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Antidiabetic Effects of Caffeic Acid on the Hippocampus of Fructose/Streptozotocin-Induced Diabetic Wistar Rats

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ABSTRACT

Diabetes mellitus is a metabolic disease that causes oxidative and inflammatory damage on the hippocampus amongst many other tissues of the body. Recent reports from developing countries show a steady increase in diabetes due to sedentary lifestyle changes, diet and weight gain. This study aimed to investigate the potential of caffeic acid (CA) as a possible treatment of oxidative, inflammatory and neurodegenerative pathology of the hippocampus in Fructose-Streptozotocin diabetic Wistar rats. Twenty (20) normoglycemic male adult Wistar rats weighing roughly 200g were used for this study. They were randomly divided into 4 groups; Control, which were given 0.1M citrate buffer daily, Fructose-Streptozotocin, which were given a single intraperitoneal injection of 50mg/kg Streptozotocin following 2 weeks of 10% fructose *ad libitum*, Fructose-Streptozotocin +CA, which were also administered the same as the aforementioned group and treated with 50mg/kg of caffeic acid daily and caffeic acid group which was treated with 50mg/kg of caffeic acid also daily. Biochemical assays showed increase in antioxidants (total thiol and protein thiol) and upregulation of anti-inflammatory markers (Interleukin-4 and Interleukin-10) in the Fructose-Streptozotocin + caffeic acid compared to the Fructose-Streptozotocin. Amyloid plaques were also observed to be lesser in the treated group relative to the untreated group. Results indicate that caffeic acid exhibits its therapeutic action mainly by upregulating protective thiol antioxidants and anti-inflammatory cytokines (IL4 and 1L10).

Keywords: Diabetes Mellitus, Hippocampus, Caffeic acid, Fructose, Streptozotocin.

INTRODUCTION

Diabetes mellitus (DM) is an inveterate pathology of the metabolic system resulting from complete or relative insulin deficiency or action.[1] The high prevalence of this disease in developed countries has been firmly established, but recent reports from developing countries also show a steady increase in diabetes due to sedentary lifestyle changes, diet and weight gain.[2,3] Around 19 million adults in Africa are living with diabetes and this number is expected to reach 47 million by 2045.[4]

Among the multi-systemic complications of DM, mild cognitive impairments and cognitive decrements have been noted from adolescence to adulthood in many cases. Although the pathophysiological mechanism remains shrouded, DM has been implicated in increasing the risk for developing dementia especially for those above 65 years.[5] Specifically, the impairment of brain glucose uptake and metabolism associated with DM has been shown to play a role in the neurodegenerative progression of Alzheimer's disease.[6] In fact, research has shown an increased risk (up to 5 times more prone) of DM patients developing Alzheimer's disease in the future and implicated the role of pancreatic amylin in the deposition of beta amyloid plaques in the hippocampus.[7] Furthermore, neuronal oxidative damage resulting from increased lipid peroxidation is also seen in DM due to high levels of circulating lipids and glucose.[6,8] In addition, Streptozotocin-diabetic rats also show high expression of tumorigenic biomarkers e.g (Lnc h19), that induces hippocampal neuronal apoptosis.[9,10]

In treatment of diabetes, herb based orthodox medicine have gotten a wide degree of attention. Caffeic acid

(CA) is a naturally occurring polyphenolic compound gotten primarily from propolis extracted from honeybee hives and also the bark of coniferous trees.[11] The chronic hyperglycemia seen in DM causes significant oxidative stress on cells and eventually leads to irreversible damages on tissues. Moreover, in diabetes the oxidative stress also concomitantly leads to decreased antioxidant defenses like catalase and glutathione.[11] CA is a free radical scavenger that proves to be useful in alleviating oxidative stress and reducing reactive oxygen and nitrogen species (RONS). Coupled with its anti-inflammatory properties, it is a promising treatment used for various neurodegenerative disease models in research.[12] Caffeic acid has also been reported to reduce the oxidative stress caused by environmental pollutants like Nickel,[13] carbon tetrachloride, cisplatin, and cadmium.[14] Furthermore, Ho *et al.*[15] found that CAPA (Caffeic acid phenyl amide), a derivative of caffeic acid improved coronary artery dilation in normal and diabetic rats and ameliorated vascular dysfunction in diabetic rats. These effects added with its strong antioxidant properties make caffeic acid a suitable treatment for diabetes.

