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Research Article

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Punicalagin Alleviates Oxidative Stress and Pathological Changes in Brain of Mice-Fed High Fat, High Fructose Diet

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Abstract: The present study investigated to know whether the pomegranate polyphenol punicalagin (PU) can ameliorate oxidative stress and pathological alterations induced by a calorie rich diet comprised of fat and fructose. Male Swiss albino mice were fed normal pellet (Control) or high fat-high fructose diet (HFFD) for 60 days. PU (30 or 60 mg/kg bw) was administered orally for the last 15 days for both dietary groups. Measurement of TBARS, AOPP and FRAP in plasma, histopathology of liver and brain, assessment of oxidative damage in brain tissues, and blood brain barrier function were carried out. HFFD mice showed an increase in the levels of TBARS and AOPP, and a decline in FRAP. PU administration to HFFD mice reduced the levels of TBARS and AOPP and improved FRAP significantly. Histopathology results showed fatty changes and inflammatory infiltration in the liver of HFFD mice. Cortex and hippocampus of HFFD mice confirmed the presence of degenerating neurons with shrunken cytoplasm and pyknotic nuclei. PU treatment caused significant hepato and neuroprotective effects, and among the two doses the high dose of PU (60 mg/kg) showed more pronounced effects than the low dose (30 mg/kg). The immunohistochemical localization of the markers of oxidative damage to proteins (3-NT), lipids (4-HNE) and DNA (8-OHG) was increased in brain tissues of HFFD mice and upon PU administration (60 mg/kg) the levels of these oxidative stress markers were significantly decreased. Our findings clearly suggest that PU can modulate oxidative stress and could be a potential candidate for treating neurodegenerative processes associated with calorie excess.

Key Words: Punicalagin, high fat-high fructose diet, oxidative stress, neurodegeneration, neuroprotective.

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1. INTRODUCTION

The influence of diet-induced metabolic abnormalities on the incidence of neurodegenerative processes has been recognized.[1-3] The histopathological hallmarks of Alzheimer's disease (AD) such as amyloid deposition and tau hyperphosphorylation were observed in brain of sucrose-fed mice having metabolic abnormalities.[4] Diet-induced metabolic stress initiates oxidative stress that underpins severe pathological alterations and neurodegenerative processes.[5] Oxidative stress in cells results from an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and removal by antioxidative defence mechanisms. The brain is vulnerable to oxidative insults than other tissues due to its high energy demand, elevated oxygen consumption rate, rich-phospholipid content and insufficient antioxidant defence.[6]Dietary phytoconstituents are being studied and are marketed as antioxidant supplements for the cure of various health ailments. Of late, increased consumption of antioxidant functional foods has been associated with mitigation of metabolic abnormalities associated with intake of fat and fructose rich diets.[7,8] Punicalagin (PU) is an ellagitannin polyphenol of pomegranate (*Punica granatum*) which has potent antioxidant and anti-inflammatory properties. The protective role of PU has been demonstrated in several studies. For instance, PU inhibits inflammation in lipopolysaccharide (LPS)-activated microglia[9] and RAW264.7 macrophages[10], and protects against ischemia/reperfusion-induced myocardial injury.[11] PU treatment has also been proved to alleviate cerebral ischemia-reperfusion in rats[12], methionine-induced hyperhomocysteinemia in mice[13]and glutamate-induced oxidative toxicity in mouse hippocampal cell line (HT22).[14] However, the recuperative effects of PU against high-calorie diet-induced oxidative stress and pathological alterations in brain of mice have not been studied so far. Therefore, the present study was undertaken to explore whether PU supplementation could ameliorate high fat-high fructose diet (HFFD)-induced oxidative changes and pathological alterations in mice brain. The results of the study will confirm the detrimental effects of HFFD on the degenerative changes in brain and the importance of including pomegranate intake as a source of PU.

2. MATERIALS AND METHODS

2.1 Chemicals, antibodies, primers and kits

Fructose was acquired from SFA Food and Pharma Ingredients Pvt. Ltd., Thane, Maharashtra and casein was obtained from Clarion Casein Pvt. Ltd., Kadi, Gujarat. PU was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Antibodies against 4-hydroxynonenal (4-HNE) and 8-hydroxyguanosine (8-OHG) were purchased from Merck (Calbiochem), Darmstadt, Germany, respectively. Antibody for 3-nitrotyrosine (3-NT) was purchased from Invitrogen, MD, USA. Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was purchased from Cell Signaling Technologies, Danvers, MA, USA. HRP-tagged anti-mouse and anti-goat secondary antibody was purchased from Genei Laboratories Pvt Ltd, Peenya, Bengaluru, India. Solvents and all other chemicals used for the study were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India unless otherwise stated.

2.2 Ethics statement

Male Swiss albino mice were purchased from Biogen Laboratory Animal Facility, Bengaluru, India. Animals were kept in polypropylene cages and maintained under standard housing conditions (room temperature 22-24 ˚C) with 12:12 light/dark cycle) and given access to food and water *ad libitum*. Animals were maintained according to the Animal Care and Use Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), in the Central Animal House, Unit of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai Nagar. Experimental protocols were approved by the Institutional Animal Ethical Committee (IEAC), Annamalai University [Reg.no.160/1999/CPCSEA, Proposal No: 1133].

