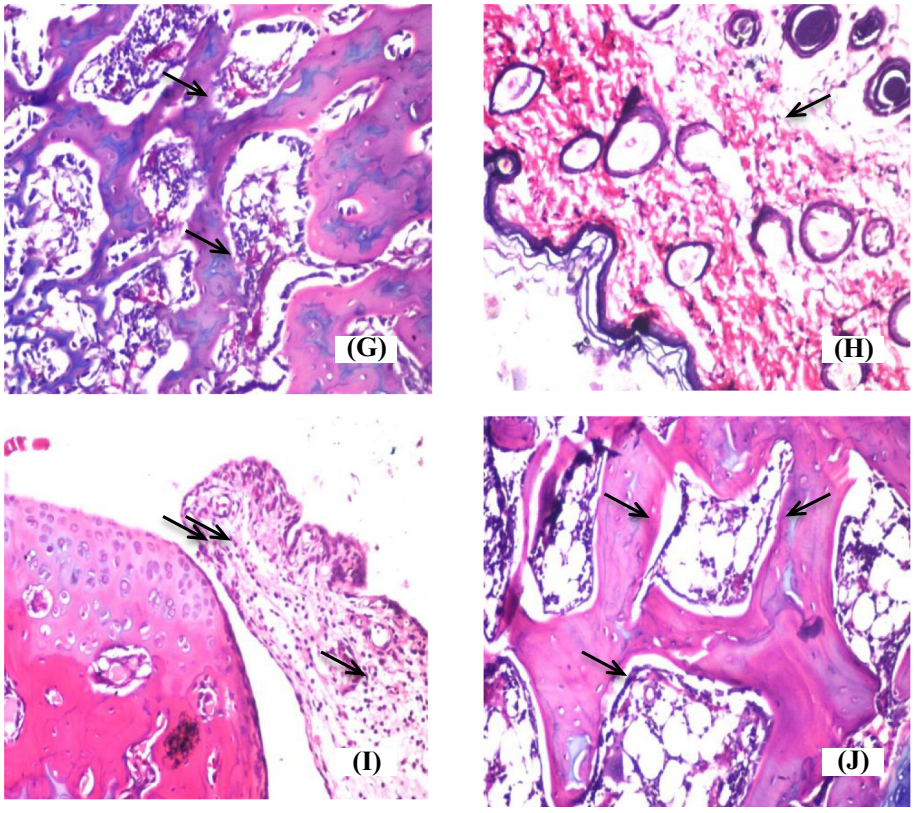

Fig. 2. (*continued*)

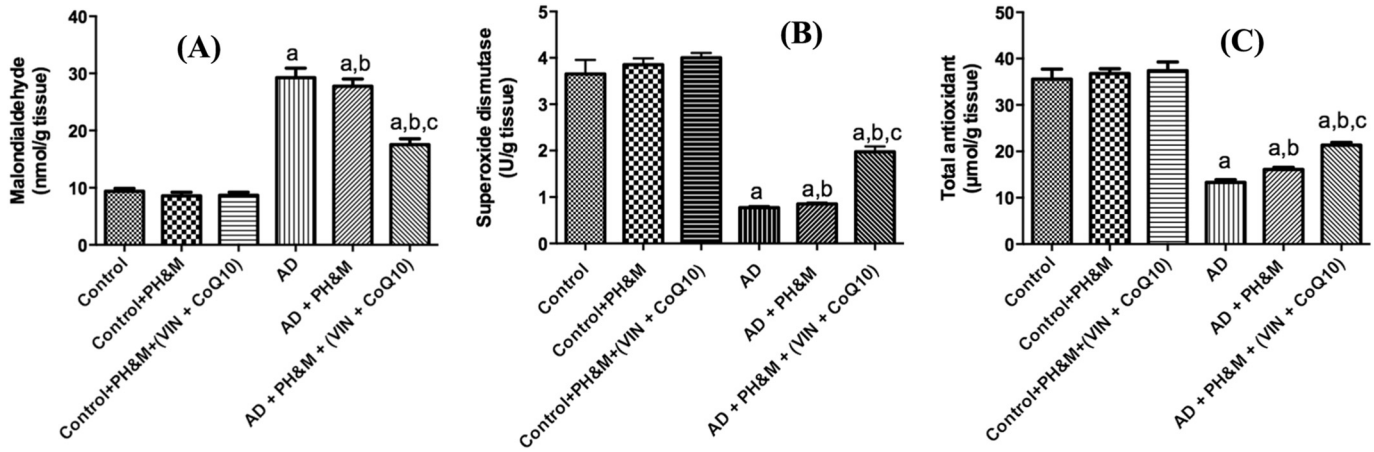

Fig. 3. The influence of physical and mental activities when combined with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) on brain Malondialdehyde (MDA), Superoxide dismutase (SOD), and Total antioxidant (TAC) level in rats. The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: * ($p < 0.001$) versus Control; # ($p < 0.001$) versus AD groups.

