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Article

Biological and Pharmacological Characterization of Ascorbic Acid and Nicotinamide Chitosan Nanoparticles against Insulin-Resistance-Induced Cognitive Defects: A Comparative Study

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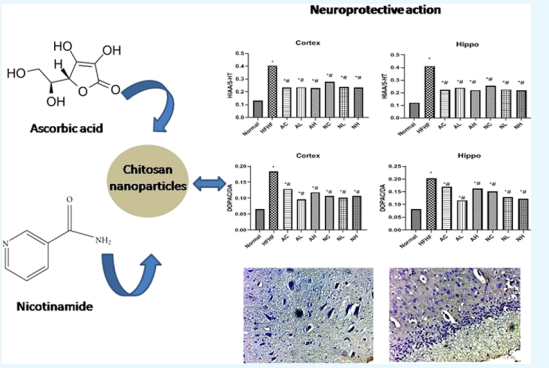
ABSTRACT: High consumption of industrialized food with high fat content is generally associated with insulin resistance, which in turn causes memory impairment and cognitive decline. Nicotinamide and ascorbic acid are among the promising neuroprotective molecules; however, an appreciable therapeutic activity necessitates the administration of a large dose of either. Therefore, the study aimed to assess if loading them in chitosan nanoparticles in doses 5−10 times lower than the unencapsulated forms would achieve comparable therapeutic results. Animals were fed a high-fat-high-fructose (HFHF) diet for 75 days. The vitamins in their conventional form (100 mg/kg) and the nanoparticles under investigation (10 and 20 mg/kg) were given orally concomitantly with the diet in the last 15 days. The intake of HFHF diet for 75 days led to an insulin-resistant state, with memory impairment, which was verified behaviorally through the object recognition test. This was accompanied by significant reduction in brain insulin-like growth factor 1 (IGF-1), increased acetylcholine esterase activity, increase in the serotonin and dopamine turnover ratio, and increase in oxidative stress and 8-OHdG, indicating cellular DNA fragmentation. Cellular energy was also decreased, and immunohistochemical examination verified the high immunoreactivity in both the cortex and hippocampus of the brain. The administration of nanoparticulated nicotinamide or ascorbic acid with a 10 times lesser dose than the unencapsulated forms managed to reverse all aforementioned harmful effects, with an even lesser immunoreactivity score than the unencapsulated form. Therefore, it can be concluded that nicotinamide or ascorbic acid chitosan nanoparticles can be recommended as daily supplements for neuroprotection in patients suffering from insulin resistance after conduction of clinical investigations.



1. INTRODUCTION

Higher consumption of industrialized food with high-fat content, along with sedentary habits, is generally associated with insulin resistance, which in turn has a hazardous impact on memory performance, increases cognitive decline, impairs spatial learning, and causes signs of depression.[1−5] Adolescence, which is a significant age-related period, is usually accompanied by major changes in brain architecture and performance.[1] Nutrition, among several other factors, definitely exerts a strong influence on adolescents' mental development.[2]

The central nervous system (CNS) is protected by a shielding barrier: the blood−brain barrier (BBB), which is formed of cellular tight junctions, as well as the extracellular matrix.[6] It limits the entry of over 98% of small-molecular-weight drugs and almost 100% of the large-molecular-weight drugs. Accordingly, one of the main challenges facing the treatment of brain disorders is delivering drugs across this barrier at an effective concentration.[7,8]

Nicotinamide (vitamin B3) has been previously reported to improve cognitive defects and possess potential neuroprotective effects in preclinical and human studies. However, high doses reaching 1500 mg/kg in preclinical animal investigations and 22.4 mg daily intake in clinical studies are usually necessary to get the requested response, which make multiple daily doses a must and is probably accompanied by several undesired side effects such as hot flushing, hepatotoxicity, as well as patient noncompliance.[9,10] On the other hand, ascorbic acid (vitamin C) is one of the strongest water-soluble antioxidants of major significance for adequate brain functioning, but the human body is incapable of its synthesis; therefore, its dietary supplementation is required to meet the brain's demand.[11] Doses ranging from 500 to 2000 mg daily in humans and 100 mg/kg in rats

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have been used to protect against ischemic strokes, neuronal damage, and the progression of Alzheimer's.[12−14] Intracellularly, ascorbic acid exhibits a vital role in maintaining integrity and function of numerous brain processes such as neuronal maturation and differentiation, synthesis of catecholamine, and modulation of neurotransmission. Therefore, it has been proposed that ascorbic acid may modulate the progression of neurological diseases and display potential therapeutic roles.[15]

Chitosan is a naturally occurring cationic polysaccharide polymer that possesses good biocompatibility, biodegradability, mucoadhesivity, and low toxicity and has been approved by the FDA for drug delivery.[16−18] Its ability to control drug release and to combine with negatively charged material, cell surface, and mucous membranes is advantageous in opening tight junctions and improving absorption by extending the residence time at the action site.[19,20]

Several approaches have been introduced to maximize the therapeutic effectiveness of drugs, especially nutraceuticals, among which is the use of nanotechnology.[21−28] Particularly for brain-related disorders, loading drugs in nanoparticles was proven to maximize their concentration in the brain[29,30] and augment their neuroprotective properties.[31] Chitosan nanoparticles present a very promising approach for delivery of neuroprotective drugs since chitosan itself as a polymer possesses membrane-fusogenic properties, which rescues glial cells from death.[32] Moreover, owing to the sustained release nature of drugs from chitosan nanoparticles, they preserve the antioxidant activity of the loaded drugs.[33] Therefore, the target of the present research was to demonstrate whether the encapsulation of nicotinamide or ascorbic acid in chitosan nanoparticles at a 5−10 times lesser dose than the unencapsulated dissolved powders would achieve a comparable effect on the cognition status and neurodegradation in juvenile male rats, by studying behavioral, biochemical, and immunohistochemical parameters.

2. EXPERIMENTAL SECTION

2.1. Materials. Nicotinamide, ascorbic acid, and sodium tripolyphosphate were purchased from Sigma-Aldrich, Germany. Highly pure chitosan (77 kDa) was kindly gifted by Primex company, Iceland. Acetic acid was purchased as analytical grade from Al-Nasr Company, Egypt.

2.2. Preparation and Characterization of Nicotinamide and Ascorbic Acid Chitosan Nanoparticles. Nicotinamide and ascorbic acid chitosan nanoparticles were prepared using the ionotropic gelation method as previously described elsewhere.[34] Four nanoparticle formulations were prepared, of low concentration (2.5 mg/mL) of either nicotinamide (NL) or ascorbic acid (AL) and another two of high concentration (5 mg/mL) of either drugs (NH and AH), respectively. The formulations were characterized for particle size, for surface charge using a Zetasizer device (Nano ZS 3600, Malvern, U.K.), and for drugs' entrapment after separation of the unentrapped drugs by centrifugation (Hermle Labortechnik GmbH, Germany) and spectrophotometric analysis at 262 and 265 nm for nicotinamide and ascorbic acid, respectively.[34]

2.3. In Vivo Experiment. *2.3.1. Animals Used.* Male Wister albino rats weighing 60−70 g and aged 4−5 weeks were provided by the National Research Centre (Dokki, Giza, Egypt) animal house. All animal experiments were approved by the Research Ethics Committee of the National Research Centre (approval number 16/313) and conducted according to the National Guidelines and Regulations. The use of juvenile rats for this model is particularly advantageous to mimic the brain developmental status of children, who are known to highly consume industrialized food with high fat content nowadays. The use of juvenile rats for brain diseases is in accordance with other authors.[35−37]