2.3 Animal maintenance and treatment schedule

The animals were allowed to acclimatize for a week after which they were divided into five groups consisting of 6 mice each (n=6). They were fed normal diet or HFFD for a period of 60 days. The animals were grouped and treated as follows: Group 1: Control; Group 2: HFFD; Group 3: HFFD+PU (30 mg/kg bw); Group 4: HFFD+PU (60 mg/kg bw) and Group 5: Control+PU (60 mg/kg bw). HFFD was freshly prepared every week in our laboratory using 45% fructose, 20% fat (10% beef tallow, 10% groundnut oil) and 22.5% casein, providing 471.25 cal/100 g. The pellet feed had 60% starch, 22.08% protein and 4.38% fat, providing 382.61 cal/100 g. PU treatment was given *via* oral gavage into esophagus once daily from 46th day till 60th day (last 15 days of the experimental period). At the end of the experimental period, mice were weighed, fasted overnight, deeply anesthetized the next day *via* ketamine injection (30 mg/kg bw, intramuscular injection) and euthanized. Blood was collected from overnight fasted animals (n=6) in EDTA containing tubes through retro-orbital venous puncture. The tubes were then centrifuged at 1500 rpm for 15 minutes to obtain plasma for biochemical analyses. Liver and brain tissues were immediately excised under sterile conditions into a dish kept over ice cubes containing cold phosphate buffered saline (PBS) (pH 7.4). The dissected liver tissues were photographed to analyze gross morphological variations and then the liver and brain tissues were processed for histopathology analysis.

2.4 Biochemical estimations

Oxidative damage to lipids and proteins was evaluated by measuring the levels of thiobarbituric acid reactive substances (TBARS)[15] and advanced oxidation protein products (AOPP)[16] respectively in plasma. TBARS react with thiobarbituric acid (TBA) under acidic conditions generates a pink coloured chromophore which was measured colorimetrically at 535 nm and the values were expressed in µmoles/dL in plasma.The total antioxidant ability was determined in plasma by ferric reducing antioxidant power (FRAP) assay[17]. FRAP assay is based on the reduction of the Fe^{3+} TPTZ (2,4,6-tri-(2-pyridyl)-s-triazine) complex to the ferrous form by antioxidants at low pH. The reduced ferrous form was measured at 593 nm.

2.5 Hematoxylin and eosin staining

Liver and brain tissues (n=3 from each group) were fixed in freshly prepared 10% neutral buffered formalin, and processed for paraffin embedding. The coronal sections of

mice brain (4 µm) were exposed to varying concentrations of ethanol and washed in distilled water. The sections were then immersed in PBS for 5 min, stained with hematoxylin and counterstained with eosin for 15 min each at 37 °C. Sections were gradually dehydrated and mounted with cover slips. Four images (40×) from liver and coronal brain sections (n= 3, tissue sections from three animals in each group) were analyzed. Images were captured using an Olympus microscope (CX41, Tokyo, Japan) with objective lens (40×) and eye piece (10×) to provide a total magnification of 400 ×.

2.6 Chromogenic immunohistochemical analysis

The paraffin sections of mice brain were deparaffinized, treated with alcohol and washed in distilled water. The slides were boiled for 20 min in citrate buffer (pH- 6.8) containing Tween-20 for antigenic epitope retrieval. The sections were then washed in Tris buffered saline (TBS) and treated with 3 % H_2O_2 for 10 min to eliminate endogenous peroxidase. After incubation, the brain sections were treated for 20 minutes with blocking solution (3% BSA in TBS-Tween-20) and then incubated at 4 °C overnight with primary antibodies for 3-NT, 4-HNE, 8-OHG and IgG (1:200). Next day, the slides were washed in TBS thrice and incubated for 2 h at room temperature with HRP-conjugated anti-rabbit, anti-goat or anti-mouse secondary antibody. Brain sections were then washed in TBS, treated with DAB chromogen and incubated for 10 minutes in dark. After washing in TBS, all the tissue sections were counterstained with hematoxylin. Photomicrographs were obtained at 400× using an Olympus microscope (CX41), with a 40× objective and 10× eyepiece. The area of DAB-staining was measured using Image J software and expressed as percentage. The values are means of four images from sections obtained from animals in each group (n=3).