AD model ($AlCl_3$ in a dose 70 mg/kg, i.p.) was represented in the following results:

3.1. Histopathological examination

3.1.1. Photomicrographs of brain tissue sections of rats stained by hematoxylin and eosin stain (magnification 50×)

Microscopic examination of the brain tissues from control group stained with H&E showed the normal histological structure of the hippocampus as well as no histopathological alteration in the cerebral cortex, striatum, and substantia nigra (Fig. 1A, B, and C). On the other hand, rats received $AlCl_3$ (70 mg/kg, i.p.) showed pyknosis, necrosis, and neuronal atrophy in the hippocampus area besides neuronal damage in cerebral cortex associated with focal gliosis (Fig. 1D, E, and F). Moreover, brain tissue sections from AD + PH&M group showed nuclear pyknosis in the hippocampus and no histopathological alteration in the cerebral cortex with a few focal plagues in the striatum (Fig. 1G, H, and I). Furthermore, using VIN and CoQ10 combination with PH&M during AD model improved these histopathological alterations, showed a few nuclear pyknosis and degeneration in the hippocampus area, no histopathological alterations in the cerebral cortex, and mild atrophy in the substantia nigra. (Fig. 1J, K, and L).

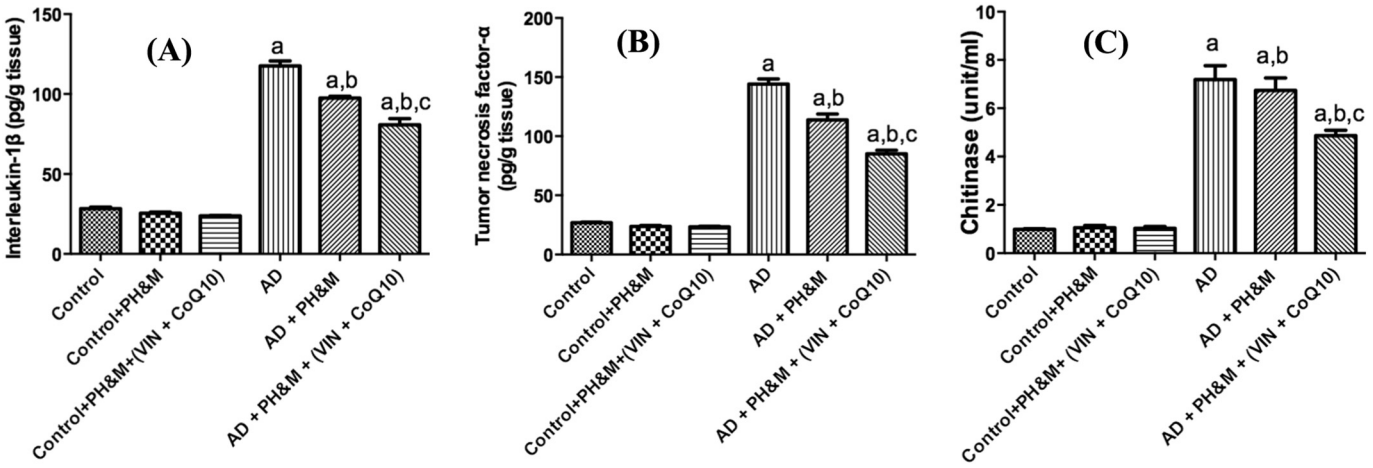

Fig. 4. The anti-inflammatory effect of the combination therapy using physical and mental activities with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) was determined by measuring Interleukin-1β (IL-1β), Tumor necrosis factor-α (TNF-α), and chitinase level in the brain tissue of different groups. The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: * ($p < 0.001$) versus Control; # ($p < 0.001$) versus AD groups.

3.1.2. Photomicrographs of hind paw sections of rats stained by hematoxylin–eosin stain (magnification 50×)

Microscopic examination of the hind paw from control groups stained with H&E showed no histopathological alteration in the skin covering the paw and knee as well as the subcutaneous and muscular tissue. The synovial membrane and bone trabeculae were histologically intact (Fig. 2A and B). AD group showed mild inflammatory cells infiltration in the dermis of the skin, while the subcutaneous tissue showed massive inflammatory cells infiltration (Fig. 2C and D). The cartilaginous articular surface showed focal erosion associated with inflammatory cells infiltration in the synovial membrane (Fig. 2E). Also, AD + PH&M group showed focal inflammatory cells infiltration in the subcutaneous tissue (Fig. 2F) accompanied by no histopathological alteration in the articular cartilaginous surface, synovial membrane and bone trabeculae (Fig. 2G). Moreover, hind paw sections from AD + PH&M + VIP and CoQ10 group showed that the skin covering the paw is histologically intact, inflammatory cells infiltration with edema in the synovial membrane, and no histopathological alteration in the bone trabeculae (Fig. 2H, I and J).

3.2. Enhancing the antioxidant effect of VIN and CoQ10 combination by PH&M during AD

As shown in Fig. 3, induction of AD by $AlCl_3$ (70 mg/kg, i.p.) showed a marked elevation in MDA (310%) besides a significant reduction in SOD (21%) and TAC (37%) levels as compared to normal control groups. On the other hand, co-administration of VIP and CoQ10 with PH&M during $AlCl_3$ administration produced a marked reduction in MDA (59.5%) as well as a significant elevation in SOD (256%) and TAC (160.5%) levels as compared to the AD group. Therefore, combing VIP and CoQ10 with PH&M during AD model improved the antioxidant status significantly with a marked reduction in lipid peroxidation ($p < 0.001$).