2.3.2. Experimental Design. Animals were randomly assigned into eight groups (n = 18 per group) after 1 week of acclimatization. They were fed either a high-fat-high-fructose diet (HFHF; 60 kcal saturated fat/100 kcal diet) with 20% fructose in the drinking water (HFHF control and treatment groups)[38] or a control diet (normal control) for 60 days. The approximate food intake for a rat ranged from 10 to 11 g of food each day for either the control or the treated group. On the 60th day, insulin resistance (IR) was verified by measuring fasting glucose and insulin levels and by calculating the fasting insulin sensitivity indices.[39] The nanoparticles under investigation were orally given concomitantly with the HFHF diet for 15 days in two dose levels (1/10 and 1/5 of the conventional dose). Two groups ingested the treatments in their conventional forms dissolved in distilled water. Therefore, the eight groups were coded as follows: the normal group receiving the control diet and daily distilled water at 5 mL/kg (normal), the group receiving the HFHF diet and daily distilled water at 5 mL/kg without treatment (HFHF), the group receiving ascorbic acid powder dissolved in distilled water of dose 100 mg/kg (AC),[40] the group receiving ascorbic acid nanoparticles of dose 10 mg/kg (AL) and another group receiving a dose of 20 mg/kg (AH), the group receiving nicotinamide powder dissolved in distilled water at a dose of 100 mg/kg (NC),[41] and the group receiving nicotinamide nanoparticles at a dose of 10 mg/kg (NL) and another group receiving a dose of 20 mg/kg (NH).

2.3.3. Behavioral Object Recognition Test. The apparatus for this test was designed by Ennaceur and Delacour, 1988. For each group, eight animals were randomly chosen, and 3 days before testing, each rat was allowed to explore the apparatus for 2 min, while on the testing day (day 74 of the experiment), a trial for each was allowed for 2 min. In the "sample" trial (T1), two identical objects were placed in two opposing corners of the apparatus. A rat was placed inside the apparatus and was left to explore these two objects. Twenty-four hours later (day 75 of the experiment), the "choice" trial (T2) was performed. In T2, a new object (N) replaced one of the objects that were present in T1, and rats were exposed again to two different objects: the familiar (F) and the new one (N). The total exploration time of the objects in T1 and T2 was calculated, and the discrimination index (DI) was calculated as previously described elsewhere.[42]

2.3.4. Assessment of Biochemical, Pathological, and Immunohistochemical Parameters. On day 75, rats were fasted overnight and on day 76 all groups were randomly divided (each group was divided into three subgroups: two subgroups had eight rats each and the third subgroup consisted of two rats). One subgroup was used for blood sample collection under phenobarbital anesthesia to compute serum glucose, insulin, and the Homeostatic Model Assessment-Insulin Resistance (HOMA-IR). The second subgroup was sacrificed by decapitation for the collection of brain samples. The cortex and hippocampus were then isolated and kept at −80 °C for further studies. The third subgroup (two rats) was stored in 10% formalin for histopathological and immunohistochemical examinations.

2.3.4.1. Serum Parameters. **Determination of HOMA-I.** After spectrophotometric determination of the serum fasting glucose level[43] and the insulin serum level by the enzyme-linked

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ACS Omega XXXX, XXX, XXX−XXX

immunosorbent assay (ELISA) kit (Sceti Medical Lab K.K., Tokyo, Japan),[44] the HOMA-IR index was calculated.[39]

$$\text{HOMA} - \text{IR} = [\text{fasting glucose(mg/dL)} \times \text{fasting insulin}(\mu\text{IU/mL})]/405$$

2.3.4.2. Brain Biochemical Assessments. **Determination of the Brain IGF-1 (ng/100 mg Tissue) Level**. Brain cortex and hippocampus levels of IGF-1 were determined by rat IGF-1 (insulin-like growth factor 1) CLIA ELISA kit Elabscience.

HPLC Assessment. After homogenization of the cortex or hippocampus in phosphate buffer and separation of the supernatant, samples were compared to reference standards and assessed using Agilent HP 1200 series HPLC apparatus.

Determination of Brain Acetylcholine Esterase: Brain cortex and hippocampus levels of acetylcholine esterase were determined according to the method of some authors[45] with modification.[46]

Determination of Brain Serotonin and Dopamine: Brain cortex and hippocampus contents of dopamine (DA), serotonin (5-HT), and their metabolites, 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), were calculated as previously described elsewhere.[47]

Determination of Brain Oxidative Stress Parameters: Brain cortex and hippocampus contents of NOx (nitrates + nitrites),[48] the MDA level,[49−51] and the ratio of thiol compounds of oxidized (GSSG) and reduced (GSH) glutathione[52,53] were calculated.

Determination of the 8-OHdG Amount (pg/g Tissue): Cortex and hippocampus 8-hydroxy-2-deoxyguanosine (8-OHdG) contents were quantified.[54]

Determination of ATP, ADP, and AMP: Brain cortex and hippocampus adenosine contents of tri-, di-, and monophosphate (ATP, ADP, and AMP) were quantified, and the total adenylate energy charge (AEC) was calculated as previously described.[55−57]

$$\text{AEC} = \frac{\text{ATP} + 0.5\ \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

2.3.4.3. Histopathological Examination. Brain samples were fixed in 10% formalin/saline. The tissue sections were placed on glass slides, deparaffinized, and stained by hematoxylin and eosin (H&E) for microscopic inspection and photography at 100× magnification, 20 μm scale bar.

2.3.4.4. Immunohistochemical Examination. For AMP-activated protein kinase semiquantitative assessment, the slides were incubated overnight at 4 °C with primary AMPK antibodies in the concentrated form consisting of rabbit IgG in phosphate buffer saline (free from Mg^{2+} and Ca^{2+}), pH 7.4, 150 mM NaCl, 0.02% sodium azide, and 50% glycerol. Detection was carried out using a Vector ABC kit (Vector Laboratories, CA) and DAB reagent (Dako Comp, Japan). All slides were examined as described in Section 2.3.4.3.

The proportions of positive cells were also calculated, and ranges were assigned from 10 to 100%. The intensity of staining was given scores of 0 (negative), 1 (very weak), 2 (weak), 3 (moderate), and 4 (intense). Lastly, the immunoreactivity score was calculated as a percentage of positive cells multiplied by the intensity of staining.[58]

2.4. Statistical Analysis. Graph Prism software (version 8) was used for statistical analysis of the effect of different treatments in the object recognition test using Student's *t*-test, while all other statistical analyses were carried out using one-way ANOVA followed by Tukey's multiple comparisons test ($P < 0.05$).

3. RESULTS

3.1. Preparation and Characterization of Nicotinamide and Ascorbic Acid Nanoparticles. Being water-soluble molecules, both nicotinamide and ascorbic acid were encapsulated in chitosan nanoparticles, with particle sizes ranging from 103 to 175 nm. The nanoparticles exhibited a positive charge ranging from +22 to +30 mV, with a high entrapment potential for both drugs, ranging from 75 to 86%.