MATERIALS AND METHODS

Animal care and management: Twenty normoglycemic (64 ± 15 mg/dl-fasting blood glucose) adult male Wistar rats weighing 236 ± 38g were purchased from the animal house of the Department of Anatomy at Ekiti State University, Ekiti, Nigeria. They were transported to the animal house of the anatomy department at the Federal university of Technology Akure, Ondo state, Nigeria where they were housed in polycarbonate cages at a controlled room temperature of about 25°c with a 12h/12h light dark-cycle. All animals were acclimatized for 2 weeks during which they had free access to standard rat chow and tap water.

Experimental Design: Two (2) random sets of 5 rats each were selected for the Control group and Caffeic acid (CA) group respectively, while experimental diabetes was induced in the other 10 by giving 10% fructose solution ad libitum for two weeks followed via a single intraperitoneal injection of 50mg/kg of streptozotocin (STZ). Diabetes was confirmed over 3 days by fasting glucose levels > 250mg/dl after which the rats were divided into two groups of 5: Fructose-Streptozotocin (FRUC-STZ) and Fructose-Streptozotocin + Caffeic Acid (FRUC-STZ +CA). The rats were randomly divided into the following experimental groups:

i. Control = 2ml citrate buffer
ii. Fructose-Streptozotocin (FRUC-STZ) = 2 weeks 10% fructose solution *ad libitum* + 50mg/kg body weight STZ
iii. Fructose-Streptozotocin + Caffeic Acid (FRUC-STZ+ CA) = 2 weeks 10% fructose solution *ad libitum* + 50mg/kg body weight STZ + 50mg/kg body weight CA
iv. Caffeic Acid (CA) = 50mg/kg body weight CA

50mg/kg of caffeic acid was dissolved in distilled water and administered orally. Citrate buffer was administered to the normal control group orally as well. Euthanasia was performed 24 hours after the last dose with the serum being collected for biochemical assay while the whole brain collected for histological evaluation.

Induction of Diabetes: After an acclimatization period of two weeks, the animals were given 10% fructose solution ad libitum for 2 weeks. The rats were then fasted overnight from the last day of fructose administration after which followed a single intraperitoneal injection of STZ at a dose of 50 mg/kg body weight on the 15th day. The STZ was dissolved in 0.1M citrate buffer at pH 4.5. The 0.1M citrate buffer was prepared by dissolving 2.94g of sodium citrate and 2.1g of citric acid in 100ml of distilled water. The pH was buffered to 4.5 by adding adequate volumes of concentrated NaOH/HCl. 48 hours after diabetes induction, fasting blood glucose was assessed by testing blood samples from the dorsal tail vein using Accu Check glucometer (Roche) and compatible glucometer strips. The diabetic rats recruited for this study were defined by blood glucose not less than 250mg/dl.[16] Treatment began after confirmation of diabetes. Animal body weight and glucose levels were checked at 3-day intervals.

Ethics statement: Study was conducted in accordance with the Guide for the care and use of laboratory animals under the approval of the Institutional Review Board of FUTA. In addition, all animal procedures in this study were in concordance with the guiding principles for research involving animals as recommended by the declaration of Helsinki and were approved by the Departmental Committee on the Use and Care of Animals. After 14 days of administration, the animals were properly euthanized.