3.3. Ameliorating the inflammatory markers and the increased chitinase level

AD disease is strongly correlated with increased chitinase level due to the neuro-inflammatory cascades in the brain. Administration of $AlCl_3$ (70 mg/kg, i.p.) induced oxidative stress that aggravated these neuro-inflammatory cascades, which evidenced by a significant elevation in IL-1β, TNF-α, and chitinase levels by approximately 416%, 535%, and 725% respectively as compared to normal control groups. On other hand, Rats protected by adding PH&M to VIP and CoQ10 combination during AD model showed a significant decrease in IL-1β, TNF-α, and chitinase levels by a rate 68.7%, 59% and 67.7% respectively as compared to $AlCl_3$ (70 mg/kg, i.p.) group ($p < 0.001$). These changes were illustrated in Fig. 4A–C respectively. Therefore, adding PH&M to VIP and CoQ10 combination reduced efficiently the inflammatory markers as well as chitinase activity when compared to PH&M or drug combination alone during AD model.

3.4. Reducing neurodegeneration in AD model by PH&M with drug combination therapy

As demonstrated in Fig. 5A–D, $AlCl_3$ (70 mg/kg, i.p.) administration for four weeks to rats showed a marked elevation in β-secretase (7.3 ± 0.4), Aβ (21.64 ± 1.8), tau protein (5 ± 0.1), and ACHE (44.5 ± 3.1) levels as compared to normal control groups that is an indicator of the neurodegeneration resulting in AD. However, combining PH&M to VIN and CoQ10 during AD model alleviated significantly the resulted neurodegeneration by reducing β-secretase (4.9 ± 0.4), Aβ (14.73 ± 0.45), tau protein (3 ± 0.9), and ACHE (36.5 ± 2.2) levels when compared to AD groups. Thus, PH&M enhanced the neuroprotective effect of VIP and CoQ10 combination during AD.

3.5. Quantification of BDNF and monoamines

The brain tissue levels of monoamines (NA, DA, and 5-HT) were estimated in addition to BDNF quantification and were illustrated in Fig. 6A–D respectively. $AlCl_3$ administration resulted in a significant decrease in brain DA (33%), NE (33%), and 5-HT (37%) levels as well as BDNF (62%) level compared to normal control groups. However, the addition of PH&M to VIP and CoQ10 co-treatment produced a maximal improvement in restoring BDNF (138.5%) and increasing monoamines levels [DA (182%), NE (175%) and 5-HT (196%)] as compared to the other groups of AD model.

3.6. Assessment of bone remodeling during AD and the impact of adding PH&M with drug combination therapy

3.6.1. Estimating serum level of ALP and minerals

We specifically tested the hypothesis that induction of AD by $AlCl_3$ (70 mg/kg, i.p.) was associated with reduced bone minerals in addition to increased ALP level as tested in the sera of animals. $AlCl_3$

