

Studying the effect of vitamin B12 and folic acid on the levels of brain-derived neurotrophic factor in the blood and the brain of obese male rats.

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Abstract

Objective: We aimed to investigate the potential benefits and mechanisms of vitamin B12 and folic acid supplementation in obesity. **Materials and methods:** Thirty two Sprague-Dawley rats were separated into four groups, eight rats each: controls were fed normal chow diet (ND), obese controls were fed high fat diet (HFD) (60% of their diet as saturated fats), vitamin B12-treated rats were fed HFD concomitant with an intraperitoneal injection of 1 mg/kg/day of vitamin B12 for 5 days in week, and folic acid-treated rats were fed HFD with a concomitant administration of 50 mg/kg day of folic acid by gastric gavage, all for 12 weeks. At the end of the experiment, behavioural tests were performed, and blood samples were gathered for lipid profiles, serum vitamin B12, folic acid, and BDNF. The hippocampus and hypothalamus were isolated for oxidative stress and BDNF testing. **Results:** Obese rats showed hypercholesterolemia, hypertriglyceridemia (TG), hyperleptinemia, elevated oxidative stress markers, and a decline in cognitive and behavioural test parameters. Obesity had a negative effect on BDNF levels in the blood, hypothalamus, and hippocampus, which was reversed by the administration of vitamin B12 and folic acid. Treatment with vitamin B12 resulted in a significant rise in BDNF levels, a decrease in serum total cholesterol and TG, and a significant improvement in cognitive functions and behavioural test parameters. However, while the folic acid improved cognitive function parameters and oxidative stress markers, it had no effect on the lipid profile and showed a non-significant increase in BDNF levels. **Conclusions:** Obesity decreased BDNF levels in the hippocampus and hypothalamus, which was reversed by vitamin B12 and folic acid treatment, they are therefore an intriguing therapeutic approach for obesity and associated cognitive decline.

INTRODUCTION

Obesity is one of the fastest spreading health issues worldwide. Obesity pathophysiology is complicated by several genetic and environmental influences, as well as their interactions. It is distinguished by an abnormal increase in fat deposition in conjunction with systemic inflammation (1).

Obesity is considered a risk factor for cognitive function disorders like dementia and Alzheimer's disease. The exact mechanism by which obesity affects the brain is unknown, however some researchers contend that the main causes of obesity-related CNS complications are increased oxidative stress levels and neuro-inflammation (2).

Through mitochondrial dysfunction, oxidative stress is thought to be a key factor in the pathogenesis of cognitive decline in obesity. It disrupts fat homeostasis in cells and increases reactive oxygen (ROS) and nitrogen species (NOS) production in brain. Obesity creates an imbalance between ROS production and antioxidant protective factors (3).

Brain-derived neurotrophic factor (BDNF) is a protein member of the neurotrophin family. Neurotrophins, mainly BDNF, are involved in neuronal survival, synaptogenesis, and synaptic plasticity in many types of mature neurons. BDNF protein and its receptor, tropomyosin receptor kinase B (Trk B), are the most prevalently expressed proteins in the hippocampus and the hypothalamus (4).

In animal models, BDNF was found essential in energy homeostasis. Recent research has established that energy status influences rat ventromedial hypothalamus BDNF gene expression. There is a substantial body of evidence linking decreased BDNF signalling to

energy balance dysregulation and extreme obesity in humans and rodents (5). In obese rodents, blood BDNF levels are strongly correlated with eating disorders such as hyperphagia and hyperglycaemia (6).

In addition, through hippocampal signalling, BDNF makes a significant contribution to a particular type of memory inhibition for food reward and food consumption inhibition. Furthermore, It is secreted in the hippocampus and is involved in memory, synaptic transmission, and cognitive function enhancement (7).

There is a growing evidence that obesity and HFD are associated with a decreased concentration of serum vitamin B12 and folic acid. Also, researchers suggest that vitamin B12 and folic acid could elevate the level of BDNF (8,9). Therefore, they may have anti-obesity effects by enhancing BDNF levels, modifying upstream inflammatory pathways, and alleviating the changes seen in obesity. The primary goal of this research is to investigate the effect of vitamin B12 and folic acid on BDNF in the hypothalamus and the hippocampus (10). In order to deduce the links between different mechanisms, it was also critical to measure lipid profile and oxidative stress parameters (11).

Material and method

Reagents

1. Vitamin B12 was provided from Sigma Chemical Co., Egypt in the form of rose to off-white powder in a 5-gm package
2. Folic acid was purchased from EL-Goumhouria Co., Cairo, Egypt in the form of yellow to orange powder in a 10-gm package.