Serum Preparation: Blood samples were collected via cardiac puncture into sterile plain bottles and allowed to stand for 2hours at room temperature to clot. Centrifugation using a C-Golden-wall centrifuge (China) followed at 2500rpm for 20minutes to obtain the sera. The sera obtained from different samples were then aspirated using micropipettes into their respective labelled sterile plastic sample bottles and immediately refrigerated for the use of biochemical assay: Insulin, TNF-α, Protein thiol, Total thiol, Nitric Oxide, Interleukin-4 and Interleukin-10.

Brain Tissue Histology: Hematoxylin and Eosin - The whole brains of the rats were collected and fixed in neutral buffered formalin for 48 hours after which they were moved to 70% alcohol for dehydration. The tissues are passed through 90% and absolute alcohol and xylene for different durations before being transferred into molten paraffin for 1 hour in each oven

at 65° for infiltration. The tissues were embedded and serial sections of 5 microns cut on a rotary microtome. The tissues were picked with albumenized slides and allowed to dry on hot plates for 2 min. the slides were dewaxed with xylene and passed through 3 changes of alcohol from absolute, 90%, 70% alcohol, 50% and then in water for 5minutes. Hematoxylin and eosin staining followed after which the slides were mounted in DPX and photomicrographed using a Leica DM750 microscope.

Brain Tissue Histochemistry: Congo Red - This staining procedure is the gold standard for the demonstration of amyloid deposition in neurodegenerative research. It detects compacted amyloid in a beta sheet structure. Staining procedure: Plates were deparaffinised and hydrated in distilled water. Plates were stained in Congo red working solution for 10 minutes after which they were rinsed in distilled water. Quick differentiation of the plates followed in alkaline alcohol solution. Plates were rinsed in tap water, counterstained in gill's hematoxylin for 30 seconds and rinsed in tap water for 2 minutes. Plates were ten dipped in ammonia water for 30seconds after which they were rinsed, dehydrated, cleared in xylene and mounted.[17]

Cresyl Fast Violet: Gelatinized slides having brain sections were immersed in xylene solution for 15 minutes and then slides were immersed in ascending grades of alcohols in the following order, absolute alcohol - 1 minute, 90 % alcohol – 2 minutes, 70 % alcohol – 2 minutes, 50 % alcohol – 2 minutes. After processing through alcohol, slides were immersed in distilled water for 10 minutes and were stained for 25–30 minutes at temperature 60° in 0.1 % cresyl violet stain and then allowed to cool at room temperature. Stained sections were again immersed in distilled water for 5 minutes and in ascending grades of alcohols (70 %, 90 %) for 2 minutes. Finally, sections were dipped in xylene for clearing and mounted with DPX.

Biochemical Assays: All biochemical assays were carried out using commercial kits. The assay for insulin was done using an ELISA (Enzyme Linked immunosorbent assay) kit by RayBio™. Seruminal pro-inflammatory marker, TNF-α was estimated using the InstantOne™ ELISA kit. The level of anti-inflammatory marker IL-4 was estimated using an ELISA kit from Cloud-Clone Corp. IL-10 was estimated using an ELISA kit from Invitrogen. The level of antioxidants, Protein thiol was estimated using the (Sigma-Aldrich) ELISA kit and Total thiol was estimated using the (Cell Biolabs) ELISA kit. NO (colorimetric) was assayed using the Sigma-Aldrich ELISA kit.

Statistics: Values were recorded as mean ± standard error of mean (S.E.M.). The statistical significance of difference in the mean and standard error of mean at $p < 0.1$ were analyzed by one-way analysis of variance (ANOVA) and the Tukey's test for multiple comparison.

RESULTS

Body Weight: Data for total body weight are expressed in Table 1. The FRUC-STZ group shows the highest decline when comparing the initial weight and final weight. The treatment groups and control group show minimal difference between the initial weight and final weight.