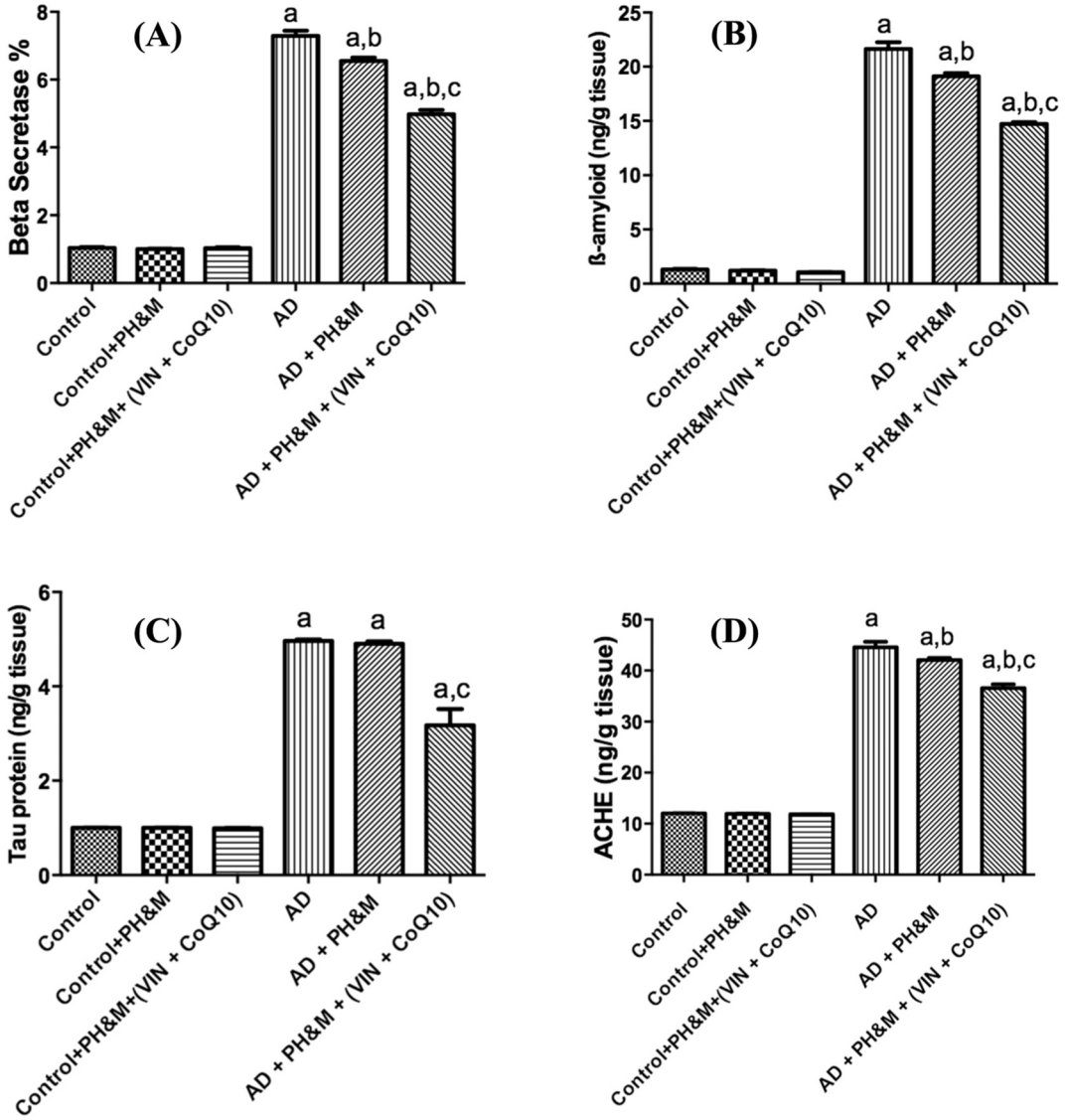

Fig. 5. Physical and mental activities enhance the neuroprotective effect of vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.). The brain levels of β-secretase (A), Beta-amyloid (B), Tau protein (C), and brain acetylcholinesterase (D) were significantly elevated in AD group as compared to normal control groups. However, combining PH&M to VIN and CoQ10 significantly attenuated the neurodegeneration by reducing β-secretase, Aβ, tau protein, and ACHE levels when compared with other groups during AD model. The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: a ($p < 0.001$) versus Control; b ($p < 0.001$) versus AD; c ($p < 0.001$) versus AD + PH&M groups.

administration resulted in a significant decrease in serum level of calcium (33%), magnesium (41%), and phosphate (63%) ions respectively accompanied by an elevated level of ALP (266%) as compared with normal control groups (Fig. 7A–D). However, AD group treated by VIN and CoQ10 in addition to PH&M showed a significant increase in the serum level of calcium (216%), magnesium (207%), and phosphate (135%) ions with a reduction in ALP (62%) level as compared to AD group ($p < 0.001$).

3.6.2. Estimating serum levels of 25-OHD and PTH

Bone impairment observed obviously in $AlCl_3$-induced AD model via a significant reduction in the serum level of 25-OHD (53%) besides a marked rise in PTH (432%) level when compared to normal control groups (Fig. 8A–B). On the other hand, co-administration of VIN and CoQ10 with PH&M during $AlCl_3$ administration showed a significant improvement in the serum levels of 25-OHD (143%) besides a significant reduction in PTH (36%) as compared to other AD groups ($p < 0.001$).

3.6.3. Estimating serum level of RANKL and OPG

Regarding the determination of RANKL and OPG levels, $AlCl_3$-induced AD significantly altered the RANKL/OPG ratio. Bone resorption was confirmed by a significant elevation in serum level of RANKL (313%) along with a marked decrease in OPG (37%) as compared to normal control groups (Fig. 9A–B). However, adding PH&M to drug combination therapy during AD model markedly improved the RANKL/OPG ratio by decreasing RANKL (63%) level with a concurrent increase in OPG (163%) level as compared to the AD group ($p < 0.001$).

4. Discussion

AD and osteoporosis are two different degenerative diseases that are commonly developed in the elderly population and are shared many risk factors [56]. AD is associated with bone loss that is considered a risk factor for osteoporosis that increased the incidence of hip fractures [57]. Based on our previous observation, both VIN and CoQ10 enhanced the protective effects of PH&M activities against brain neuronal