Preparation of vitamin B12 and folic acid solution

Vitamin B12 was dissolved in normal saline. Each 10 mg of vitamin B12 was dissolved in 1 ml of normal saline and given at a dose of

0.1 ml/kg of body weight (1 mg Vitamin B12/kg).

Folic acid was dissolved in 0.5 M sodium bicarbonate solution. Each 500 mg of Folic acid was dissolved in 10 ml of 0.5 M sodium bicarbonate solution and given at a dose of 1 ml/kg of body weight (50 mg folic acid/kg).

Experimental animals

Thirty two male Sprague-Dawley rats, weighing approximately 200 ± 20 g, were used after a week of acclimation at the Mansoura experimental research centre (MERC), University of Mansoura. Animals were kept in clean plastic cages with wood chippings as bedding. Each group of rats was placed in a separate cage. All animals had free access to water and were fed regular commercial rat chow pellets for those on the normal diet or a formulated high-fat diet for the other experimental groups.

All research protocols were authorised by our local animal care and ethics committee (**Code# MS.21.02.1368**).

Basal diet and experimental high fat diet composition

The regular diet consisted of normal rat chow diet obtained from MERC (Mansoura, Egypt) with the following composition: fibre (15%), Fat (8%), protein (13%), phosphorus (0.35%), Calcium (0.9%), energy (2600 kcal/kg). The high fat diet composed of animal feed supplemented with 0.3% methionine and 44% animal fat (lard) (**12**).

Study design

Thirty-two male rats, were divided into four groups (eight each); controls fed normal rodent diet (ND), obese controls fed HFD (**13**), vitamin B12-treated rats fed HFD with concomitant IP injection of 1 mg/kg/day of vitamin B12 for 5 days a week (**14**), and folic acid-treated rats fed HFD with concomitant administration of 50 mg/kg/day of folic acid by gastric gavage (**8**), all

for 12 weeks. The control group received normal saline in place of vitamin B12 and distilled water in place of folic acid.

In the week preceding the sacrifice, behavioural tests (open field and Y maze tests) were performed. Prior to sacrifice (at 12 weeks), blood was drawn from the retro-orbital plexus using a capillary tube after overnight fasting. Different serum parameters (total cholesterol, triacylglycerol (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), and BDNF) were measured. Total cholesterol, TG, and HDL were measured using Bio diagnostics Egypt kits. Rats were sacrificed one day after the last vitamin B12 and folic acid dose at the end of week 12. Rats were anaesthetized with thiopental and manually decapitated. Autoclaved dissection tools were used to open the skull. The brain was placed on a Petri dish with phosphate buffer and isolated as necessary. The hippocampus and the hypothalamus were placed on a separate dish with the same buffer. The hippocampus and the hypothalamus were homogenised in order to measure BDNF, MDA, and GSH levels using supernatant. By the end of study, all tissue were packed, syringes were put in safety boxes, all of them were sent to incineration.

Behavioral procedure

Test for locomotor activity and anxiety behavior using open field test

The open field test was used to determine whether vitamin B12 and folic acid affected the locomotor activity in rats fed on a high fat diet. Each rat was placed in the center of the open field apparatus and given 5 minutes for free exploration. The number of center entries, time spent at the center, rearing, and defecation boli were all recorded. The arena was cleaned with 70% ethanol after each test to remove any animal

hints. When the animals' four paws were completely in the center, an entry was scored (15). Test for memory performance using Y-maze paradigm

The Y-maze paradigm was used to examine the impact of vitamin B12 and folic acid on spatial working memory in HFD-fed rats. Rats were each assigned to the center of Y-maze and given 5 minutes to freely explore all three arms. After each test, the apparatus was cleaned with 70% ethanol to eliminate animal hints and the number and sequence of arm entries were recorded. When the animals' four paws were completely in the arm of the Y-maze, they received a point. To calculate the percentage alternation, divide the total number of correct alternations by the total number of arm entries minus two, and multiply by 100 (16).

Biochemical assays

Rats were sacrificed 24 h after the last dose of vitamin B12 and folic acid, at the end of week 12, after an overnight fast. Blood was collected from the retro-orbital plexus before scarify to obtain serum for lipid profile, vitamin B12, folic acid and BDNF parameters, blood was centrifuged for 15 minutes at 4000 rpm at room temperature. Using a Teflon homogenizer, the hypothalamus and the hippocampus were homogenized in 0.1 M cold sodium phosphate buffer (pH 7.4). The homogenate was centrifuged for 10 minutes at 10,000 rpm. The supernatant was separated for later analysis of oxidative stress markers and BDNF.

Estimation of serum lipid profile

Randox diagnostic kits were used to calculate total cholesterol, triglyceride (TG), HDL, and LDL (Randox Laboratories Limited, Antrim, United Kingdom). LDL cholesterol was calculated as $LDL = (TC - HDL) - (TG/5)$.