3.2. Behavioral Object Recognition Test. As shown in Figure 1A, the memory impairment caused by HFHF did not significantly affect the total exploration time in T1 and T2. Oral administration of different treatments did not also show any difference in the total exploration time in T1 and T2. As shown in Figure 1B, during T2, HFHF-induced memory-impaired rats did not reveal any significant difference in the exploration time of N as compared to their exploration time of F. HFHF-induced memory-impaired rats explored N and F objects similarly, while rats treated with different treatments explored the N object significantly more than F except for NL. DI indicated that all rats, except for HFHF rats, significantly discriminated N better than F (Figure 2).

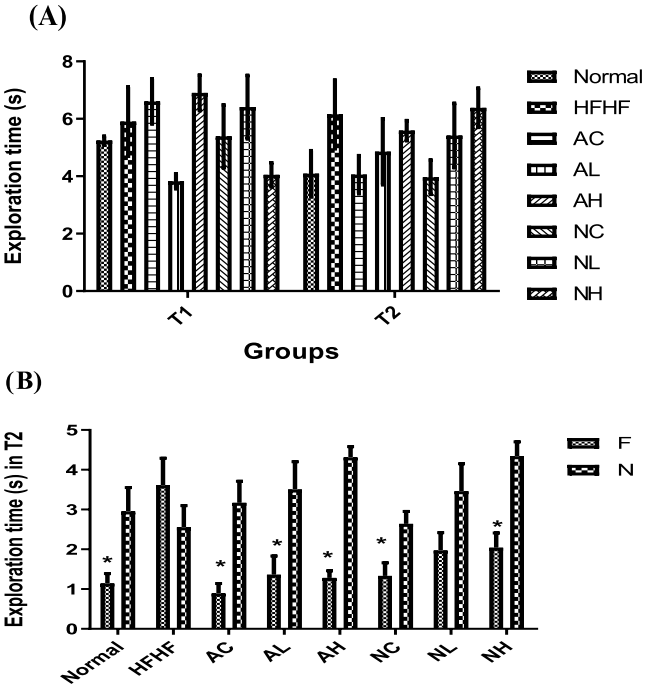

Figure 1. Effect of different treatments on the (A) exploration time in T1 (sample trial) and T2 (choice trial) and the (B) exploration time in T2 for the familiar object (F) versus the novel object (N) in the HFHF-induced memory-impaired model using the object recognition test. Asterisk represents significant difference from N ($P < 0.05$).

3.3. Effect on HOMA-IR. Serum fasting blood glucose and insulin were measured to determine HOMA-IR. Results revealed that HFHF diet ingestion for 75 days resulted in significant elevation in HOMA-IR compared to normal control. Concomitant administration of all treatment groups with the diet during the last 15 days resulted in a significant decrease in HOMA-IR as compared to the HFHF control (Table 1).

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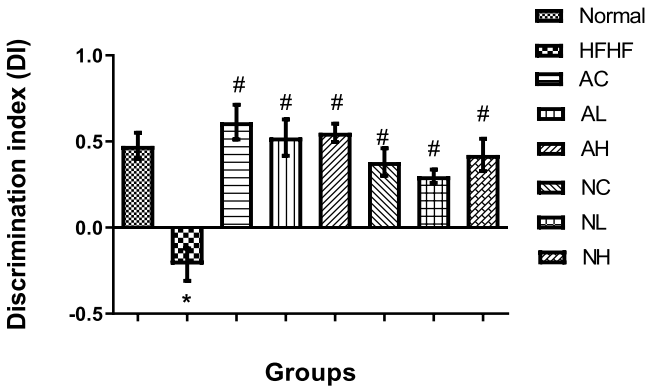

Figure 2. Effect of different treatments on DI of HFHF-induced memory impairment using the object recognition test. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

Table 1. HOMA-IR Values for the Different Treatment Groups

group	HOMA-IR
normal	0.89 ± 0.018
HFHF	2.64 ± 0.076[a]
AC	1.19 ± 0.045[a,b]
AL	1.20 ± 0.045[a,b]
AH	1.08 ± 0.037[b]
NC	0.97 ± 0.042[b]
NL	1.02 ± 0.0298[b]
NH	0.93 ± 0.0289[b]

[a]Significantly different from the normal control. [b]Significantly different from the HFHF control ($P < 0.05$).

3.4. Brain Biochemical Assessment. *3.4.1. Effect on the Brain IGF-1 Level.* The HFHF diet significantly reduced IGF-1 in both brain regions. Both dose levels of the ascorbic acid formulations normalized the IGF-1 cortical level, while nicotinamide formulation was effective only at the higher dose level. All of the treatments under investigation normalized the IGF-1 level in the hippocampus region (Figure 3).

3.4.2. Effect on Brain Acetylcholine Esterase Activity. As shown in Figure 4, the acetylcholine esterase enzyme activity increased in both regions with the HFHF diet. The most prominent observations were that ascorbic acid formulations at both dose levels significantly reduced the acetylcholine esterase activity in the hippocampus, and the higher dose level normalized the enzyme activity in the cortex region. On the other hand, nicotinamide groups normalized the enzyme activity in both regions.

3.4.3. Effect on Brain Serotonin and Dopamine. As shown in Figure 5 and Table S1, the HFHF diet significantly decreased serotonin and dopamine levels. Moreover, it increased both cortical and hippocampal serotonin and dopamine turnover as compared to the normal control. Both formulations under investigation had a significant impact on the serotonin and dopamine turnover ratio.

3.4.4. Effect on Brain Antioxidant Activity. HFHF induced a significant oxidative stress status represented by an elevation in both cortical and hippocampal MDA, NOx, and GSSG values in comparison with the normal control, with an increase in the GSSH/GSH ratio and a decrease in the level (Figures 6 and 7).

3.4.5. Effect on 8-OHdG. HFHF caused a significant elevation in the 8-OHdG content compared to the normal control, while both formulations caused a significant reduction in 8-OHdG in comparison with the HFHF control (Figure 8).

3.4.6. Effect on Cellular Energy Status. HFHF caused a significant decline in AEC as well as a significant elevation in both AMP/ATP and ADP/ATP ratios compared to the normal control. Both formulations improved the cellular energy status (Figure 9).

3.5. Histopathological and Immunohistochemical Examination. The observations recorded after histopathological examination of brain tissues in both the cortex and hippocampus for the different groups and the corresponding photographs are tabulated in Table 2.

Upon immunohistochemical examination of tissue slides, the normal group revealed negative staining for AMPK and the HFHF group exhibited the highest immune-reactivity score. All treatments resulted in reduction of the immune-reactivity score, and particularly AL and NL groups resulted in the lowest immune-reactivity score: $T = 20$ at both regions under investigation. Results are shown in Tables 3 and 4.