3. STATISTICAL ANALYSIS

Results were analyzed using SPSS software, version 20 (SPSS Inc., Chicago, IL, USA) and comparisons were made using One Way ANOVA followed by Tukey's post hoc test. The values represent mean ± SD (n=6 for biochemical estimations). Percentage area of immunoreactivity was obtained using Image J software (National Institute of Health Bethesda, MD, USA) and the values represent mean ± SD of four images from cortex and hippocampus. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1 PU ameliorates HFFD- induced histopathological changes

Gross examination of HFFD-fed mice liver revealed pale colour due to the presence of fat deposits within hepatocytes, whereas the tissues from control and treatment groups displayed brown to reddish brown colour. Microscopic examinations showed occurrence of micro- and macrovesicular steatosis, ballooning degeneration of hepatocytes and inflammatory infiltration in HFFD-fed mice. In HFFD mice administered PU the manifestation of fatty infiltration (intracytoplasmic triglycerides/neutral fats) was reverted. Treatment with high dose showed features of mild microvesicular steatosis when compared to low dose and the liver tissues from control and control+PU group showed normal morphology of central vein and hepatic architecture (Figure 1). The impact of HFFD-induced metabolic abnormalities on the integrity of cerebral cortex and hippocampus was analyzed. Interestingly, we observed marked degenerative changes in cortex and hippocampus after 60 days HFFD feeding. Tissues of HFFD mice display shrunken degenerating neurons, pyknotic nucleus, foci of mononuclear inflammation, reactive gliosis, vascular congestion and vacuolation. Cellular damage inflicted by HFFD was alleviated by PU treatment (30 and 60 mg/kg bw), and the brain sections resemble that of control+PU (60 mg/kg). Control (group 1) and Control animals treated with PU (group 5) show compact layer of brain cells with normal nuclei structure (Figure 2)

4.2 PU relieves HFFD-induced oxidative stress and improve FRAP

The status of oxidative stress in plasma was assessed by measuring the indices of lipid peroxidation and protein oxidation. The levels of TBARS and AOPP were significantly increased in plasma of HFFD mice when compared to control mice. PU treatment to HFFD mice for 15 days significantly decreased the levels of TBARS and AOPP when compared to mice fed HFFD alone (Table 1). Among the two PU-treated groups, the high dose of PU (60 mg/kg bw) was observed to exert more pronounced effect than the low dose of PU (30 mg/kg bw). Control and Control+PU group did not show significant difference between the groups. FRAP was found to be decreased in HFFD mice when compared to control mice. PU treatment to HFFD mice at 30 and 60 mg/kg considerably improved FRAP, however the high dose of PU was comparably more effective than the low dose. Control+PU group showed higher values in FRAP assay when compared to control group, however no significant difference was observed between the groups (Table 1). Since the high dose of PU showed profound effects on plasma oxidative stress markers and histopathology examinations, we used high dose of PU for further experiments in brain tissue.

4.3 PU rescues oxidative stress in brain tissues of HFFD mice

Oxidative stress in brain tissues was evaluated by visualizing the DAB-peroxidase reaction adducts for 3-NT, 4-HNE and 8-OHG by immunohistochemistry. The immunoreactivity (DAB-staining) for 3-NT was notably increased in cortex (Figure 3B) and hippocampus (Figure 3F) of HFFD mice when compared to cortex (Figure 3A) and hippocampus from control mice (Figure 3E). In PU-treated HFFD-mice, the immunohistochemical localization of 3-NT was found to be decreased in cortex (Figure 3C) and hippocampus (Figure 3G) when compared to mice-fed HFFD alone. No significant difference observed between Control and Control+PU (Figure 3D, 3H) groups. The presence of 4-HNE was intense in cortex (Figure 4B) and hippocampus (Figure 4F) from HFFD group, when compared to the cortex (Figure 4A) and hippocampus (Figure 4E) from control group. In PU-treated HFFD-mice, the presence of 4-HNE adducts was found to be reduced in both cortex (Figure 4C) and hippocampus (Figure 4G) when compared to mice fed HFFD alone. No significant difference was observed between Control and Control+PU (Figure 4D, 4H) groups. The localization of 8-OHG was found to be increased in cortex (Figure 5B) and hippocampus (Figure 5F) of HFFD mice when compared to cortex (Figure 5A) and hippocampus (Figure 5E) from control mice. Cortex (Figure 5C) and hippocampus (Figure 5G) from PU-treated

HFFD mice showed significant reduction in the intensity of DAB when compared to sections from HFFD mice. Our results confirm that HFFD consumption for 60 days not only induces systemic oxidative stress but also develops oxidative stress in brain.

4.4 PU protects BBB integrity in HFFD mice

To study oxidative stress-induced blood brain barrier (BBB) damage, we have done immunohistochemical analysis for IgG. Intense DAB-staining pattern for IgG extravasation to brain was observed in HFFD mice, indicating the loss of integrity of BBB. In control mice there was rare or no IgG immunoreactivity. Upon treatment with PU, the presence of IgG was notably decreased signifying that PU could recuperate BBB damage (Figure 6).