Estimation of serum vitamin B12 and folic acid

Both vitamins are measured by using the #Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radio-assay kit.

Estimation of oxidative stress parameters in the hippocampus and hypothalamus

The concentration of the lipid peroxidation end product malondialdehyde (MDA) in brain supernatant was determined using the thiobarbituric reacting substance (TBARS) assay. The concentration of reduced glutathione (GSH) in brain supernatant was determined using (BiokitsUSA).

Estimation of BDNF levels in serum and hippocampus and hypothalamus

The concentration of BDNF (BiokitsUSA) in the serum and brain supernatant (hypothalamus and hippocampus) was ascertained per the manufacturers' instructions. BDNF was measured using ELISA with a sensitivity limit of 6 pg/mL (Biokits ELISA MAXTM Deluxe kit, USA). All measurements were performed at room temperature using a microplate reader with a 450 nm filter, as guided by the Biokits (Micro READ 1000, Belgium). The BDNF concentration in the tissue and serum was determined by applying the curve of BDNF standards included in the assay kits. BDNF levels in the serum and brain were expressed as pg/ml and pg/mg protein, respectively.

Statistical analysis

Data was gathered, tabulated, subjected to statistical analysis, and plotted on a graph. The Statistical Package for Social Sciences (SPSS) version 23 was used. To compare groups, the one-way ANOVA test with Tukey Post-hoc multiple comparisons was used. In the case of variables with non-normal distribution, to compare groups, the Kruskal-Wallis test was used. The findings are presented as Mean \pm SD or median and range in

all tables P value equal to or less than 0.05 was considered significant.

Results

The effect of vitamin B12 and folic acid on body weight gain

HFD group seemed to have the highest body weight gain after 12 weeks of administration, which was significantly different from the normal diet group (ND). Furthermore, when compared to the HFD group, the vitamin B12 and folic acid treated groups showed a significant decrease ($P= 0.0003$) in body weight gain as shown in table 1.

Effect of vitamin B12 and folic acid on memory performance in normal diet and high fat diet-fed rats

Figure 2 depicts the impact of vitamin B12 and folic acid on spatial working memory in normal diet fed rats or HFD-fed ones. The HFD group showed a significantly worsened spatial memory when compared to the ND group, as evidenced by a lower percentage of correct alternation in the Y-maze test ($p < 0.001$). Treatment with vitamin B12 and folic acid on the other hand, significantly

enhanced the impaired spatial memory related to HFD in rats.

Impact of vitamin B12 and folic acid on locomotors activity and anxiety in normal diet and high fat diet-fed rats

The results presented in Fig. 3(A-D) showed that HFD fed-rats had a significant rise on the total time spent in the centre ($P<0.01$), number of entries in centre ($P<0.01$) and number of defecations boli ($P<0.01$) in the open field test in comparison with ND group which indicate anxiety and change in locomotion. Moreover, both vitamin B12 and folic acid administrations with high fat diet induced changes in the spontaneous locomotion in mice. However, the total time spent in the centre of the apparatus and the total number of the entries to the centre were increased by vitamin B12, in a way similar to folic acid (Fig 3 A and B, respectively), The one-way ANOVA revealed no significant effects of both vitamin B12 and folic acid treatment on the number of the rearing ($P=0.15$).

Table (1): Effect of vitamin B12 and folic acid on body weight gain.

Measure mean±SD	Control group N=8	High fat diet group N=8	Vit B12 group N=8	Folic acid group N=8	Test of significance ANOVA
Weight Gain during 12 weeks	78.1±21.2	153.8±19.2 ^a	126.3± 20 ^{a,b}	122.5±42 ^{a,b}	F=7.5 P= 0.0003

a= any significance with control group b= any significance with high fat diet group

c= any significance with vit b12 group

P: Probability: a, b, c significance <0.05

Test used: One way ANOVA followed by Tukey posthoc test.

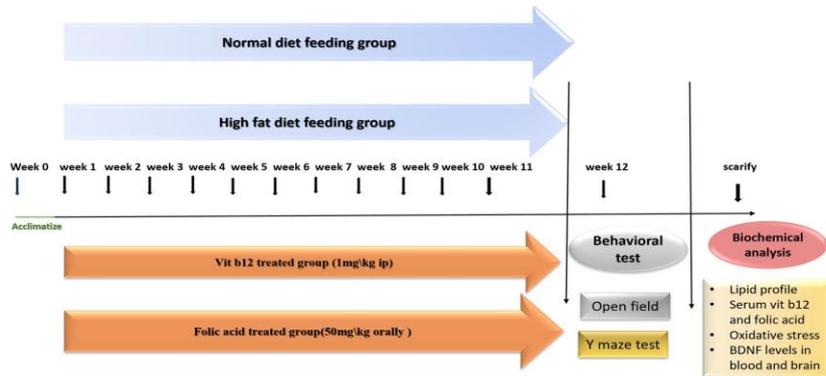


Fig 1: study design showing timeline of the study groups and biochemical analysis.