4. DISCUSSION

Nowadays, diets in countries are characterized by a high fat content and are usually associated with excessive energy intake.[59] Excessive caloric intake is considered one of the major reasons contributing to the increased risk of developing memory and cognitive impairment as well as neurodegeneration.[60]

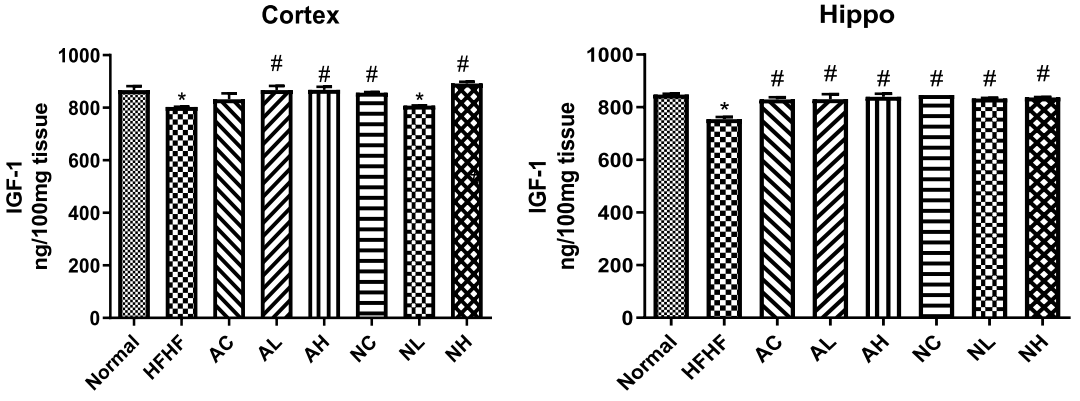

Figure 3. IGF-1 levels in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

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ACS Omega XXXX, XXX, XXX−XXX

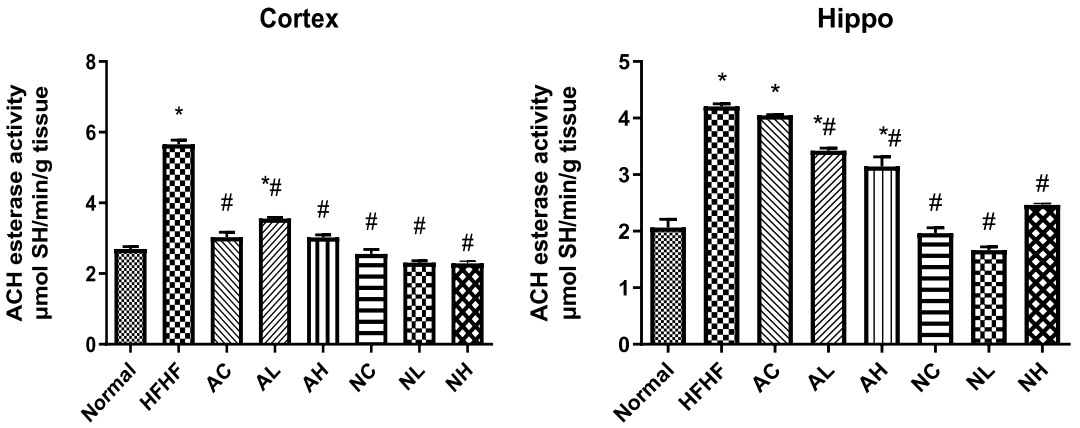

Figure 4. Acetylcholine esterase activity in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

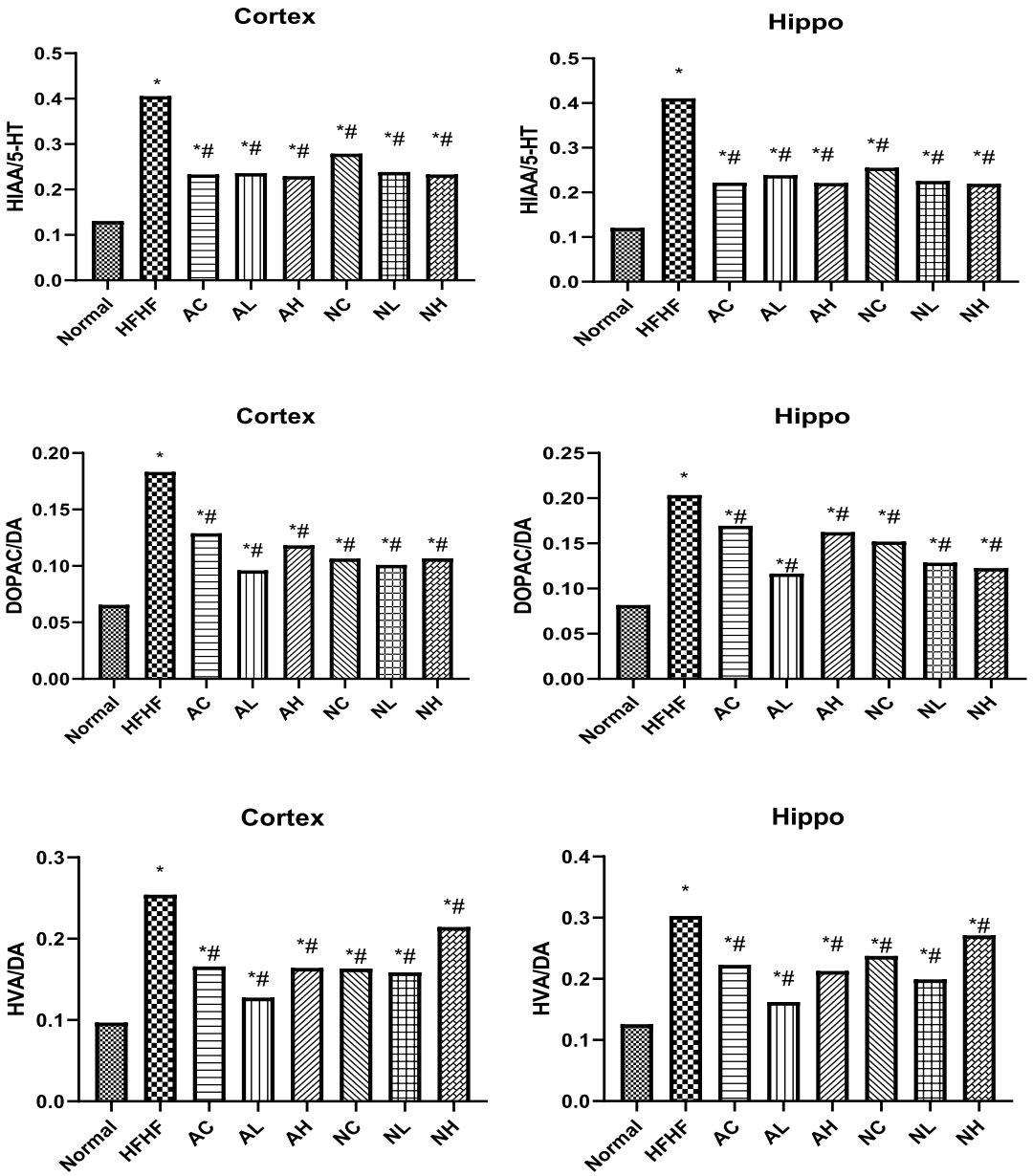

Figure 5. Turnover of serotonin and dopamine in the cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