Table 1 Measurement of oxidative stress markers in plasma

Parameters	Control	HFFD	HFFD+PU30	HFFD+PU60	Control+PU60
TBARS (µmol/dL)	0.68±0.03	2.47±0.21$^{\#}$	1.88±0.07$^{\#,\clubsuit}$	0.88±0.07$^{\#,\clubsuit,*}$	0.71±0.04
AOPP (µmol/L)	84.3±5.0	135.1±11.6$^{\#}$	116.8±6.7$^{\#,\clubsuit}$	97.8±5.9$^{\#,\clubsuit,*}$	85.2±5.9
FRAP (µmol/L)	936±48.3	578±32.5$^{\#}$	775±50.1$^{\#,\clubsuit}$	856±48.1$^{\#,\clubsuit,*}$	945±49.3

Values are means ± SD of six mice from each group. One way ANOVA followed by Tukey's post hoc test. p<0.05 were considered as statistically significant. HFFD = high fat-high fructose diet; HFFD+PU30 = high fat-high fructose diet + punicalagin (30 mg/kg bw); HFFD + PU60 = high fat-high fructose diet + punicalagin (60 mg/kg bw); Control + PU60 = Control + punicalagin (60 mg/kg bw). $^{\#}$Significant vs Control; $^{\clubsuit}$Significant vs HFFD; *Significant vs HFFD+PU30

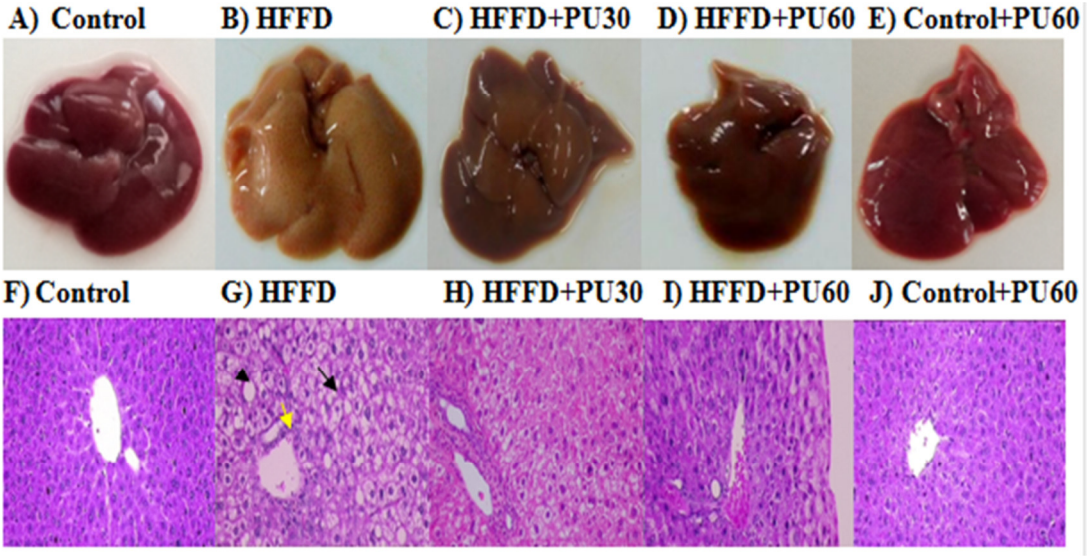


Fig 1. Representative images of the gross morphology of liver (A-E). HFFD-fed mice liver (B) show pale colour due to the presence of fat deposits within hepatocytes, whereas the tissues from control (A) and treatment groups (C,D,E) display brown to reddish brown colour. Representative images of Hematoxylin and eosin stained liver (40×) (F-J). HFFD group show fatty changes and inflammatory infiltration (G). PU-treated HFFD groups (H, I) shows reduction in number and size of fat vacuoles within hepatocytes. Control (F) and Control+PU (J) group show normal liver architecture. Microvesicular steatosis (black arrow), macrovesicular steatosis (arrow head) and inflammatory cells (yellow arrow).