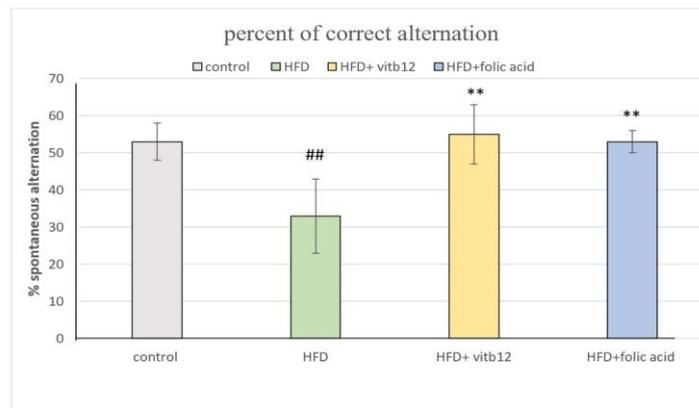


Fig (2): Effect of vit b12 and folic acid on spatial working memory of high fat diet-fed rats measured in YMT Value: (correct alternation) in different groups, in different groups are expressed as mean \pm SD (n =8). ##p < 0.05 compared to ND group, **p < 0.05 compared with HFD group, \$\$ < 0.05 compared with vit b12 group, Test used: One way ANOVA followed by Tukey posthoc test.