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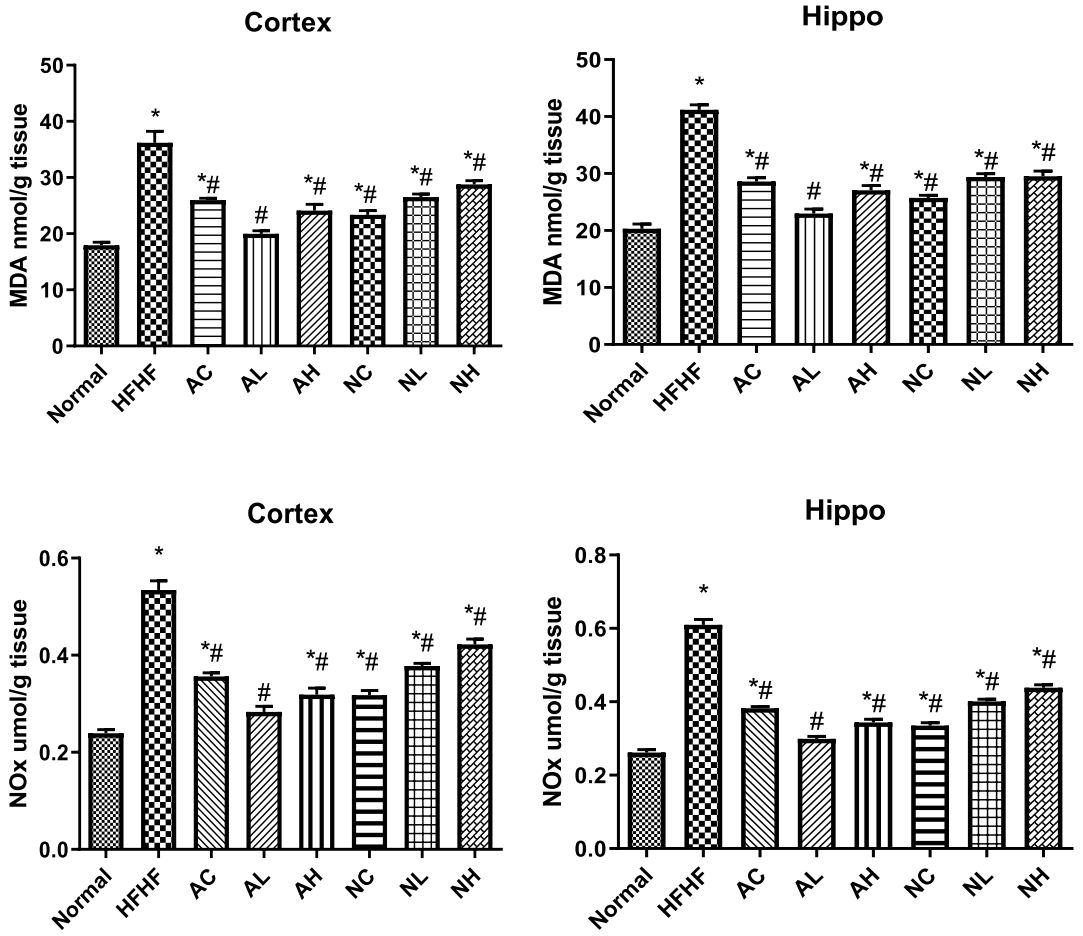

Figure 6. MDA and NOx levels in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

In the present work, the consumption of HFHF diet for 75 days led to a significant elevation of serum HOMA-IR, indicating an insulin-resistant state (IR). This IR state led to memory impairment, which was verified behaviorally through the object recognition test. Furthermore, IR significantly reduced brain insulin-like growth factor 1 (IGF-1) and significantly increased the acetylcholine esterase activity along with a significant decrease in the neurotransmitter levels accompanied by a pronounced elevation in both serotonin and dopamine turnover. Oxidative stress was demonstrated, alongside a significant increase in 8-OHdG, indicating cellular DNA fragmentation. Cellular energy was reduced, as confirmed by the significant reduction in AEC as well as the significant increase in the ADP/ATP and AMP/ATP ratios. Finally, AMPK was increased as confirmed by immunohistochemical examination.

HFHF diet has previously been implicated in the progression of insulin resistance, which in turn is positively correlated to neurodegeneration, as well as the increase in monoamine turnover, and generalized brain oxidative stress status.[36] It was previously demonstrated that ingestion of high fat with added sugars such as sucrose and fructose is a major causative factor for cognitive impairment.[61] Moreover, oxidative stress and neuroinflammation are considered to be crucial mediators of such mental disorders.[59] In addition, the brain is regarded as an insulin-sensitive organ since insulin is crucial for normal brain functioning as well as for stimulating the release of neurotransmitters like catecholamines.[62] The binding of insulin to receptors induces a complex intracellular signaling cascade affecting several neural functions such as learning and memory. Development of brain-insulin resistance has been confirmed to be a major contributor in cognitive disorders. On the contrary, renovation of brain-insulin signaling may improve cognition.[59,62]

The insulin superfamily of peptides was delineated as an essential key element in the growth and development of the central nervous system (CNS). Insulin-like growth factor 1 (IGF-1) is one vital member of this family and is a potent growth factor in the CNS.[63] It is expressed in the cerebral cortex and hippocampus and is strongly involved in neurogenesis and synaptogenesis. Moreover, it has been previously marked as a neuroprotective agent in brain injuries.[64−66] Altered insulin and/or IGF-1 signaling in the brain is connected with increased risk for Alzheimer's disease, premature cognitive decline, and dementia.[67]

Additionally, acetylcholine plays an important role in the regulation of cognition and behavior.[62] The brain level of acetylcholine esterase (AChE), which is the enzyme responsible for breaking down acetylcholine, is usually considered as a reliable marker enzyme of the cholinergic activity.[68] An increased AChE activity in the brain triggers memory deficits and oxidative stress. It has been previously reported that during insulin signaling disorders, the AChE level is increased.[69−71]

Adding to this cascade, neurotransmitters are important for learning and memory processes. A decrease in dopamine and serotonin contents has been proposed to cause memory

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ACS Omega XXXX, XXX, XXX−XXX