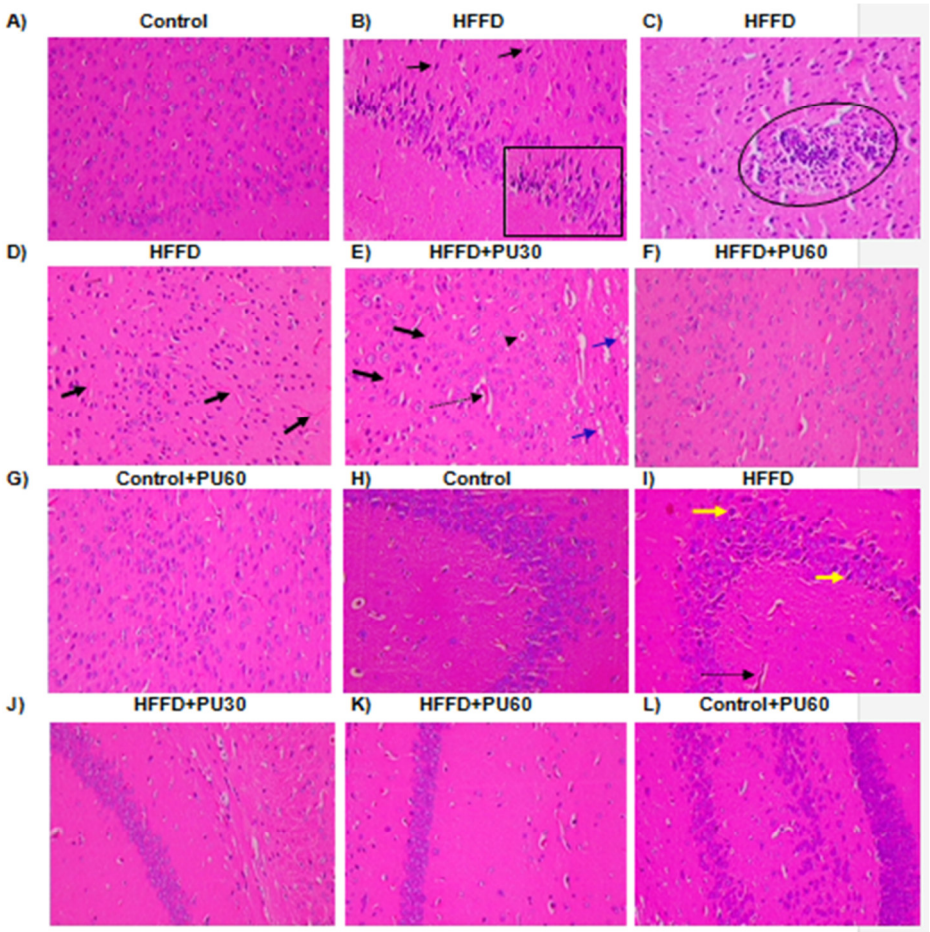
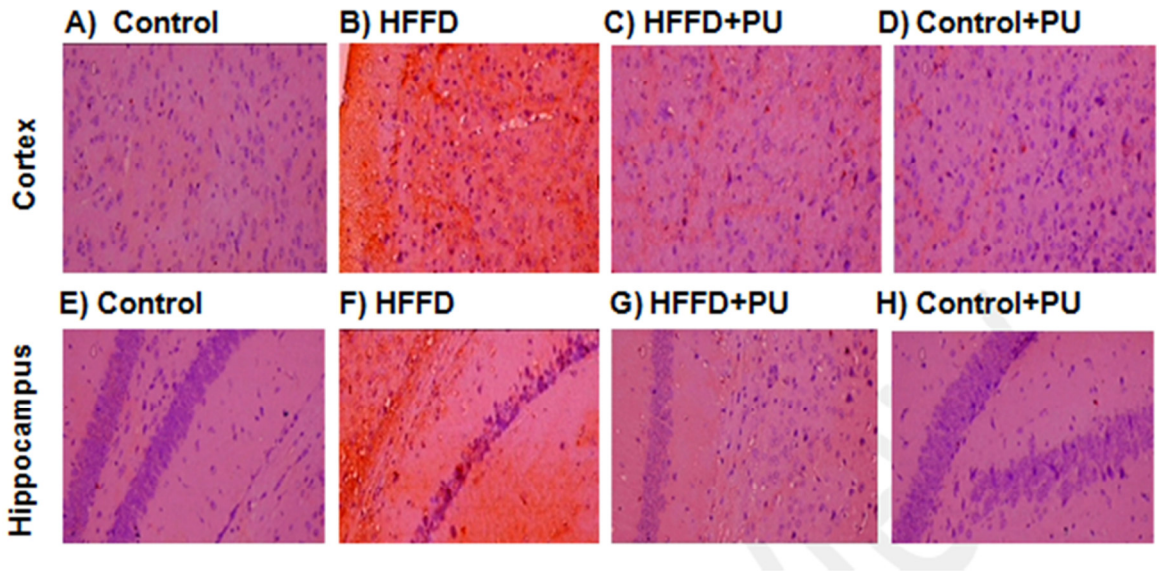


Fig 2. Hematoxylin and eosin staining of cortex (40×) (A-G) and hippocampus (H-L). Sections from HFFD mice show shrunken neurons with dark nuclei (picture insert), degenerating neurons (black thin arrow), inflammatory foci of mononuclear cells (area marked in circle), reactive gliosis (D), active microglia (black thick arrow), pericellular hallos (arrow head), vacuolation (blue arrows), vascular congestion (black dotted arrows), dark neurons (yellow thick arrows) (B,C,D,I). HFFD mice treated with PU show few vacuolations in cytoplasm, pyknotic nuclei and degenerating neurons (E,F,J,K). Control (A,H) and Control+PU treated groups (G,L) show cells with normal nuclei showing dispersed chromatin and prominent nucleoli.



