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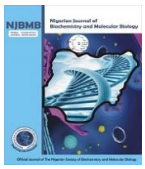

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Research Article

Upregulation of Bdnf-Trkb and Mapk1/Erk Gene Expressions Improve Skeletal Muscle Strength in High Fat Diet-Fed Swiss Mice

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ABSTRACT

Persistent consumption of foods rich in fats, sugars and calories have been implicated in a number of diseases including those of the nervous systems. This study investigated the role of co-administration of cannabidiol (CBD) and omega-3 in mice fed on high fat diet (HFD) for a period of 12 weeks. The animals were grouped as follows: Control (water and feed), HFD fed, HFD+CBD, HFD+omega-3, HFD+CBD+omega-3 and Omega-3 for 2 weeks post-HFD. They were subjected to wire hanging testing after the interventions. In the end, the animals fed HFD alone, had a statistically significantly shorter time hanging on the wire ($p<0.05$), when compared to the control, CBD and Omega treated animals. The amyloid deposits in the brains of the HFD-fed mice, after congo staining, was also significantly higher. The combination of CBD and omega-3 significantly cleared the brain of amyloid plaques, which is a pathological protein, that causes neuromuscular damage. qPCR gene expressions, for BDNF and MAPK, were significantly higher in the CBD plus omega-3 treated group compared to the HFD-fed group. This benefit was more pronounced in the post HFD group which had omega-3 for two weeks. Co-administration of CBD and Omega-3, in HFD-induced neuromuscular injury, is highly ameliorative. They are noted to increase endurance levels, during strenuous muscular exercise in mice. This is due to the clearance of amyloid proteins from the brain and upregulation of BDNF and MAPK genes. Also, stoppage of the HFD and administering omega-3 is of immense benefit.

Keywords: *High fat diet, Cannabidiol, Omega-3, BDNF, MAPK, Amyloids*

INTRODUCTION

Muscle anabolic resistance has been attributed to chronic diseases and aging; this resistance is a metabolic perturbation that is associated with decreased muscle protein sensitivity to physiological anabolic stimuli, like nutrients and exercise (Poggiogalle et al., 2022). Unhealthy body mass index (BMI), has been linked to impaired skeletal muscle performance, predominantly from altered insulin signaling (Beals et al., 2018; Chevalier et al., 2015). Our previous work demonstrated that, chronic high fat diet ingestion, induced hyperglycaemia and significantly increased body weight in mice (Abi et al., 2020). Following a wire hang test experiment, mice fed on high fat diet, were found to have a significantly higher number of falls, less number of reaches and less wire hanging time compared to control group (Tam et al., 2015). Cannabidiol is the non-psychoactive component of cannabis sativa plant, which appears to possess weight loss potentials via anorexigenic mechanisms (Pinto et al., 2022).

Similarly, omega-3 (a polyunsaturated fatty acid) has been shown to reduce adiposity, food intake and weight loss both in human and animal studies (Delpino et al., 2021; Salman et al., 2022). Studies investigating the synergy between CBD and omega-3, are quite limited. Also, it is a known fact that multi-therapeutic approach tends to give better outcomes in managing most illnesses due to etiological diversity. Thus, this research seeks to assess the effect of these two important biomolecules, on muscle strength, in mice that were chronically fed on high fat diet and to identify the roles of BDNF-Tropomyosin-related kinases B (TrkB) and MAPK in the entire process.

MATERIALS AND METHODS

Animal management

A total of 30 young albino mice (6-8 weeks) weighing between 18 to 25g were used for this experiment. They were housed in polypropylene cages in the animal room of the Department of Physiology, College of Health Sciences, Benue State University, Makurdi. They were grouped as follows (n=5):

Group 1 (Negative control): Normal chow and water *ad libitum* for 12 weeks

Group 2 (Positive control): HFD and water *ad libitum* for 12 weeks

Group 3: HFD *ad libitum* and then administered CBD (10 mg/kg b.w) orally for 12 weeks

Group 4: HFD *ad libitum* and administered Omega-3 (200 mg/kg b.w) orally for 12 weeks