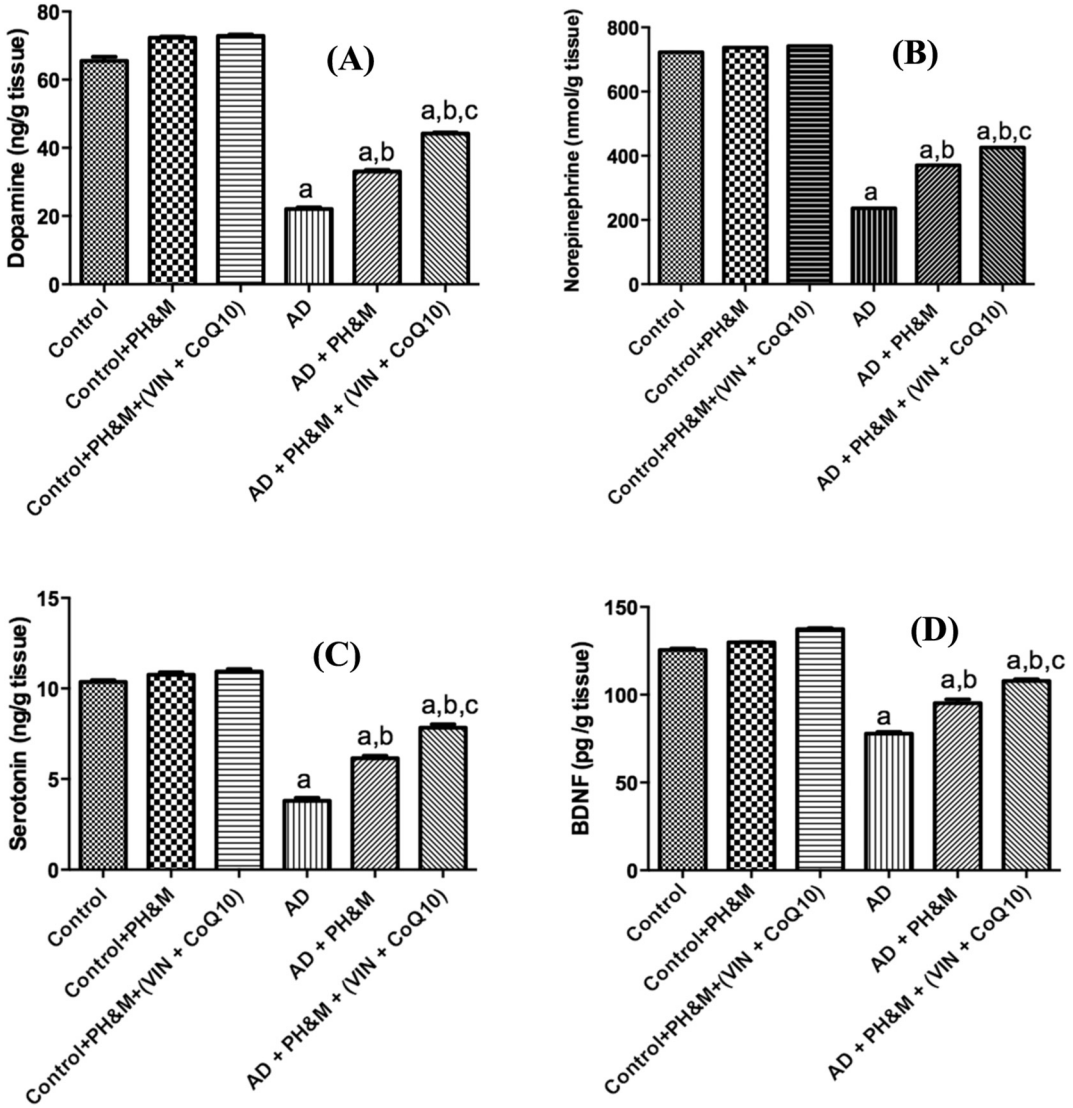

Fig. 6. The effect of physical and mental activities when combined with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) on Dopamine (A), Norepinephrine (B), and Serotonin (C) levels as well as the Brain-derived neurotrophic factor (D) level in brain tissue. The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: a ($p < 0.001$) versus Control; b ($p < 0.001$) versus AD; c ($p < 0.001$) versus AD + PH&M groups.

degeneration associated with the development of AD [58]. Therefore, this study was constructed to spot the light on the efficacy of combining PH&M with VIN and CoQ10 co-administration against neurodegeneration as well as bone loss associated with AD. Al is considered a common environmental risk factor for both osteoporosis and AD progression. Al is a potentially neurotoxic metal, accumulates in bone, replaces calcium in hydroxylapatite, and is the main mineral component of bone [59,60]. In the present study, AD model was induced by the injection of $AlCl_3$ (70 mg/kg, i.p.) daily for four weeks subsequently in rats and this is in accordance with Ali et al., [61]. Al is a prooxidant that mediates cellular injury by impairing antioxidant defense system thus initiating neurodegenerative processes [62]. The significant increase in MDA level in our study reflects the efficacy of $AlCl_3$ administration to induce lipid peroxidation in brain tissue. Moreover, SOD and TAC levels in the brain tissues of Al-treated rats were reduced to indicate impaired antioxidant defense system by $AlCl_3$ administration. Our finding is in accordance with Abdel Moneim [63] and Liaquat et al. [62] who reported that oxidative stress induced by $AlCl_3$ may result in neuronal damage.

Additionally, the imbalance between inflammatory control and unregulated inflammation are highly associated with AD incidence [64]. Neuroinflammation is one of the pathological factors in AD incidence that results from the action of inflammatory proteins such as TNF-α and IL-1β [65]. Chitinase enzyme activity, a marker of neuroinflammation, is elevated in the cerebrospinal fluid of patients with AD [66]. In the current study, $AlCl_3$ exposure groups revealed a significant increase in inflammatory cytokines (IL-1β and TNF-α) and chitinase enzyme activity, reflecting the increased DNA damages in the brain [67]. Moreover, several studies reported that $AlCl_3$-induced oxidative stress and inflammatory response that cause bone deterioration [68,69]. Both oxidative stress and inflammatory response were significantly reduced by combining PH&M with VIN and CoQ10 co-administration during AD model by decreasing MDA, TNF-α, IL-1β levels as well as chitinase enzyme activity with significant elevation SOD and TAC content.

In the brain, the cholinergic neurotransmission system and ACHE activity play an important role in conserving the brain normal function and on the other hand they participate in AD etiology [70,71]. Our investigation has inferred that chronic $AlCl_3$ administration elevates the ACHE activity because of enhanced lipid oxidation process due to free