Table 1: Initial and final body weight changes in the experimental animals after a period of 14 days

Groups	Initial weight (g)	Final weight (g)	|Weight difference (g)|
Control	195.7±12.6	204.7±16.7	9.0±4.1
FRUC-STZ	206.8±12.8	167.3±8.2*	39.5±1.6
FRUC-STZ+CA	213.6±9.3	210.2±11.7	3.4±2.4
CA	228.8±12.7	209.6±10.0	19.2±2.7

Values represent mean ± SEM; $N = 5$. * denotes significant decrease at $p < 0.1$ to the Control, FRUC-STZ+CA, and CA groups.

Blood Glucose: Table 2 contains data for the total blood glucose of the animals. The FRUC-STZ group showed a high average final glucose level in the diabetic range (234.0±32.6mg/dL) while the FRUC-STZ+CA (116.3±38.6mg/dL) group showed final blood glucose levels closer to the control (76.8±3.6 mg/dL) group and CA group (72.6±3.7 mg/dL).

Table 2: Initial and final blood glucose changes in the experimental animals after a period of 14 days**.**

Groups	Initial glucose (mg/dL)	Final glucose (mg/dL)	|Glucose difference (mg/dL)|
Control	47.5±2.9	76.8±3.6 #	29.3±0.7
FRUC-STZ	306.3±16.5 *	234.0±32.6	72.3±16.1
FRUC-STZ+CA	340.3±31.0 *	116.3±38.6 #	224.0±7.6
CA	57.2±2.0	72.6±3.7 #	15.4±1.7

Values represent mean ± SEM; N=5. * denotes significant increase at $p < 0.1$ relative to the Control group. # denotes significant decrease at $p < 0.1$ relative to the FRUC-STZ group.

Insulin: Serum insulin levels are displayed in Figure 1. The FRUC-STZ+ CA (1.120 ± 0.075) shows the highest levels of insulin while the FRUC-STZ (0.973±0.074) group shows the lowest level of insulin when compared to the Control (1.112±0.032) and CA (0.979±0.728) groups. There was no significant difference ($p < 0.1$) amongst the groups.

Markers of Inflammation: Tissue Necrosis Factor-Alpha **-** Serum levels of Tissue Necrosis Factor-alpha are represented in Figure 2. Analysis shows the highest levels of TNF α in FRUC-STZ group (27.830±5.709) while the CA group (20.960±1.254) has the lowest levels. There is an observable decrease in the FRUC-STZ+CA (26.670 ± 2.803) group compared to the FRUC-STZ group (27.830 ± 5.709) although this is not statistically significant at $p < 0.1$.

Interleukin-4: Serum levels of IL-4 are represented in Figure 3. The FRUC-STZ + CA group (364.700±39.650) is significantly higher than the FRUC-STZ group (256.100±16.200) ($P < 0.1$). The control (300.200±10.910) and CA group (301.200±19.180) did not vary significantly at $p < 0.1$

Interleukin-10: Serum levels of IL-10 are represented in Figure 4. The FRUC-STZ + CA (256.000±14.810) group showed the highest levels of IL-10 but did not vary significantly at $p < 0.1$ from the Control group (240.800±6.162), FRUC-STZ group (230.700±14.100) and CA group (193.600±9.054).

Anti-oxidant Markers: Total Thiol - The levels of total thiol are represented in Figure 5. There is a significant increase ($p < 0.1$) in the total thiol of the Control group (1.060 ± 0.104) relative to the FRUC-STZ (0.584 ± 0.028), FRUC-STZ + CA (0.694 ± 0.036) and CA group (0.778 ± 0.044). However, the diabetic group had the lowest value relative to the control, therapeutic (FRUC-STZ + CA) and CA group though with no statistical difference at $p < 0.1$.

Protein Thiol: Serum levels of Protein Thiol are represented in Figure 6. The diabetic group had the lowest value relative to the control, therapeutic (FRUC-STZ + CA) and CA group though with no statistical difference at $p < 0.1$. The Control group (0.652±0.145) showed the highest levels relative to the FRUC-STZ (0.492±0.082), FRUC-STZ + CA (0.631±0.058) and CA (0.596±0.095) groups.