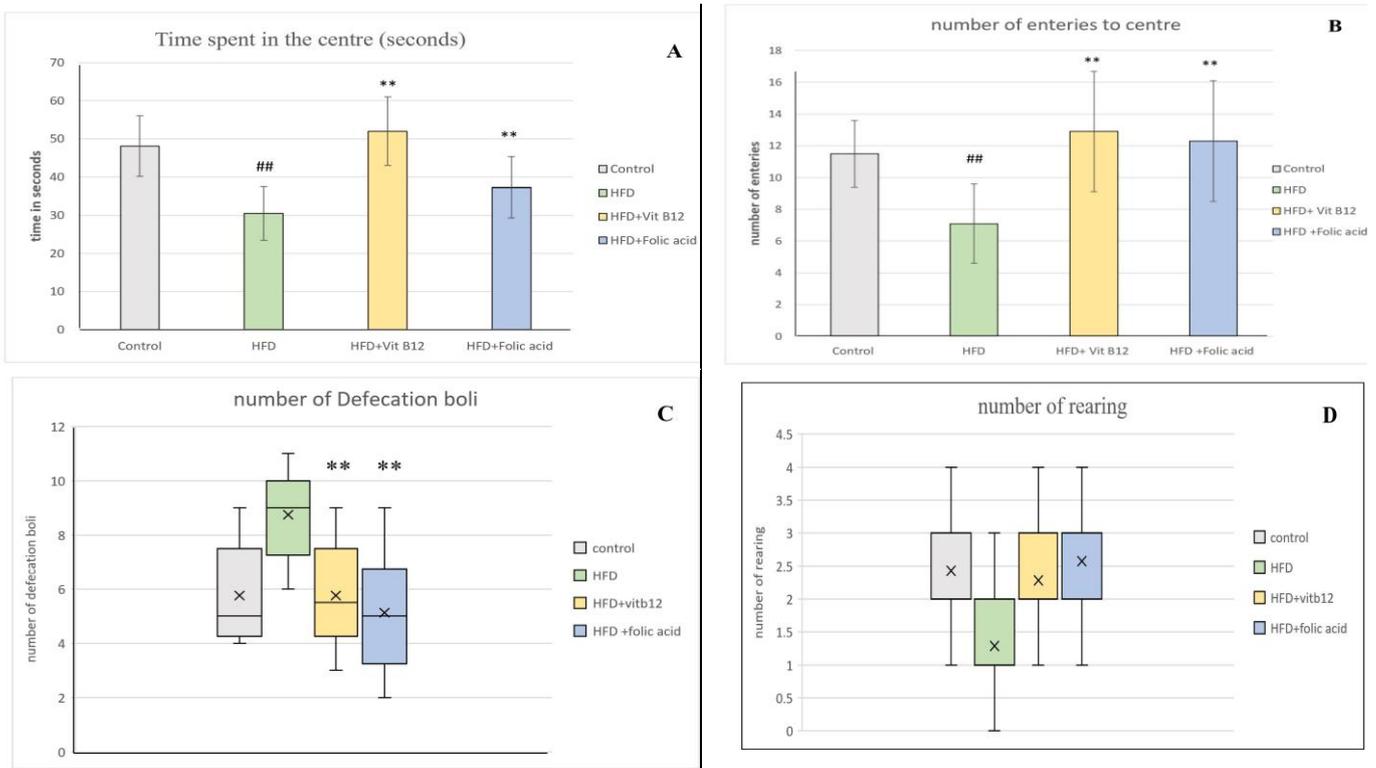


Fig 3: Effect of vitamin b12 and folic acid on locomotors activity and anxiety behaviour of high fat diet-fed rats measured in open field test.

Assay of serum vitamin B12 and serum folic acid

As shown in Fig.4 A&B, there was a slight decline in serum level of vitamin B12 in the HFD group as compared to the ND group. However, there was an insignificant difference in serum folic acid in the HFD group when compared to the ND group. Moreover, there was a significant increase in the level of vitamin B12 and folic acid in the vitamin B12 and the folic acid treated groups respectively which reassured that treated groups reached the optimum level of both vitamin B12 and folic acid.

Effect of vitamin B12 and folic acid on lipid profile in normal diet and high fat diet-fed rats

The impact of vitamin B12 and folic acid on lipid profile is shown in table 2; Total cholesterol [$p = 0.002$], TG [$p = 0.003$] and LDL [$p = 0.016$] were significantly elevated in HFD-fed rats and the folic acid treated group when compared to normal diet fed-rats. However, treatment with vitamin B12 significantly improved TG and LDL levels in HFD-fed animals. No significant effect was noticed in HDL [$p = 0.2$] among study groups.

Effect of vitamin B12 and folic acid on brain oxidative stress parameters in normal diet and high fat diet-fed rats

As depicted in Fig.5 A&B and table 3, there was a significant rise in the index of lipid peroxidation (MDA) in the hippocampus of the HFD group [$p < 0.009$; Fig.5 A] when compared to the ND group. Vitamin B12 and folic acid

supplementation significantly improved HFD-induced lipid peroxidation. (GSH) was significantly lower in the hippocampus of the HFD group than in the ND group [$p = 0.002$; Fig.5 B]. GSH levels in the brain of the HFD group was significantly increased by vitamin B12. However, the folic acid-treated group showed an increase in GSH levels, but it was not statistically significant.

Effect of vitamin B12 and folic acid on serum and brain BDNF levels in normal diet and high fat diet-fed rats

BDNF level in the serum:

Fig.6 shows the impact of vitamin B12 and folic acid on the serum BDNF of HFD-fed rats. When compared to the normal diet, there was a significant decrease ($p < 0.03$) in BDNF in the HFD group. However, treatment with vitamin B12 and folic acid depicted a slight increase in BDNF levels in treated-HFD rats when compared to the HFD group.

BDNF level in the brain :

Figure 7 demonstrated the contribution of vitamin B12 and folic acid on the level of BDNF the brains of high fat diet fed rats. There was a significant decline in BDNF levels in the hypothalamus as well as the hippocampus of the HFD group compared to the ND group [$p < 0.05$]. However, as compared to the HFD group, vitamin B12 treatment significantly increased BDNF levels in both the hypothalamus and the hippocampus in HFD-fed rats. In the folic acid-treated group, there was a slight increase in brain BDNF levels yet it was statistically insignificant.

Table (2): comparison of lipid profile results among study groups

Measure	Control group N=8	High fat diet group N=8	Vit B12 group N=8	Folic acid group N=8	Test of significance
Cholesterol	88.3±11.6	131±40.5 ^a	108.5±11.1	130.2±13.9 ^a	F=6.3, p=0.002
Triglycerides	149.3±18.9	235.6±72.1 ^a	189.5±20 ^b	196.9±26.7 ^a	F=6, p=0.003
HDL	36.2±8	30.6±3.9	36.8±7.5	32.3±6.5	F=1.6, p=0.2
LDL	22 (12.4-39)	31.7 (23-44) ^a	25.9 (22-56)	45.5 (20-60) ^{ab}	p=0.016*

All measures are expressed as mean±SD except LDL as median (range)

F of ANOVA test

*Kruskal-Wallis test

Table (3): brain oxidative stress parameters among study groups

Measure	Control group N=8	High fat diet group N=8	Vit B12 group N=8	Folic acid group N=8	Test of significance
MDA median (range)	55 (11.8-287)	126.5 (58-474) ^a	71.5 (50-101) ^b	58.5 (50-74.7) ^b	Kruskal-Wallis test p=0.009
GSH mean±SD	60±7	42.4±8 ^a	66.1±17.2 ^b	49.4±12.5	ANOVA F=6.4, p=0.002