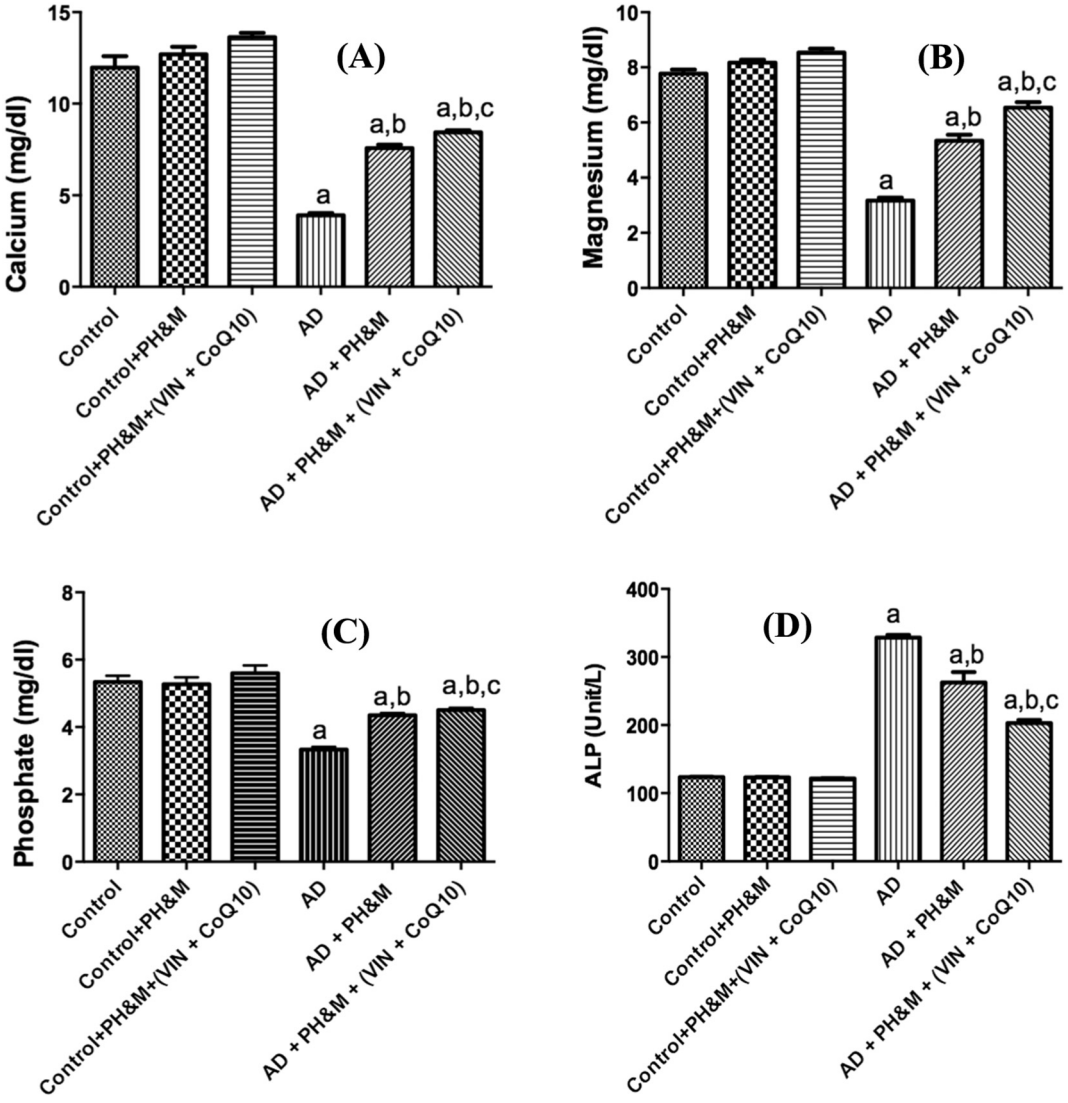

Fig. 7. The effect of physical and mental activities when combined with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination during Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.) on the serum levels of Calcium (A), Magnesium (B), Phosphate (C) ions, and Alkaline phosphatase (D) respectively. The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: a ($p < 0.001$) versus Control; b ($p < 0.001$) versus AD; c ($p < 0.001$) versus AD + PH&M groups.

radical and ROS generation resulting in a reduction of acetylcholine (Ach) neurotransmitter thus impairing cognition and memory [68–70,72,73].

Amyloid and tau aggregation are implicated as the main markers for the progression of AD [74]. In the view of the present results, $AlCl_3$ exposure resulted in a marked elevation in Aβ deposition and tau protein in the brain tissue, which is in accordance with Huang et al. study [59]. Chronic exposure to Al results in its accumulation in the brain tissue that in turn increases the APP over expression within damaged neuritis. This APP undergoes amyloidosis pathway via sequential cleavage by β- and γ–secretases that lead to Aβ generation [75]. Therefore, Aβ deposition is related to the expression of APP, β- and γ–secretases enzymes in Al-treated groups. Earlier observations indicated that Al exposure could increase the expression of amyloidogenic proteins such as APP, β-secretase, γ-secretase, and Aβ [76–78]. Also, $AlCl_3$ exposure groups revealed an increase in β-secretase activity, which is in accordance with Justin Thenmozhi et al. [79]. The elevation in β-secretase enzyme activity enhances the formation of amyloid plaques that aggravates further inflammation and oxidative stress damage in AD [80,81].

Brain-derived neurotrophic factor (BDNF) plays important role in neuronal growth, differentiation and regeneration of various neuronal cells in CNS due to its structure that is related to nerve growth factor, also its wide distribution in brain tissues [82,83]. Notably, our results have shown a decrease in BDNF level following Al intoxication that agrees with several studies [84–86]. Various animal studies reported that Al exposure increases Al level in brain tissue that subsequently followed by a decline in cognition and memory [87]. Additionally, decreased BDNF levels have been linked to the rapid decrease in cognitive and memory performance that accompanied AD [88,89]. Also, Al intoxication decreases 5-HT level in the brain either by reducing its synthesis directly or indirectly through downregulation of BDNF level that has trophic effects on serotonergic neurons [90]. Moreover, NE level declined in Al intoxication groups and this correlated with the reduction in BDNF levels [91]. BDNF is synthesized by noradrenergic neurons and is trophic for catecholaminergic and cholinergic neurons [92]. According to our results, combining PH&M with VIN and CoQ10 co-administration during AD model induced by $AlCl_3$ improved BDNF and monoamine levels.