Group 5: HFD *ad libitum* and administered Omega-3 (200 mg/kg b.w) plus CBD (10 mg/kg b.w) orally for 12 weeks

Group 6: HFD *ad libitum* for 12 weeks then followed by administration of Omega-3 (200 mg/kg b.w) orally for 2 weeks

Materials

The reagents and solvents (all of analytical grade) used for the research, were supplied by the CAMRET lab Sokoto but purchased from Merck (Darmstadt, Germany). Omega-3 fatty acids (1000 mg/gelatin capsule) were purchased from a local pharmaceutical store in Makurdi-Benue State. Manufactured by Gujarat Liqui pharmacapspvt ltd, Gujarat-India (NAFDAC Number: A4-8330; License Number: G/1565, Mfg. date: 10/2018; Exp. date: 09/2021). Pure cannabidiol oil (1000 mg) was obtained from Results RNA, LLC., Orem, Utah, USA (License Number: 1103-1). Chloroform (100 mls) and formalin (10%, 100 ml) of analytical grades were obtained from the laboratory of the Department of Physiology, College of Health Sciences, Benue state University (CHS-BSU). Dimethyl Sufoxide (DSMO) of analytical grade was gotten from a local store in Makurdi. Forty sample bottles of RCL2 fixatives were purchased from the DNA labs, Kaduna-Nigeria. SYBR Green PCR master mix was obtained from Thermofisher USA.

Ethical approval

This study was done in accordance with the laid down procedures of the Basic Science Research Ethics Committee of the College of Health Sciences, Benue State University, Makurdi, Nigeria. Ethical approval was obtained from same committee (Approval ID: CREC/001).

High fat diet (HFD) composition

The HFD was constituted using chow, tallow and soy oil at inclusion rates of 60%, 25% and 15% respectively. The total caloric content from fats (unsaturated and saturated) was 35% of the total calorie (Bradley *et al.,* 2020). The saturated and unsaturated fat percentages were 60% and 40% respectively; with a total caloric value of 5340 kcal/kg of which caloric contribution from fat was about 3670 kcal/kg.

Wire hang test (WHT)

Wire hang test is a simple method for determining neuromuscular integrity in experimental animals (Emma and Steven, 2016). It non-invasively measures the limb tension against gravitational pull. A 2mm copper cable wire was used. The length was 1.5m and suspended across two fixed points and raised 35cm above the ground. The floor had clothing and tissue paper to prevent traumatic falls. Each mouse was gently placed on the wire around the middle and allowed to hang on all four limbs as described by Sergio *et al* (2017). The test was repeated three times for each animal with a 1 min rest interval in between trials for a maximum period of 3 min trial. The longest average suspension time was then measured.

Primer's design

The primers for the genes of interest (BDNF and MAPK) and the suitable housekeeping gene (RPL) were designed using *Rattus norvegicus* gene sequence from the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nucleotide/). They were purchased from Tomy Kogyo Company Ltd, Tokyo Japan. The name of the gene, the accession number and primer sequence are as shown in Table 1.

Brain RNA extraction

Brain tissues were washed, weighed and placed in a microcentrifuge tube; followed by addition of 200 µL of Lysis buffer using an eppendorf micropipette and gently homogenized. Chloroform (200 µL) was then added, vortexed and centrifuged at 21952 rcf for 1 minute. Then 200 µL of the aqueous layer (uppermost layer) was transferred into a new tube and 200 µL of 70% ethanol was then added and vortexed. The content was then transferred into a spin column and centrifuged at 21952 rcf for 1 minute and flowthrough discarded. Wash buffer 1 was added and centrifuged at 21952 rcf for 1 minute and flowthrough discarded. Wash buffer 2 was then added and centrifuged again at 21952 rcf for 1 minute and flowthrough discarded. Column was spin-dried for 3 minutes at 21952 rcf and flowthrough discarded. Column

was transferred into a new microcentrifuge tube and 30 µL of elusion buffer was added to soak RNA for 3 minutes. This was centrifuged for 1 minute at 21952 rcf before storage at -20 ˚C.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

A solution composed of SYBR green master mix, Primers (reverse and forward), RNA extracts and Manganese II acetate tetrahydrate was constituted. This mixture was used for RT-PCR in an XP Thermal Cycler with the following specification: Initial Denaturation at 90˚C for 30 seconds, Reverse transcription at 61˚C for 20 minutes, Final denaturation at 95˚C for 15 seconds, Annealing at 59˚C for 15 seconds and Extension at 74˚C for 45 seconds. The last three phases were programmed to run for 45 cycles.