Nitric Oxide: Serum levels of Nitric Oxide are represented in Figure 7. The Control group showed the highest levels (6.275±2.009) relative to the FRUC-STZ (2.478±1.109), FRUC-STZ + CA (2.797±1.236) and CA (2.797±1.057) groups. Although this is not statistically significant at $p < 0.1$. The diabetic group had the lowest value relative to the control, therapeutic (FRUC-STZ + CA) and CA group though with no statistical difference at $p < 0.1$.

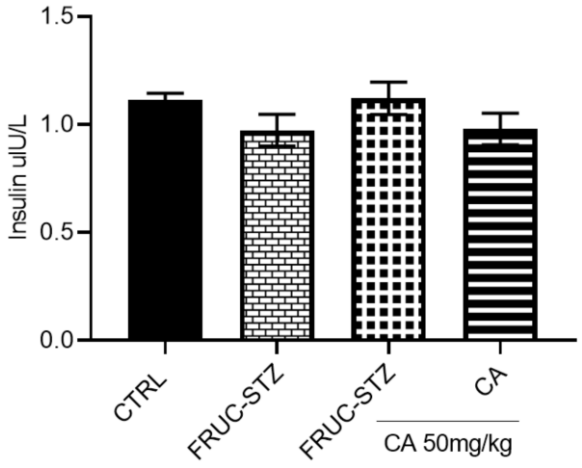

Figure 1: Serum insulin levels

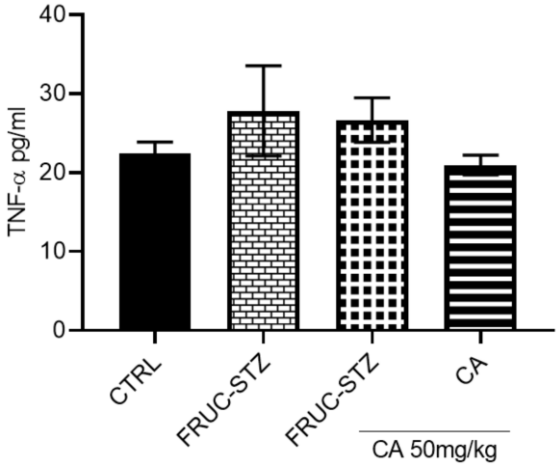

Figure 2: Serum levels of Tissue Necrosis Factor-alpha

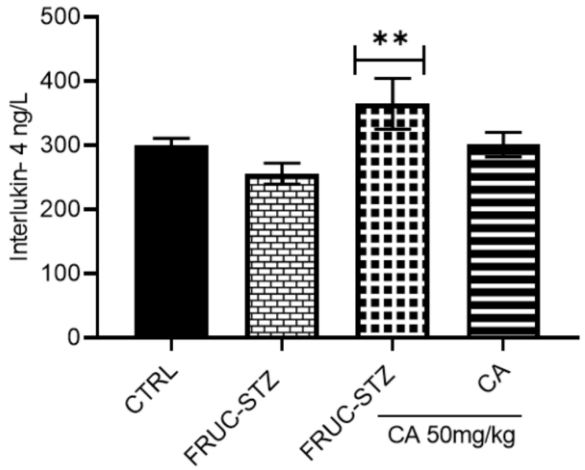