Physical activities increase the levels of dopamine, serotonin, acetylcholine as well as muscarinic receptor density [93]. Nahid et al. [94] documented that PH&M cause an increase in the memory function of

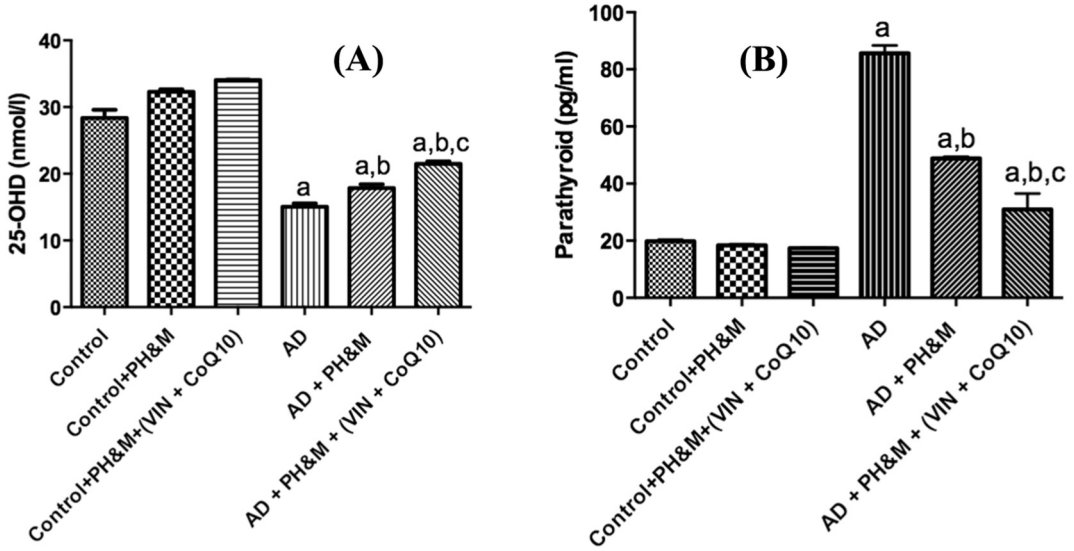

Fig. 8. Physical and mental activities together with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination therapy improved the bone impairment effect in Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.). $AlCl_3$ administration induced a significant reduction in the serum level of 25-OHD besides a marked rise in PTH level when compared to normal control groups. On the other hand, co-administration of VIN and CoQ10 with PH&M during AD model significantly improved the serum levels of 25-OHD accompanied by a significant reduction in PTH as compared to other AD groups ($p < 0.001$). The data are expressed as the mean values ± SD ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: a ($p < 0.001$) versus Control; b ($p < 0.001$) versus AD; c ($p < 0.001$) versus AD + PH&M groups.

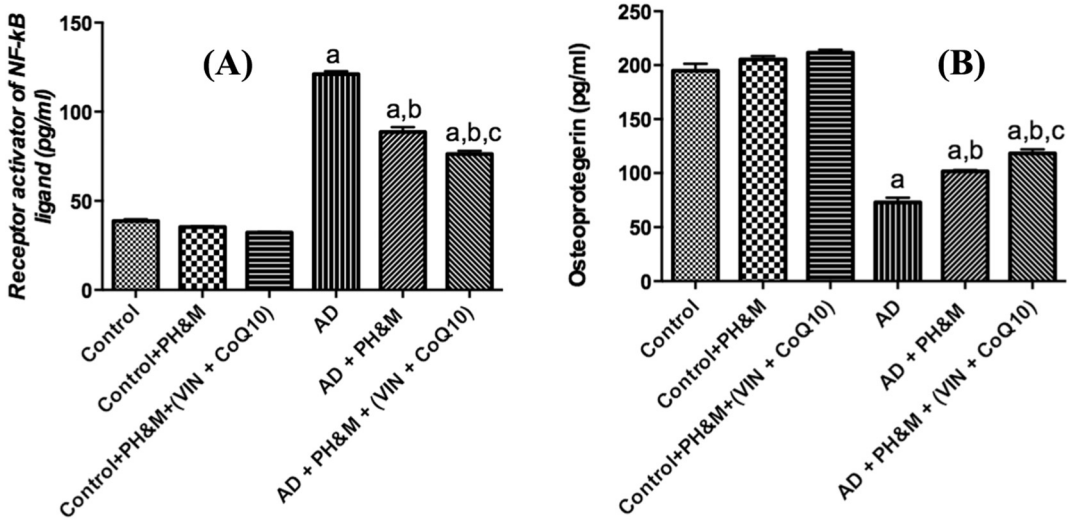

Fig. 9. Physical and mental activities together with vinpocetine (20 mg/kg, p.o.) and Coenzyme Q10 (200 mg/kg, p.o.) combination therapy attenuated the bone resorption activity in Alzheimer model ($AlCl_3$ in a dose 70 mg/kg, i.p.). The data are expressed as the mean values ± SD (n = 8). Statistical analysis was performed using one-way ANOVA followed by Tukey Kramer as a post hoc test. Significance: a ($p < 0.001$) versus Control; b ($p < 0.001$) versus AD; c ($p < 0.001$) versus AD + PH&M groups.