Data analysis

Data obtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparisons using SPSS statistical tool version 22 (IBM corp.). Values of $p < 0.05$ were considered significant. For the RT-qPCR, Livak method ($2^{-\Delta\Delta Ct}$) was used to measure difference in gene expression at the respective Ct values using the Microsoft Excel 2010 software.

Table 1. Primer Sequence used in Gexp Multiplex Analysis of BDNF Gene in Frontal Cortex of Mice Brain Tissues

Gene name and accession number	Primer sequences	
	Forward	**Reverse**
1. BDNF NM_007540.4	TTGTTTTGTGCCGTTTACCA	GGTAAGAGAGCCAGCCACTG
2. MAPK	AGAAGTCAGAGGCAGGTGGA	GGTGCCATCATCAACATCTG
2. RPL* M8523	TGAAGACAGTGCGAAAGGCA	CACCTTTGGGCTCACTCCAT

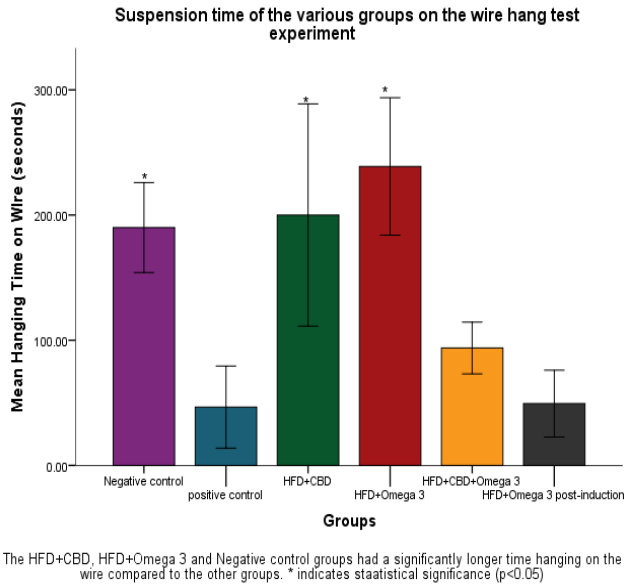

Figure 1. Suspension Time of the Different Groups on the Wire Hanging Test Experiment.

The HFD+CBD, HFD+Omega 3 and negative control groups had a significantly longer time hanging on the wire compared to the other groups. *indicates statistical significance (p<0.05)

Plate 1. A proposed mechanism of action by which Omega-3 promotes neurogenesis and enhanced neuromuscular strength via brain elevation in BDNF