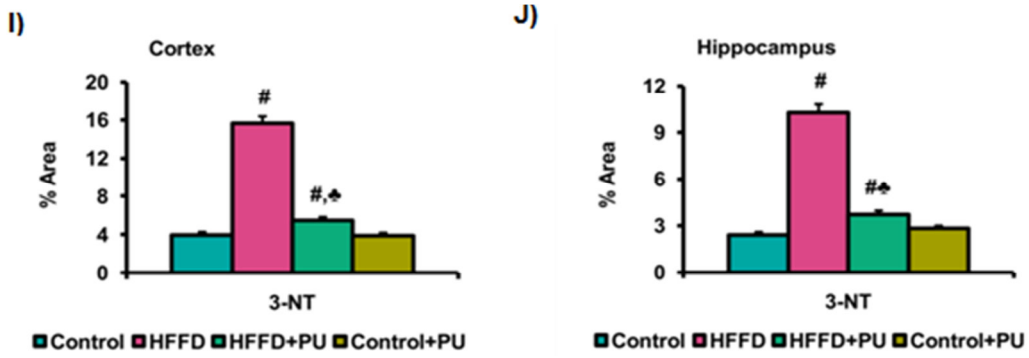


Fig 3. Representative images for immunohistochemical localization of 3-NT in cortex (A-D) and hippocampus (40×) (E-H). The brown peroxidase-DAB reaction product indicates the presence of 3-NT adducts. Percentage area occupied by 3-NT immunoreactivity in cortex (I) and hippocampus (J) was obtained using Image J software. Mean ± SD of 4 view fields/coronal brain section [n = 3, tissue sections from three animals in each group]. One way ANOVA followed by Tukey test, $p<0.05$. #Significant *vs* Control; ♣Significant *vs* HFFD.

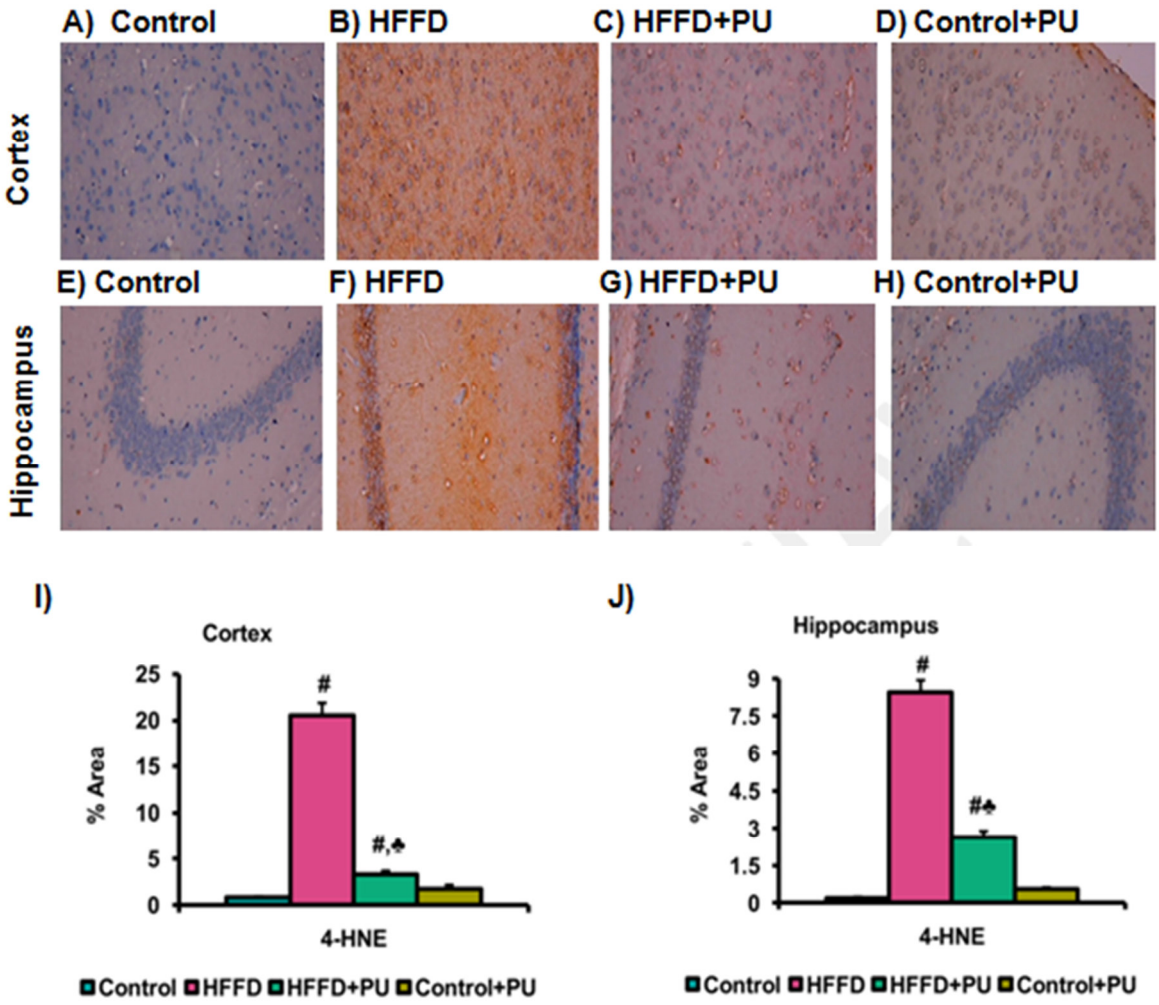


Fig 4. Representative images for immunohistochemical localization of 4-HNE in cortex (A-D) and hippocampus (40×) (E-H). The brown peroxidase-DAB reaction product indicates the presence of 4-HNE adducts. Percentage area occupied by 4-HNE immunoreactivity in cortex (I) and hippocampus (J) was obtained using Image J software. Mean ± SD of 4 view fields/coronal brain section [n = 3, tissue sections from three animals in each group]. One way ANOVA followed by Tukey test, $p<0.05$. #Significant *vs* Control;♣Significant *vs* HFFD.