Figure 3: Serum levels of IL-4

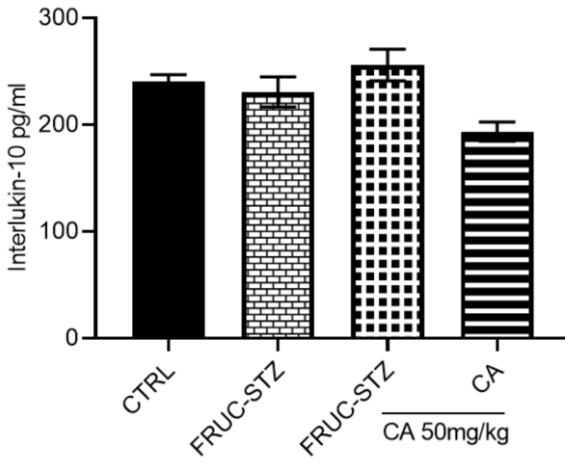

Figure 4: Serum levels of IL-10

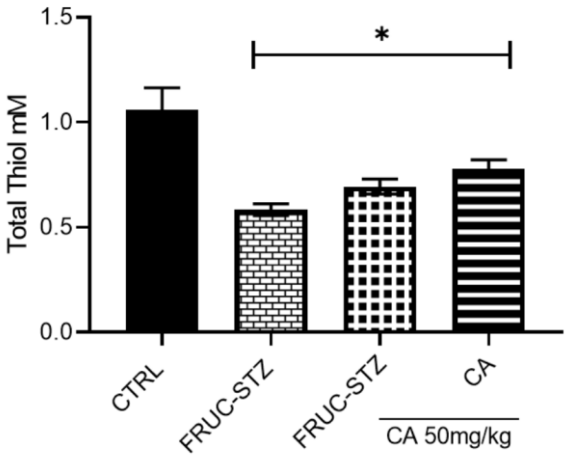

Figure 5: The levels of total thiol

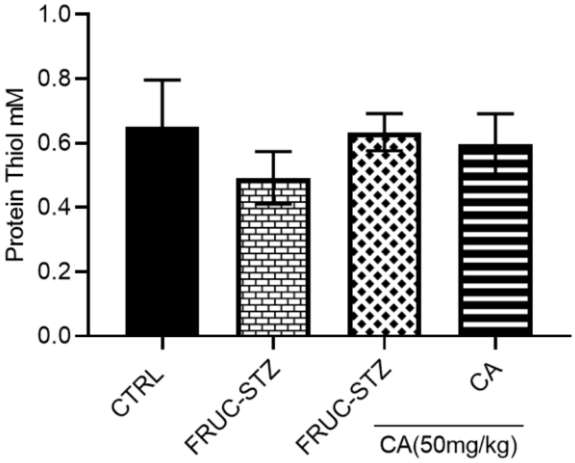

Figure 6: Serum levels of Protein Thiol

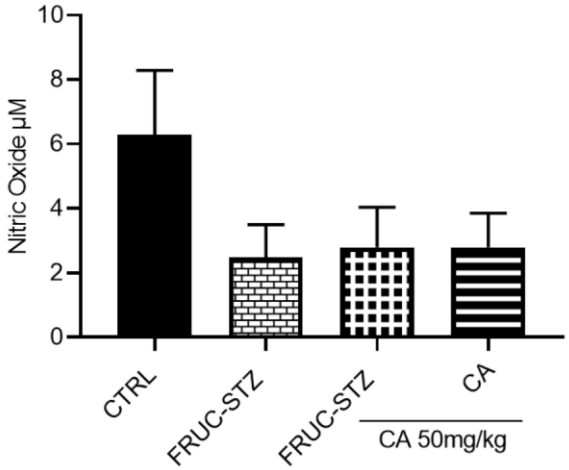

Figure 7: Serum levels of Nitric Oxide

Histological Evaluation: Hematoxylin and Eosin - Figure 8 shows representative photomicrographs of the hippocampus showing the state of neurons in the CA3 subregion. The FRUC-STZ group showed vacoulating and chromatolytic cells compared to the recovery state seen in the therapeutic FRUC-STZ + CA group.