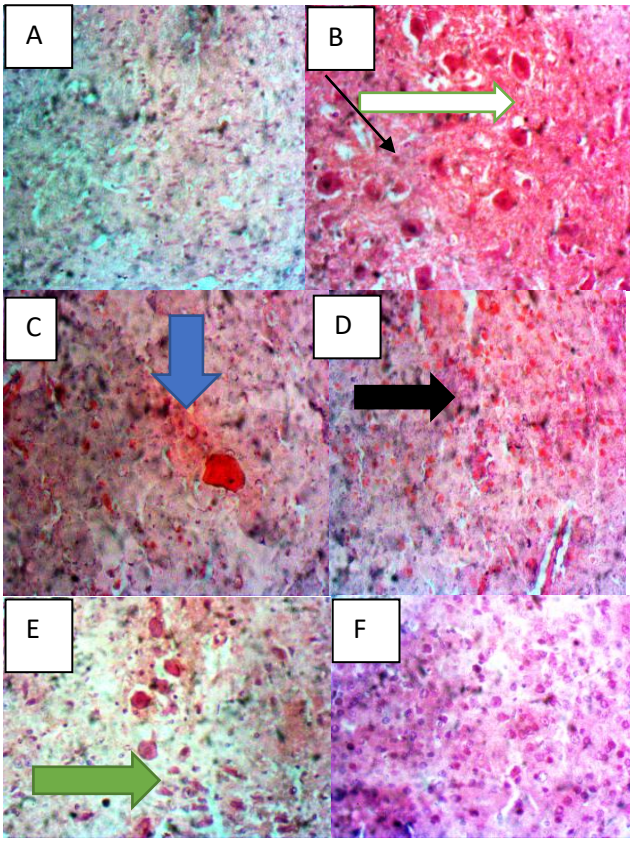

Figure 2. Control shows normal brain architecture
Figure 3. HFD fed mice shows multiple regions of amyloid plaque deposits (white arrow)
Figure 4. HFD+CBD shows reduced levels of amyloid plaques (blue arrow)
Figure 5. HFD+Omega-3 shows reduced levels of amyloid plaques (black arrow)

Figure 6. HFD+CBD+Omega-3 shows increase evidence of neurogenesis (green arrow) and significantly reduced amyloid deposits
Figure 7. HFD+Omega-3 for 2 weeks post induction shows almost total clearance of amyloid plaques.

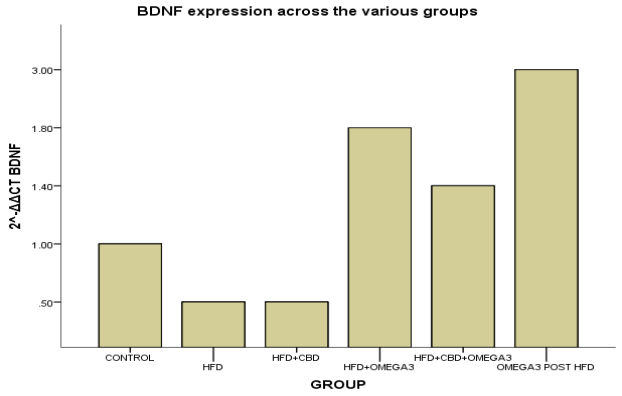

Figure 8. BDNF Expression Across the Various Groups.

The HFD+Omega, HFD+CBD+Omega-3 and Omega-3 post induction groups had statistically significant fold increase in brain BDNF levels compared to control and HFD groups.

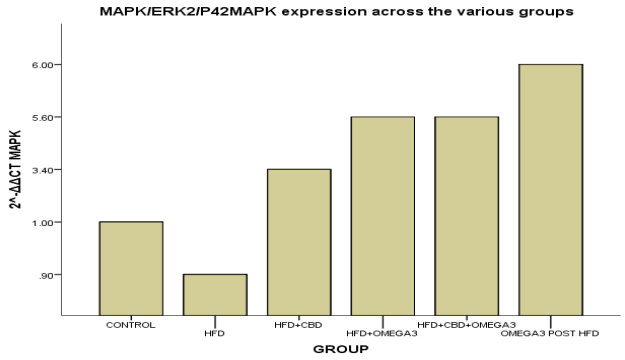

Figure 9: MAPK/ERK2/P42MAPK Expression Across the Various Groups.

All treated groups had statistically significant fold increase in brain MAPK levels compared to control and HFD groups.

In Figure 9 all treated groups had statistically significant fold increase in brain MAPK levels compared to control and HFD groups