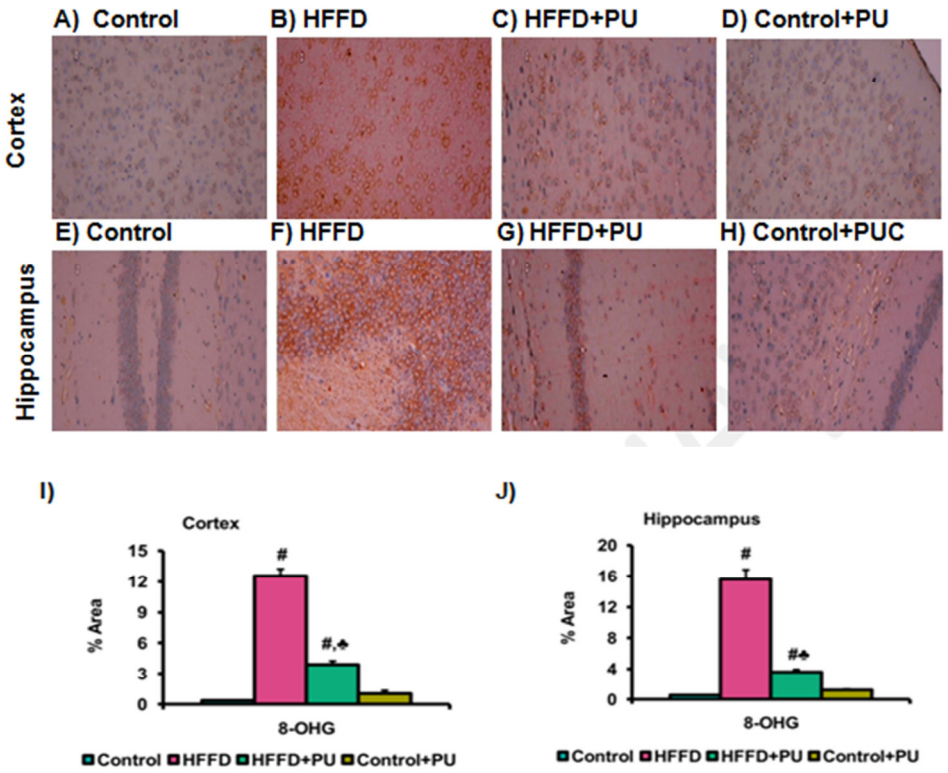


Fig 5. Representative images for immunohistochemical localization of 8-OHG in cortex (A-D) and hippocampus (40×) (E-H). The brown peroxidase-DAB reaction product indicates the presence of 8-OHG adducts. Percentage area occupied by 8-OHG immunoreactivity in cortex (I) and hippocampus (J) was obtained using Image J software. Mean ± SD of 4 view fields/coronal brain section [n = 3, tissue sections from three animals in each group]. One way ANOVA followed by Tukey test, p<0.05.#Significant *vs* Control; ♣Significant *vs* HFFD.