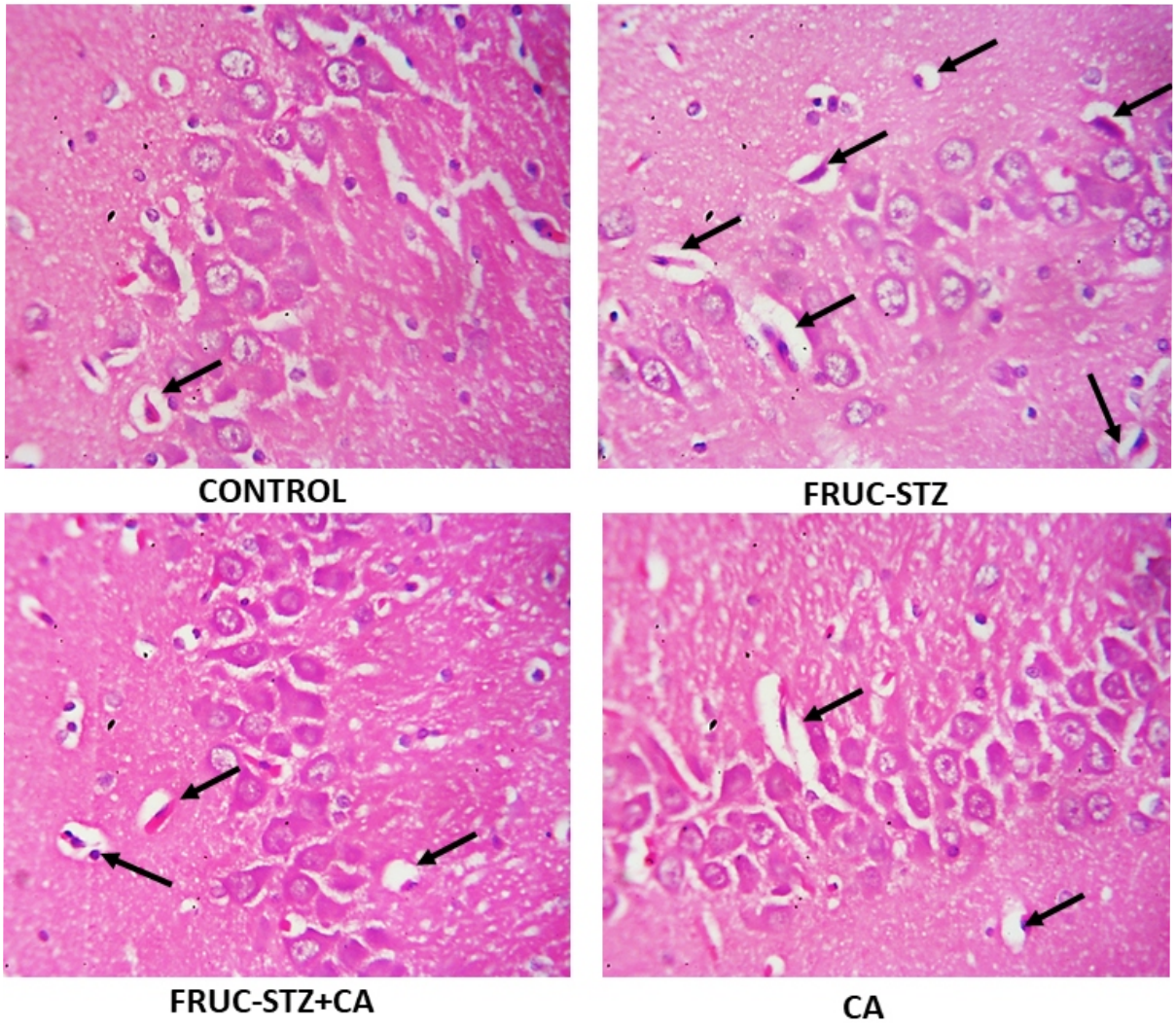

Figure 8: Representative photomicrographs of the hippocampus (H&E)

Congo Red: Figure 9-10 are representative photomicrographs of the hippocampus showing the amount of amyloid beta deposits in the CA3 subregion and parietal cortex. Both the control and CA groups show negligible number of these deposits. The FRUC-STZ group showed numerous amyloid beta deposits (indicated by black arrows) compared to the therapeutic FRUC-STZ +CA group.