50–70-year old women who suffered memory impairment. Therefore, adding PH&M to VIN and CoQ10 combination during AD enhances the neuroprotective effect of these drugs more efficiently than VIN and CoQ10 combination. Recently it is reported that AD alters neural and neurohumoral regulatory systems of bone remodeling leading to bone loss in early AD [95]. Accordingly, we evaluated the parameters of bone remodeling in this Study. Total ALP is one of the most commonly used serologic markers to screen and diagnose metabolic bone disease. It is found on the membrane of osteoblasts and elevates during the increased bone turnover rates [96,97]. Furthermore, the ALP level is increased in brain injury and cerebrovascular disease, which reflects neuronal loss due to its presence on neuronal membranes [98]. Serum ALP was elevated in $AlCl_3$-induced AD reflecting neuronal damage as well as bone loss in the AD group, this finding is in line with earlier investigators who reported the ALP elevation in AD is associated with the neurotoxic effect of extracellular tau protein [99].

Regarding the hypothesis of this study, combining PH&M with VIN and CoQ10 co-administration exerts considerable neuroprotection against $AlCl_3$-induced AD as well as reducing bone loss associated with AD. Therefore, we evaluated the bone remodeling process by estimating the RANKL and OPG levels as well as serum levels of Ca^{2+}, Mg^{2+}, PO_4, PTH, and vitamin D. Our results showed a decrease in Ca^2/PO_4 levels in $AlCl_3$-induced AD group indicating bone loss and inhibition of bone formation because $AlCl_3$ administration inhibits osteoblastic differentiation leading to bone impairment [23]. Al administration disrupts calcium homeostasis and signal transduction that occurs in aging and AD disease [100]. Also, we observed a significant decrease in Mg^{2+} level in the AD group and this is in line with Grober et al. [101].

Moreover, Mg^{2+} has an important vital role in neuronal properties and synaptic plasticity [102].

25-OHD is a critical regulator of both calcium and phosphate homeostasis, providing adequate mineral for normal bone formation [103]. However, the 25-OHD deficiency is a common problem associated with aging and cognitive dysfunction [104,105]. Recent studies found that 25-OHD receptors are localized with amyloid precursor protein on the neuronal plasma membrane [106], as it can decrease Aβ plaques deposition and hyper-phosphorylated tau protein in AD [107]. In the present study, our results showed a decrease in 25-OHD levels in the AD group, this is due to the suppression of 25-OHD activation via the kidney in response to PTH [108]. As a result of low serum 25-OHD and mineral levels in the AD group, PTH secretion is increased. PTH is a hormone that regulates intracellular calcium levels, induces bone resorption, and mobilizes bone calcium into the blood to balance low calcium levels in the blood by activating receptor activator of nuclear factor-κB (NF-κB) ligand/receptor activator of NF-κB (RANKL/RANK) signaling pathway. Thus, high levels of PTH are associated with a higher bone resorption rate [109,110], since the binding of RANKL to its receptor on the surface of osteoclast cell is essential for osteoclast cells activation and osteoclastogenesis process [111,112]. On the other hand, osteoprotegerin (OPG) is known as osteoclasts inhibitor factor (OCIF) and acts as an antagonistic endogenous receptor for RANKL [113]. OPG protects against excessive bone resorption by binding with RANKL inhibiting the downstream signaling of RANKL/RANK pathway, therefore, the RANKL/OPG ratio is thus an important determinant of bone mass [114]. In the present study, an increase in the RANKL/OPG ratio in AlCl3-induced AD group indicating a bone loss. Recently it was documented that AlCl3 administration inhibits the formation of hydroxyapatite crystals, synthesis, secretion, and mineralization of bone matrix that results in the inhibition of bone formation and induction of osteoporosis [115]. To the best of our knowledge, this is the first study to address the levels of RANKL and OPG in AD to support the hypothesis of relating cognitive dysfunction and AD with bone resorption activation in addition to the impacts of treating AD by drug combination with PH&M and its impression on bone remodeling. Our results showed a significant improvement in bone measured parameters in AD groups that treated with a combination therapy of VIN and CoQ10 in addition to PH&M represented in restoring blood minerals and decreased the RANKL/OPG ratio.

Finally, the histopathological examination of bone tissue in AlCl3-induced AD group revealed focal erosion in the cartilaginous articular surface associated with inflammatory cells infiltration in the synovial membrane. These data collectively indicate the bone damaging effect of AlCl3 administration and the strong relation between this AD model and its coexistent bone degenerative effect. Additionally, using VIN and CoQ10 combination with PH&M during AD model improved this bone histopathological damage and showed no histopathological alteration in the bone trabeculae with intact skin covers the paw.

5. Conclusion

Alzheimer's disease is a neurodegenerative disorder associated with a bone loss, low bone mineral density, and subsequent fracture. Co-administration of VIN and CoQ10 with PH&M is more significant in reducing the biochemical and histopathological changes in AlCl3-induced AD model. The addition of PH&M to VIN and CoQ10 combination enhanced the neuroprotective, anti-oxidant, and anti-inflammatory effects of both neuroprotective drugs. Also, this combination therapy improves the low bone mineral density associated with AlCl3-induced AD model, which indicates the strong correlation between their neuroprotective effects and bone remodeling. Further studies are required to evaluate the possibility of clinical application.

Conflict of interest

The authors declare no potential conflicts of interest.

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