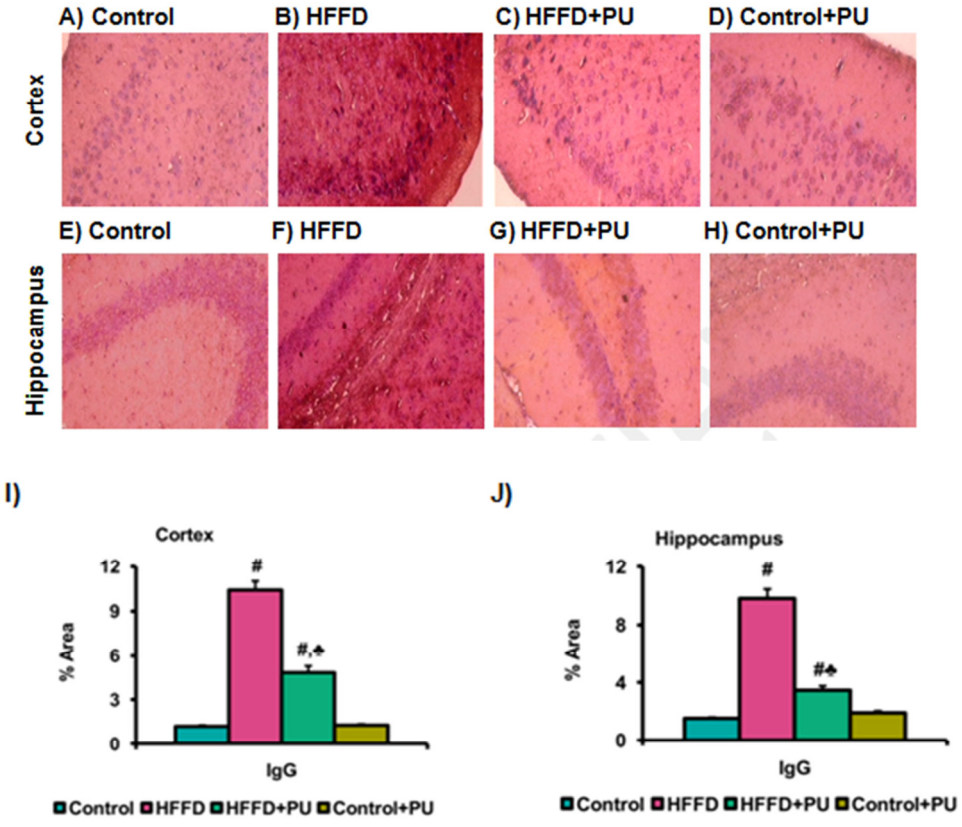


Fig 6. Representative images for immunohistochemical localization of IgG in cortex (A-D) and hippocampus (40×) (E-H). The brown peroxidase-DAB reaction product indicates the presence of IgG extravasation. Percentage area occupied by IgG immunoreactivity in cortex (I) and hippocampus (J) was obtained using Image J software. Mean ± SD of 4 view fields/coronal brain section [n = 3, tissue sections from three animals in each group]. One way ANOVA followed by Tukey test, p<0.05. #Significantvs Control; ♣Significant *vs* HFFD.

5. DISCUSSION

The major findings of the present study are (i) PU suppresses oxidative stress in brain tissues of HFFD mice and improves BBB function, and (ii) PU offers protection against diet-induced pathological changes through its antioxidative effects. HFFD is known to induce lipid abnormalities[18] and incite damage to peripheral tissues such as liver, kidney and heart.[19-21]Severe pathological changes and existence of a metainflammatory state in liver and kidney tissues of HFFD mice was observed in this study. Non-alcoholic fatty liver (NAFLD) is associated with altered cerebral volume in middle aged adults[22] and induce pathological signs of AD in both wild type and amyloid precursor protein transgenic mice.[23]In our study, HFFD feeding for 60 days elicited pathological alterations in brain, confirmed by the presence of degenerating neurons showing shrunken cytoplasm and pyknotic nuclei along with fatty liver. Chronic oxidative injury results in neurodegeneration. The effect of diet enriched with both fat and fructose in brain oxidative stress has been reported.[24]In this study, the development of oxidative stress in brain tissues of HFFD mice was evident from the presence of adducts of oxidative damage to proteins (3-NT), lipids (4-HNE) and nucleic acids (8-OHG) in cortical and hippocampal regions. The BBB is a special microvessel structure in the central nervous system, which protects the brain from blood-borne substances. Oxidative stress during nutrient excess can increase BBB permeability that precedes neurodegeneration.[25]Extravasation of IgG and albumin to brain serves as an index of alterations in vascular permeability.[26,27] The increased extravasation of blood-borne IgG to brain of HFFD mice signifies loss of BBB integrity which can be attributed to increased oxidative stress. PU is a polyphenol found in the rind, peel, seeds and fruit juices of pomegranate at varying concentrations. Traditionally, the fruit is used for the treatment of various diseases such as ulcers, fever, diarrhea, and microbial infections.[28] Studies documenting the protective effects of PU in both *in vitro* and *in vivo* models has been emerged in recent years. PU show high absorption rate of up to 95% and into smaller polyphenols such as ellagic acid which is further metabolized by colonic microflora. PU can be a potent antioxidant in living systems due to its water-soluble nature and hence could be a promising nutraceutical candidate. PU treatment to SH-SY5Y cells prior to 6-hydroxydopamine exposure inhibits oxidative stress and restores mitochondrial function.[31]PU alleviates doxorubicin-induced toxicity in H9c2 cardiomyoblasts[32] and ameliorates palmitate-induced lipotoxicity in HepG2 cells[33] *via* Nrf2 antioxidant defence system. The antioxidant and hepatoprotective effects of PU in acetaminophen-induced rats have also been reported.[34] PU-administration to HFFD mice improved hepatic and brain architecture, whereas no pathological signs were exhibited by the control group treated with PU. Further, we observed a marked decrease in the levels of oxidative stress markers in plasma as well as brain tissue which signify the anti-oxidative properties of PU.

6. CONCLUSION

Taken together, our findings suggest that PU administration curtails HFFD induced oxidative stress and pathological changes in mice brain. PU exerts beneficial effects by preserving blood brain barrier function. Hence, PU supplementation may be recommended to prevent or delay the detrimental consequences of HFFD as well as the associated neurodegenerative changes.

7. AUTHORS CONTRIBUTION STATEMENT

C.V.A. and C.S.P. conceived and designed the experiments. C.S.P. and R.V. performed the experiments. C.S.P. and K.J. analyzed the data. C.V.A. and C.S.P. contributed to the writing of the manuscript.

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9. CONFLICTS OF INTEREST

Conflict of interest declared none.

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