RESULTS AND DISCUSSION

The wire hang test (WHT) showed that CBD and omega-3 administered individually significantly improved muscle strength comparable to the control group (Fig 1). The HFD was shown to reduce muscle strength significantly compared to every other group. This could not be sufficiently reversed by omega-3 after 12 weeks of HFD despite returning to normal diet. This is corroborated by existing research that has established the fact that HFD causes impaired muscle functions on WHT and muscular atrophy via the activation of ubiquitin proteasome pathway, oxidative stress, generation of myonuclear apoptosis and extracellular matrix remodeling as a result of elevated COL3 and 6 genes (Tam *et al.,* 2015; Johanna *et al.,* 2016). Research has also shown that, Intake of HFD, causes enhanced adipokinetic hormone signaling, shortened lifespan and age-related behavioural senescence (Liao et al., 2020). The upregulation of BDNF could lead to affinity binding with tropomyosin kinase B (Trk-B) receptor leading to enhanced neuromuscular function, muscular strength and integrity (Chart 1). This is corroborated by a recent work by Forgaty *et al* (2022). So far, no data to compare the effect of CBD or omega-3 on this model thus, making this the first experiment in that regard.

Congo staining revealed significant amyloid deposits in HFD, HFD + CBD and HFD + omega-3 groups (Figure 2, 3 & 4) but absent in the control, HFD + CBD + omega-3 and HFD + omega-3 post induction groups (Figure 1, 5, & 6). This study proved for the first-time histological evidence of amyloid plaques in the brains of young/middle aged mice chronically fed with HFD. This may be as a result of our own modified HFD composition. The study showed that CBD and omega-3 administered alone could not effectively clear the brain of amyloid plaques. However, a combination of CBD and omega-3 effectively cleared the brain of amyloid plaques. Another novel finding is the fact that, stoppage of the HFD for 2 weeks followed by administration of omega-3 alone was able to clear the brain of plaques. This is corroborated by a study which demonstrated that omega 3 could clear amyloid proteins in rat brain endothelium (Wang et al., 2018). Our study, also demonstrates that the mere presence or absence of amyloid deposits alone does not determine the cause of neuromuscular damage, rather the total restoration of all neural damage that occurred due to various neuro-inflammatory processes in the nervous system. It also shows that stoppage of the causal factor and administering of omega-3 will halt the progression of the neuro-degenerative process. This can be proven by the significant upregulation of BDNF and MAPK (Fig. 8 and Fig. 9) in the post induction group when compared with the HFD group. Even though in the past a study proposed that amyloid plaques deposits in skeletal muscles results in cytoplasmic inclusion body and vacuolation myopathies (Ken-ichiro et al., 1998). A recent study however showed that even though amyloid deposits are detrimental to neuromuscular functions, resistant trainings could sufficiently restore impairments (Masoud et al., 2023). Another recent study found that CBD in mice model of AD helped restore the functions of TREM2 and IL-33 which are essential brain proteins necessary for reducing beta-amyloid plaque accumulation and also improved neuronal functions (Khodadadi et al., 2021).

Another study using omega-3 showed that it stimulated microglial phagocytosis of beta amyloid plaques in a biphasic manner i.e initial period of sustained stimulation followed by a period of inertness (Hjorth *et al.,* 2013). Ren *et al* (2017) also discovered that omega-3 could potentiate glymphatic system and consequently promote interstitial amyloid clearance from the brain. The postulation from this research is that a combination of CBD and Omega-3 gives a sustained clearance of amyloid beta plaques from the brain via multiple synergistic mechanisms. Also this research has shown the role of CBD and Omega-3 in restoring neural function via the upregulation of the BDNF and MAPK genes;

which are requirements for neurogenesis, axonogenesis and synaptogenesis amongst other key functions.

CONCLUSION

The role of the synergistic combination of CBD and Omega-3, in enhancing muscle strength in HFD fed mice, was evaluated in this research. The combination of CBD and omega-3 was found to prolong the wire hanging time in treated mice, akin to enhanced endurance during strenuous exercise. Theese compounds were also able to clear the brains of amyloid plaques and enhanced the expressions of BDNF and MAPK genes. The role of a synergistic bio molecular approach in promoting skeletal muscle strength is required especially amongst athletes and in endurance training. A translational study will buttress this fact in the future.

AUTHORS' CONTRIBUTIONS

IA: Conceptualization, methodology, writing – original draft. OSA: Formal analysis, Supervision, Interpretation, review & editing. MUI: Conceptualization, Supervision, Methodology, writing – review and editing.

CONFLICT OF INTEREST

None to declare

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