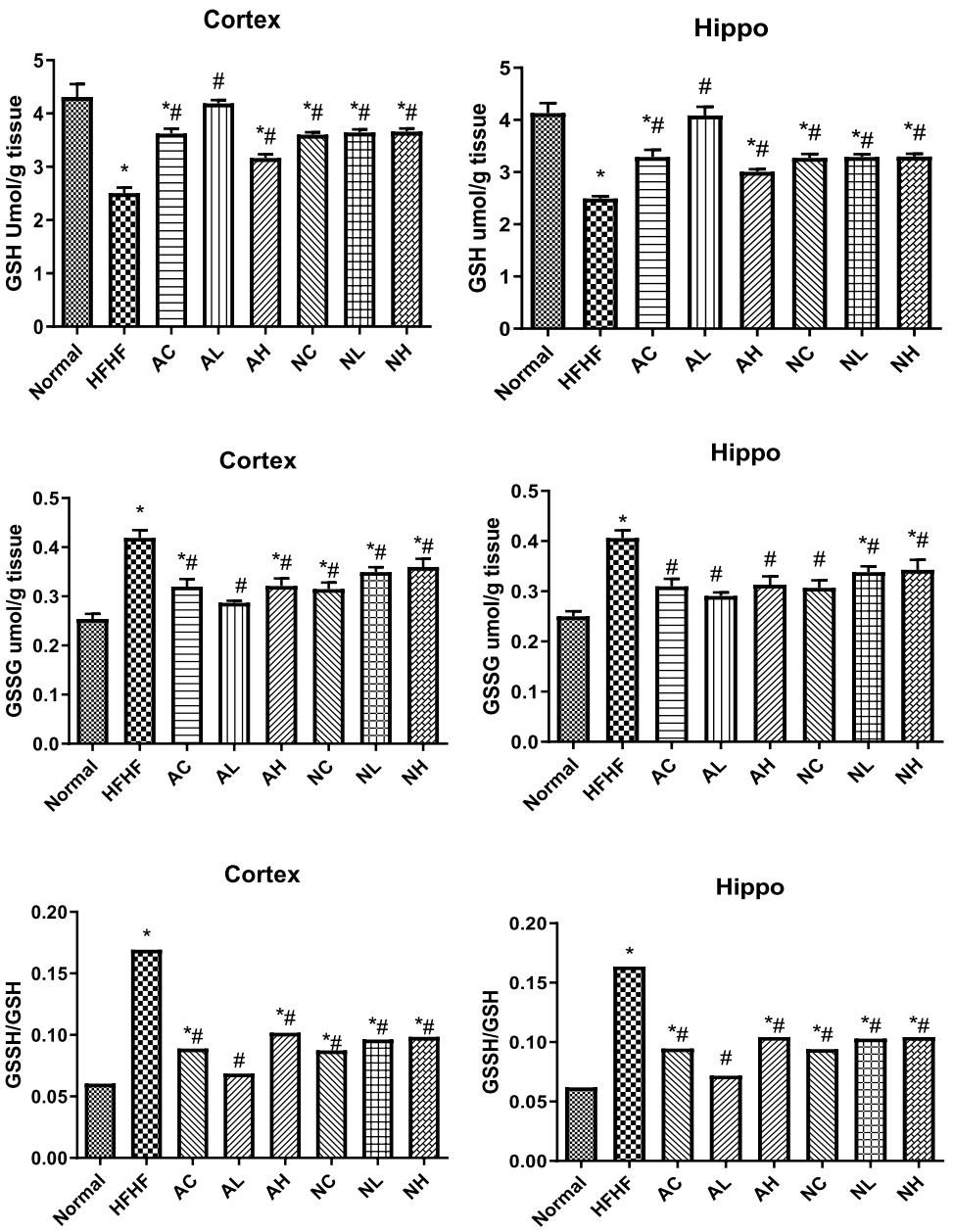

Figure 7. GSH and GSSG levels and the GSSG/GSH ratio in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control ($P < 0.05$).

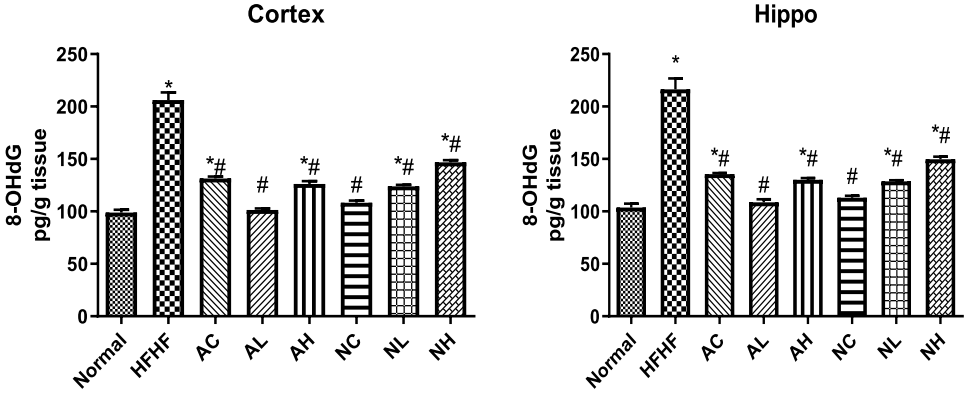

Figure 8. Levels of 8-OHdG in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control $P < 0.05$.

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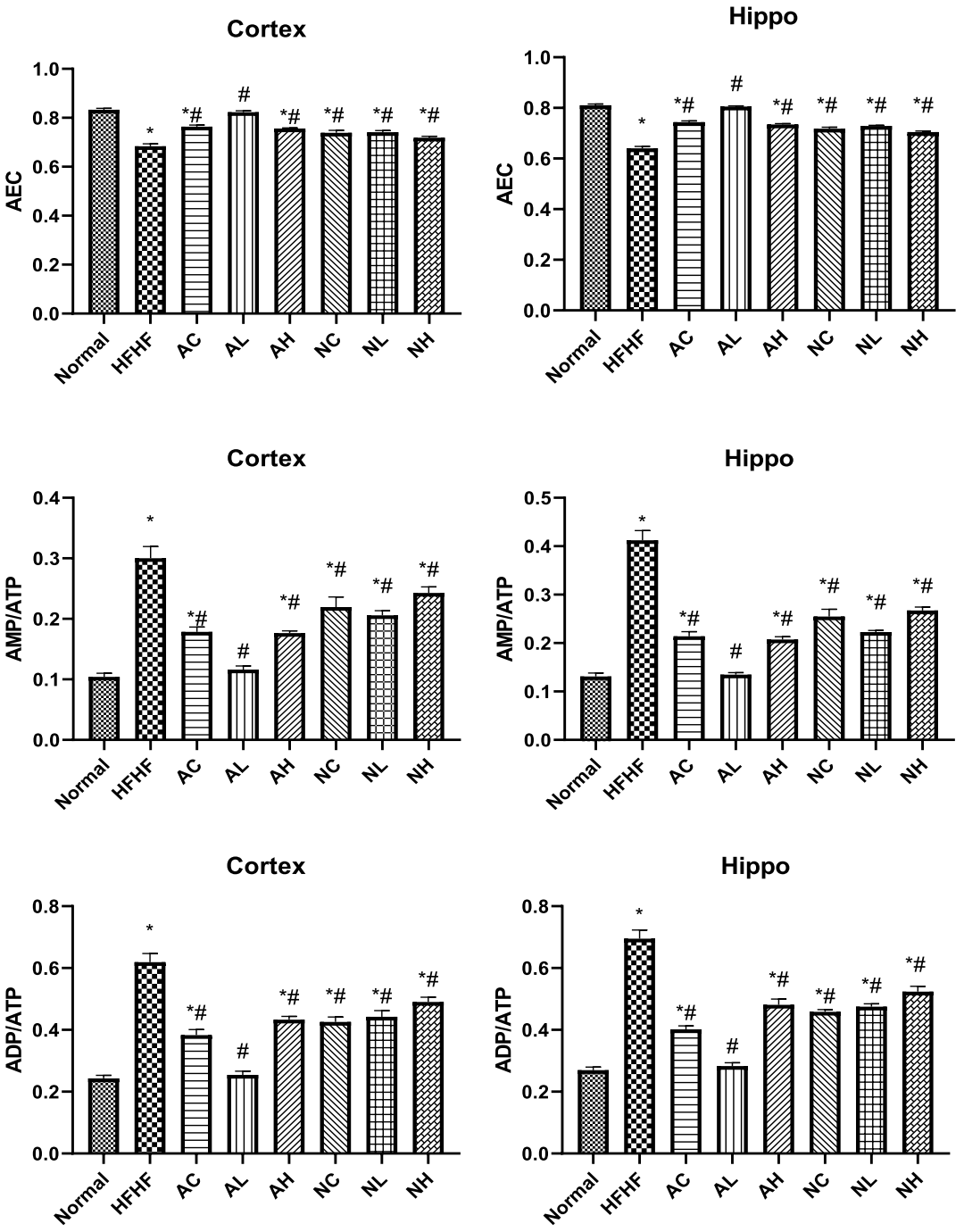

Figure 9. Cellular energy status in cortex and hippocampus brain regions for different groups. * represents significant difference from the normal control, # represents significant difference from the HFHF control $P < 0.05$.

impairment,[61,72] and enhancement of dopaminergic signaling has been implicated in the regulation of cognitive flexibility.[73] Serotonergic transmission was proven to modulate decision making, working memory, and attention. Low brain serotonin levels were found to be associated with poor memory.[74,75]