GSH measures are expressed as mean±SD and MDA as median (range)

F of ANOVA test

*Kruskal-Wallis test

a= any significance with control group

b= any significance with high fat diet group

c= any significance with vit b12 group

P: Probability: a, b, c significance <0.05

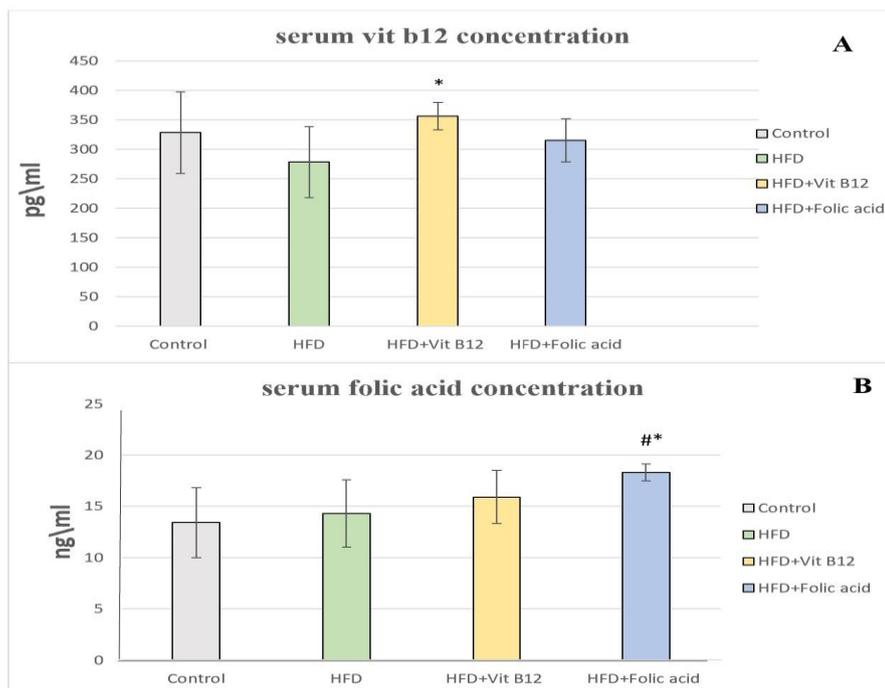


Fig (4): serum vit b12 and folic acid A: (vitb12) in different groups, B: (folic acid) in different groups are expressed as mean ± SD (n =8). #p < 0.05 compared to ND group, *p < 0.05 compared with HFD group, \$\$ compared with vit b12 group, Test used: One way ANOVA followed by Tukey posthoc test.

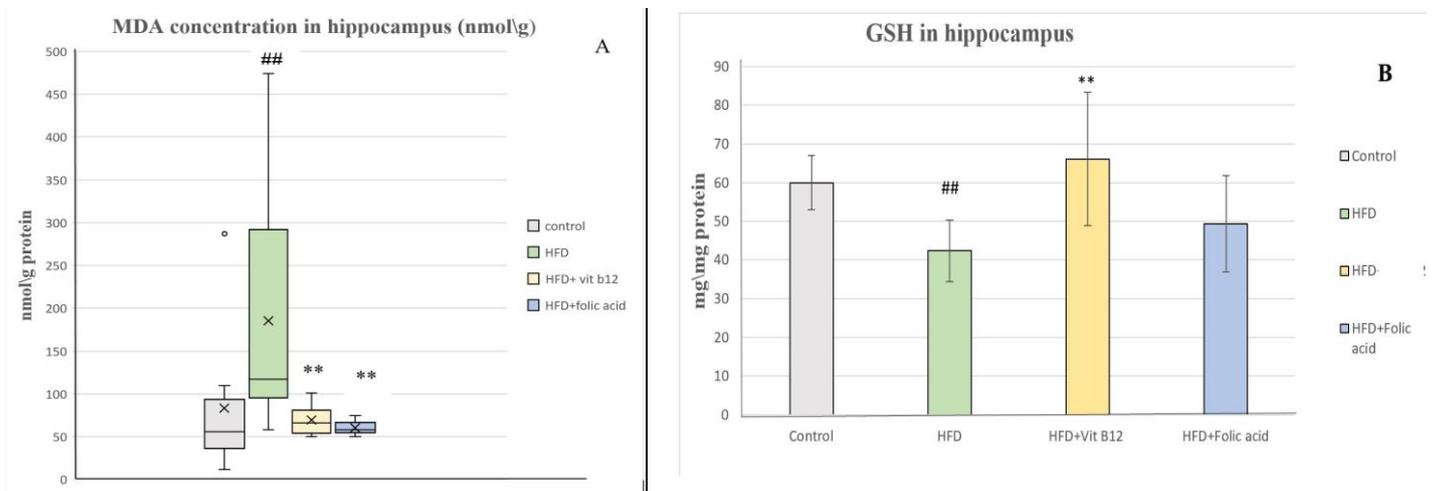


Fig (5): Effect of vitamin b12 and folic acid on oxidative stress A: (MDA) in different groups, B:(GSH) in different groups.