Oxidative stress and cellular oxidative DNA fragmentation have previously been positively correlated with IR and HOMA-IR elevation.[35] In the brain, an increased oxidative stress can induce lipid peroxidation, hence producing the MDA and numerous free radicals and leading to neuronal cell membrane deformation causing cell death and resulting in long-term complications and cognitive decline. Thus, elevated MDA levels indicate neuronal degeneration and, similarly, an altered ratio of the total GSH to oxidized GSH is biologically utilized to indicate oxidative cell damage.[70,76,77] Overproduction of nitric oxide was also proven to be implicated in cellular oxidative stress, potential mitochondrial damage, and apoptotic neuronal cell death.[78,79] Finally, 8-OHdG, which is a repair product of oxidized guanine lesions, has been linked to increased oxidative stress or disease states and can be taken as a reliable biomarker of oxidative DNA and RNA damage and repair.[80−82]

The AMP-activated protein kinase (AMPK) is indicative of the cellular energy status. It is activated by an increase in AMP/ATP or ADP/ATP ratios as a result of cellular energy status disruption in response to metabolic stress that either interferes with ATP production or accelerates ATP consumption.[83]

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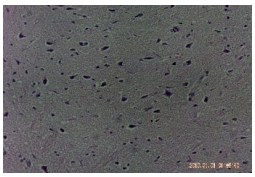
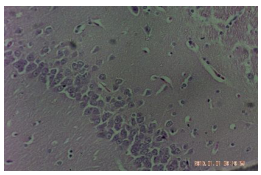
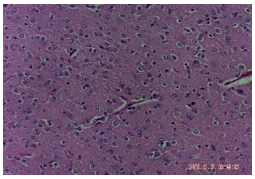
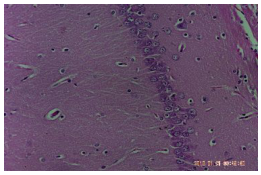
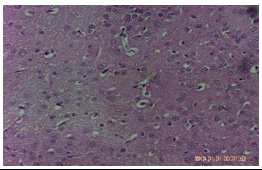
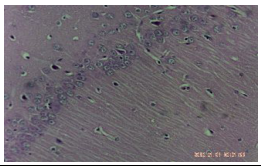
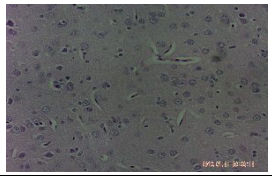
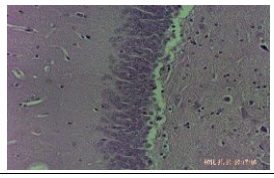
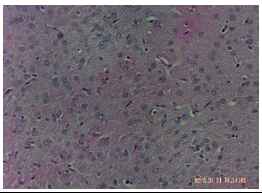
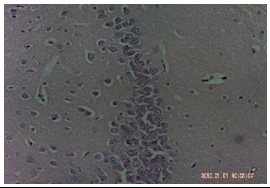
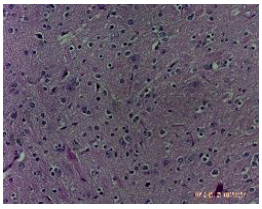
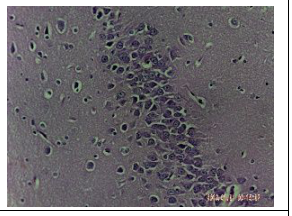
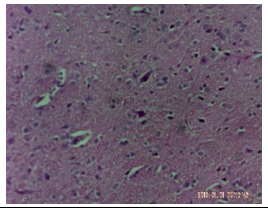
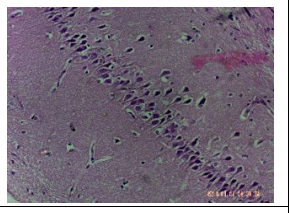
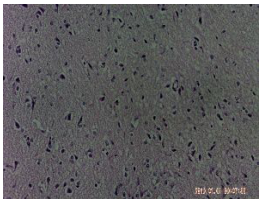
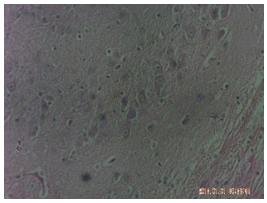
ACS Omega XXXX, XXX, XXX−XXX

Table 2. Histopathological Examination of the Cortex and Hippocampus Brain Regions of Different Treatment Groups

Group	Findings	Cortex	Hippocampus
Normal	Normal appearance of cortex, and hippocampus with nerve cells and fibers completely arranged and density of neuronal fibers well maintained		
HFHF	Both regions showed neuronal damage with increased number of pyknotic nuclei and neurons at various stages of degeneration as well as vacuolated ones especially at hippocampus level		
AC	Variable degrees of neuronal damage including eosinophilic degeneration and fragmentation of nuclei		
AL	Few pyknotic nuclei at cortical and hippocampus levels		
AH	Light edema at cortical level with degenerated neurons at variable stages. Gemistocytic cell recognition is indicative of prolonged inflammatory status.		
NC	Vacuolation and eosinophilic degeneration of neurons as well as pyknotic nuclei on both cortical and hippocampus areas		
NL	Few damaged nerve cells with subsequent pyknotic nuclei and few fragmented ones		
NH	Cortical edema and degenerated neurons most of them showing pyknotic nuclei and areas of gliosis.		

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ACS Omega XXXX, XXX, XXX−XXX

Table 3. Percentage of Positively Stained Cells, Intensity of Staining, and Immune-Reactivity Score of the Cortex and Hippocampus Brain Regions of Different Groups

	cortex			hippocampus		
group	% of positively stained cells	intensity of staining	immunoreactivity score	% of positively stained cells	intensity of staining	immunoreactivity score
normal						
HFHF	25%	4	$T = 100$	40%	3	$T = 120$
AC	25%	3	$T = 75$	20%	3	$T = 60$
AL	10%	2	$T = 20$	10%	2	$T = 20$
AH	15%	3	$T = 45$	30%	3	$T = 90$
NC	30%	3	$T = 90$	30%	3	$T = 90$
NL	10%	2	$T = 20$	10%	2	$T = 20$
NH	20%	3	$T = 60$	20%	3	$T = 60$

AMPK activation is also mediated by reactive oxygen species (ROS) independent of the ADP/ATP ratio.[84]

Previous investigations have reported that ascorbic acid is vital for attenuating oxidative stress as well as neuronal differentiation, maturation, myelin formation, and modulation of the cholinergic and catecholamininergic systems. It also controls catecholamines' release and reuptake, hence serving as a cofactor for neurotransmitters' synthesis and inducing synaptic release of acetylcholine (Ach). Moreover, it was reported to increase thymidine incorporation into the DNA and potentiate the stimulatory effect of IGF-1 on cellular DNA synthesis.[85−87] Ascorbic acid was proven to be effective in the recovery of memory impairments, with prevention of neurodegeneration and neuroinflammation especially in high doses. Patients administrated ascorbic acid supplements presented a reduced risk of cognitive decline,[15,88−90] and although it is generally reported that ascorbic acid is safe, large doses of ascorbic acid could result in GIT disturbances (nausea, pyrosis, and diarrhea) and increased frequency of urination with burning sensation. By prompting severe urine acidification, such high doses of ascorbic acid impair the excretion of weak acids and bases, leading to the precipitation of cystinate and urate deposition in the urinary tract and the formation of renal calculi. Hence, it would be favorable to find a way to decrease such a dose while keeping the favorable neuroprotective effect.[91,92]

Nicotinamide is another antioxidant molecule that has been reported as a probable neuroprotective agent in cellular, animal, and human studies. In the body, it serves as the precursor of nicotinamide adenine dinucleotide (NAD(+)), which is a key coenzyme in the production of adenosine triphosphate (ATP) or cellular energy. It also inhibits polyADP-ribose polymerase-1 (PARP-1), an enzyme activated by DNA damage, causing depletion of both NAD+ and ATP. Consequently, treatment with nicotinamide could be favorable to reduce cell death.[9,93] It was previously reported that administration of nicotinamide elevated brain tissue ATP concentrations and attenuated the ATP depletion as well as 5-HT and dopamine depletion, reduced the MDA and nitrite levels, and increased GSH, thus reversing the neurodegenerative effects of several neurotoxins such as amphetamines, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 3-nitropropionic acid (3- NP).[94−97] Unfortunately, the body could absorb a limited amount of nicotinamide at a time, hence requiring the administration of multiple daily doses,[10] which could cause neurotoxicity instead of neuroprotective actions if administered at high doses. It worth mentioning that nicotinamide hypervitaminosis toxicity state is common if the dose is not maintained.[98]

Therefore, in an attempt to decrease the doses of both ascorbic acid and nicotinamide, they were formulated in chitosan nanoparticles in the current study, to a dose up to 10 times less than that of the conventional dissolved powder dose.

The discrimination indices assessed during the object recognition test indicated that all rats, except HFHF rats, discriminated N significantly better than F, indicating that ascorbic acid and nicotinamide ameliorated HFHF-induced memory impairment in rats. HOMA-IR was significantly reduced, leading to improvement of all biochemical, pathological, and immunohistochemical assessments. Interestingly, despite administering the nanoparticles of ascorbic acid and nicotinamide at a dose of 5 or 10 times less than that of the conventional dissolved powder, a comparable effect was achieved in terms of behavioral response. Similarly, this correlated with a comparable decrease in HOMA-IR, increase in brain IGF-1 levels, decrease in brain acetylcholine esterase activity, decrease in the brain serotonin and dopamine turnover ratios, decrease in oxidative stress, and increase in cellular energy. The nanoparticles were even superior in decreasing the immune-reactivity score compared to the conventional dissolved powder. This improved therapeutic efficacy of chitosan nanoparticles containing a low dose of nicotinamide and ascorbic acid compared to the high conventional dose is probably attributed to their small particle size, which enables them to permeate in a better way across the intestinal membranes, in addition to their mucoadhesive potential, which intimates contact with the mucus layer of the intestinal epithelium, hence leading to enhancement in the absorption of the drugs.[16] Chitosan as such being a positively charged polymer is well-reported to decrease the transepithelial electrical resistance of cells, in addition to increasing the paracellular permeability by interaction with tight junction proteins.[99,100] Regarding chitosan in nanoparticles, they were reported to be even more efficient in enhancing epithelial uptake than chitosan solution.[101−104] The penetration of the enterocytes' mucus layer by chitosan nanoparticles and their internalization in intestinal cells were confirmed and reported,[105] probably following the clathrin-dependent endocytosis uptake mechanism,[106,107] with the possibility of being absorbed in an intact form to the systemic circulation.[16] Taking into consideration the possibility of reaching the systemic circulation intact, chitosan nanoparticles were also reported to cross the blood−brain barrier by virtue of their positive charges, which interact with the negatively charged sites on the cell membranes and tight junctions, hence facilitating their crossing across the blood−brain barrier.[108,109] It was reported that chitosan is specifically able to interact with the tight junctions of the brain endothelial cells[110] and to be internalized via adsorptive mediated transcytosis.[111−113] All of the aforementioned discussions suggest that chitosan nanoparticles could be a valuable delivery

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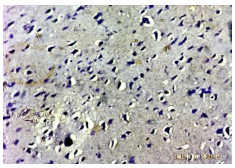
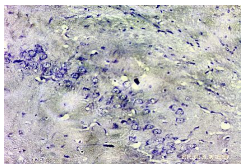
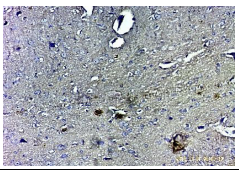
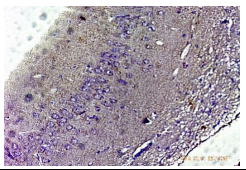
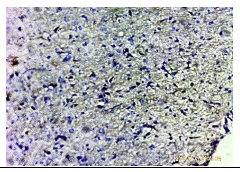
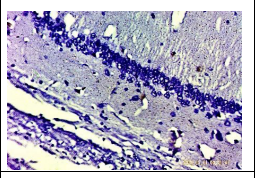
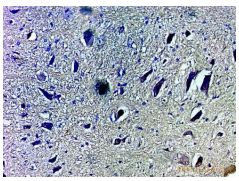
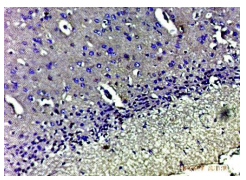
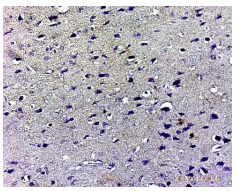
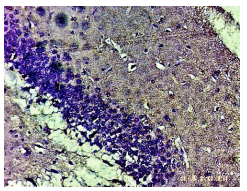
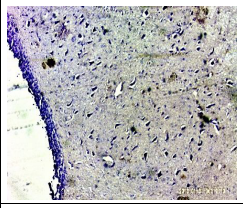
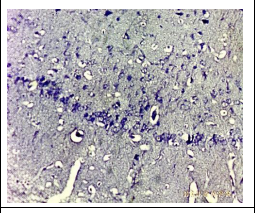
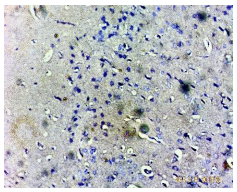
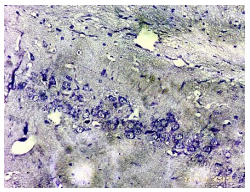
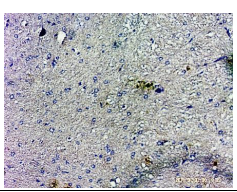
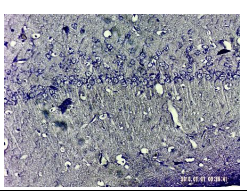
ACS Omega XXXX, XXX, XXX−XXX

Table 4. Immunohistochemical Morphological Appearance of the Cortex and Hippocampus Brain Regions of Different Groups

Group	Cortex	Hippocampus
Normal		
HFHF		
AC		
AL		
AH		
NC		
NL		
NH		

system for enhancing the bioavailability and therapeutic efficacy of nicotinamide and ascorbic acid.

5. CONCLUSIONS

The overall results of the present study revealed that encapsulation of nicotinamide and ascorbic acid in nanoparticles was proven to achieve comparable and in some instances superior therapeutic effects over the unencapsulated drugs in counteracting insulin-resistance-induced cognitive and neurodegenerative defects, presumably by increasing their bioavailability. There were no significant differences detected in the results obtained from both dose levels investigated for either ascorbic acid or nicotinamide. Hence, a daily supplement of single-dose (10 mg/kg) nanoencapsulated formulation in the management of insulin resistance would be recommended for further clinical investigations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05096.

Effect of different treatments on serotonin, dopamine, and their metabolites (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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ACS Omega XXXX, XXX, XXX−XXX

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