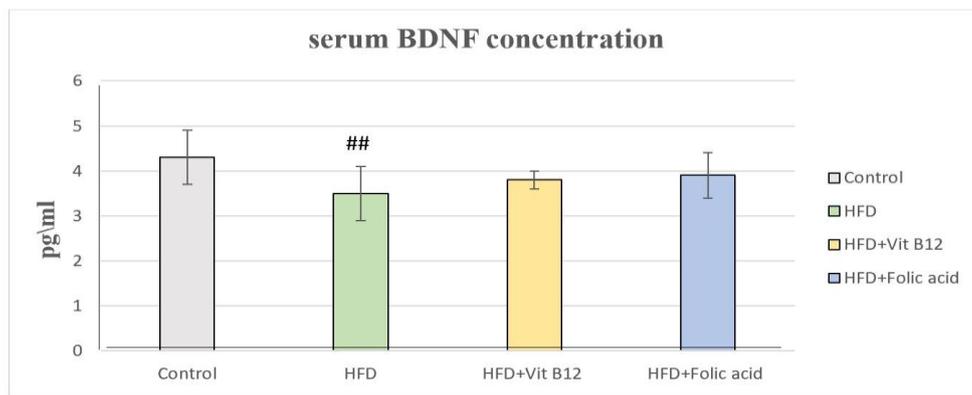


Fig (6): Effect of vit B 12 and folic acid on serum BDNF level of high fat diet-fed rats, BDNF among study groups are expressed as mean \pm SD (n =8). ##p < 0.05 compared to ND group, **p < 0.05 compared with HFD group, \$\$p < 0.05 compared with vit b12 group. Test used: One way ANOVA followed by Tukey posthoc test.

##= any significance with control group
 **= any significance with high fat diet group
 \$\$=any significant with vit b12 group

P: Probability: #, *, \$ significance <0.05

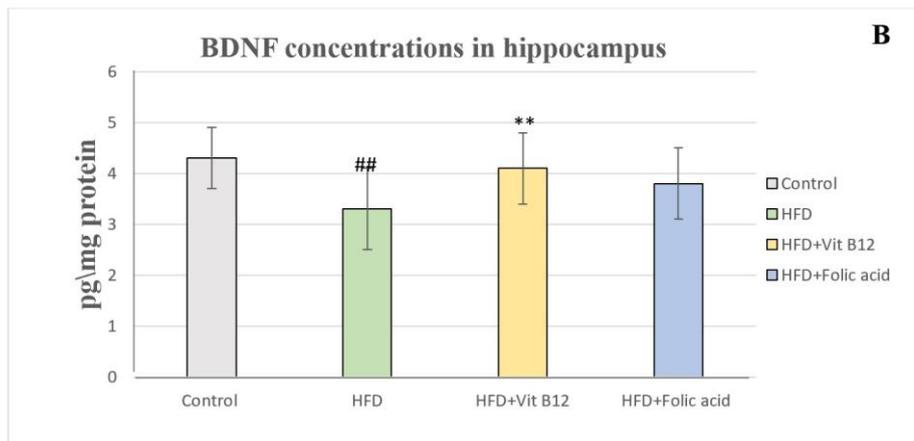


Fig (7): effect of vit b12 and folic acid on brain BDNF, A: (hypothalamus), B: (hippocampus) in different groups, values are expressed as mean \pm SD (n =8). ##p < 0.05 compared to ND group, **p < 0.05 compared with HFD group, \$\$ p < 0.05 compared with vit b12 group, Test used: One way ANOVA followed by Tukey posthoc test.

##= any significance with control group
 **= any significance with high fat diet group
 \$\$=any significant with vit b12 group

Discussion

Obesity is becoming a modern day spreading plague with many complications. Recent evidence demonstrates that Obesity and cognitive dysfunction are strongly correlated. Consumption of high-calorie diets was proven to impair spatial and working memory in rodents (2). The atheroprotective, anti-amnesic, anti-anxiety, and antioxidant effects of vitamin B12 and folic acid in a HFD rat model were investigated in this study

(12). Our results revealed that vitamin B12 and folic acid improved lipid profile, behavioural paradigms, oxidative stress markers and BDNF levels.

In this study, as depicted by various literature HFD administration to rats resulted in dyslipidaemia, evidenced by elevated total cholesterol, triglycerides, and LDL, and decreased HDL (7,17). Hypercholesterolemia has been linked to a higher risk of developing dementia

later in life which is consistent with our behavioural test results. However, vitamin B12 administration to HFD-fed rats significantly reduced cholesterol, triglycerides and LDL. It appears that vitamin B12 may be beneficial in the treatment of hypercholesterolemia patients. Earlier **Boachie *et al.*, 2020** has shown that vitamin B12 has anti-hyperlipidaemic properties. A reduction in the atherogenic effect of HFD is a beneficial physiological effect. As a result, it is plausible to speculate that vitamin B12 has atheroprotective properties **(18)**. In adverse, the folic acid treated group showed no improvement in lipid profile results.

In this study, twelve weeks of HFD feeding resulted in behavioral impairment in rats on the Y-maze test and open field test (OFT). HFD-fed rats had a lower percentage of correct alternation and time spent at the center. Previously, high-fat diets were proven to inhibit spatial learning on the Y-maze paradigm as well as anxiety behaviors on the OFT which is consistent with **(16)**. In our study, supplementation of vitamin B12 and folic acid reversed the HFD-induced memory impairment and anxiety behavior patterns in rats. A previous study found that vitamin B12 and folic acid improved cognitive function in a model of cerebral palsy and depression **(8,9)**.

OFT, one of the most widespread used anxiety models in animal testing, is based on the findings that rats avert the central area and the presumption that this evasion is triggered by fear **(15)**. Our findings revealed that a HFD increased anxiety symptoms in tested animals which are in consistent with **(13)**, and administration of vitamin B12 and folic acid significantly increased time spent at center and number of center entries, implying anxiolytic effects **(8)(19)**. Spatial

memory is completely reliant on a functioning hippocampus, and we assume that the HFD is deteriorating this region **(2)**. Those findings could be explained by our results that a high fat diet for 12 weeks disrupted the neurotrophin system, whereas HFD-fed rats receiving vitamin B12 and folic acid had a high BDNF level in the brain.

In our study, the HFD deteriorated serum GSH significantly, whereas vitamin B12 and folic acid administration dramatically improved GSH. GSH aids in the eradication of free radicals and serves as a substrate for glutathione peroxidase, which neutralizes organic hydroperoxides and hydrogen peroxide in lipid of cell membranes to protect them from ROS. Vitamin B12 may have enhanced GSH content by up-regulating glutathione reductase activity **(6)**. MDA level in the hippocampus was increased in the HFD group, whereas supplementation of vitamin B12 and folic acid decreased it. The improvement observed after vitamin B12 and folic acid treatment of HFD-fed rats suggested a therapeutic influence on HFD-related oxidative changes via the normalization of ROS **(7)**.

In HFD studies, oxidative stress markers and antioxidant concentrations were assessed to investigate the role of oxidative stress in anxiety and amnesia **(20)**. Because of its high oxygen consumption and high lipid, the brain is mostly vulnerable to oxidative damage. The current study found that rats fed HFD for twelve weeks had significantly higher lipid peroxidation and lower antioxidant levels similar to **(11,21)**. Treatment with vitamin B12 or folic acid limited oxidative stress in the brain, implying that treatment could scavenge ROS excessive production to counteract the negative effects of an HFD.

As revealed in our results, HFD considerably lowered BDNF levels in the brain which is relevant to **Jin Y.J et al., 2015 (19)** while administrating vitamin B12 and folic acid significantly elevated the levels of BDNF in HFD-fed rats especially in the hippocampus, a brain area concerned with the memory and anxiogenic/depressive phenotype, and in the hypothalamus, a brain region involved in energy homeostasis **(5)**. Our inability to investigate the actual mechanism by which BDNF appears to work is a drawback of this research, however, some earlier reports have shown the presence of important molecular pathways for BDNF action such as Ras/MAPK/ERK pathway **(22)**. Further research is needed to elucidate the precise pathway of BDNF in enhancing memory and suppressing appetite in case of folic acid and vitamin B12 supplementation with HFD .

To conclude, the current study shows improvement of memory impairment and anxiogenic effects of high-fat diet using vitamin B12 and folic acid which also display atheroprotective, anti-inflammatory, and antioxidant effects. As a result, we recommend vitamin B12 and folic acid as a potential natural source that could help prevent obesity-linked complications.

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Conflict of interest:

There is no conflict of interest in this manuscript.

Ethics approval:

Mansoura University Ethics Committee authorised the paper.

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