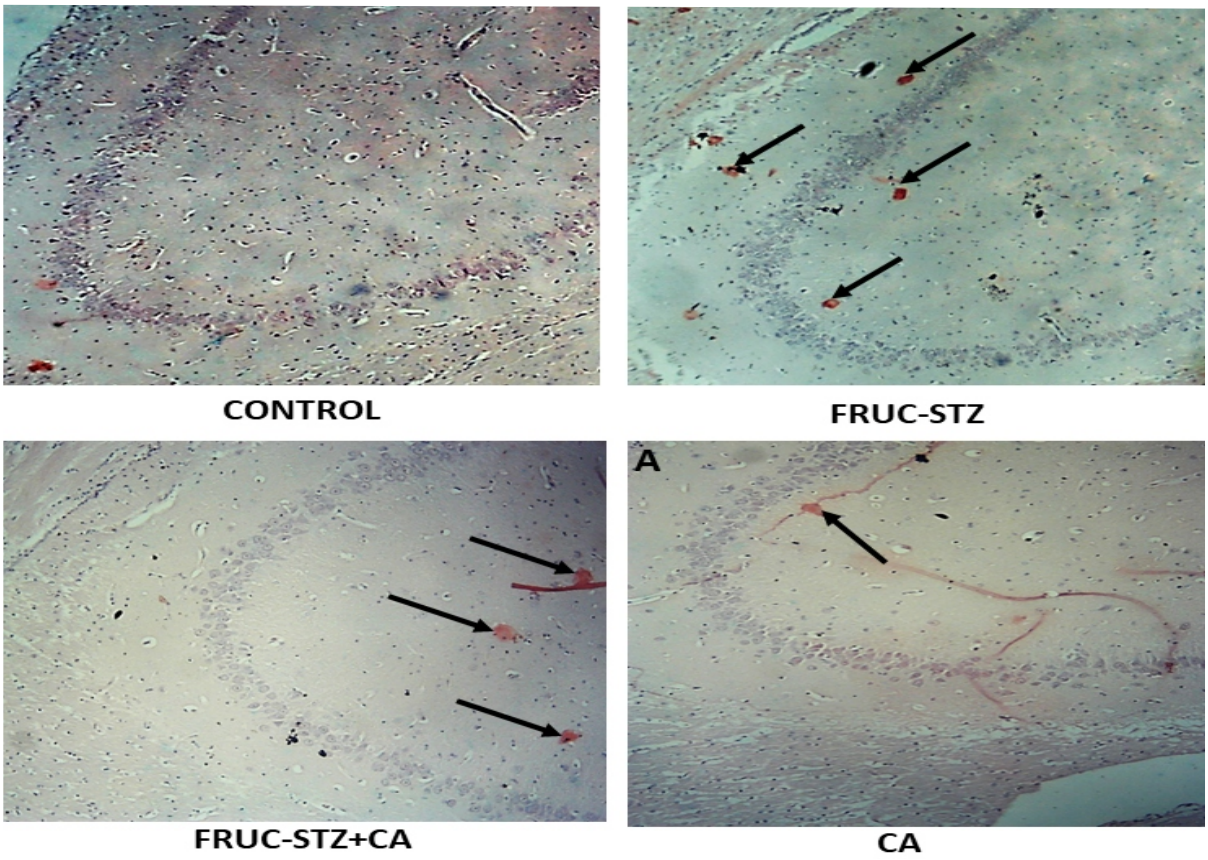

Figure 9: Representative photomicrographs of the hippocampus (Congo Red)

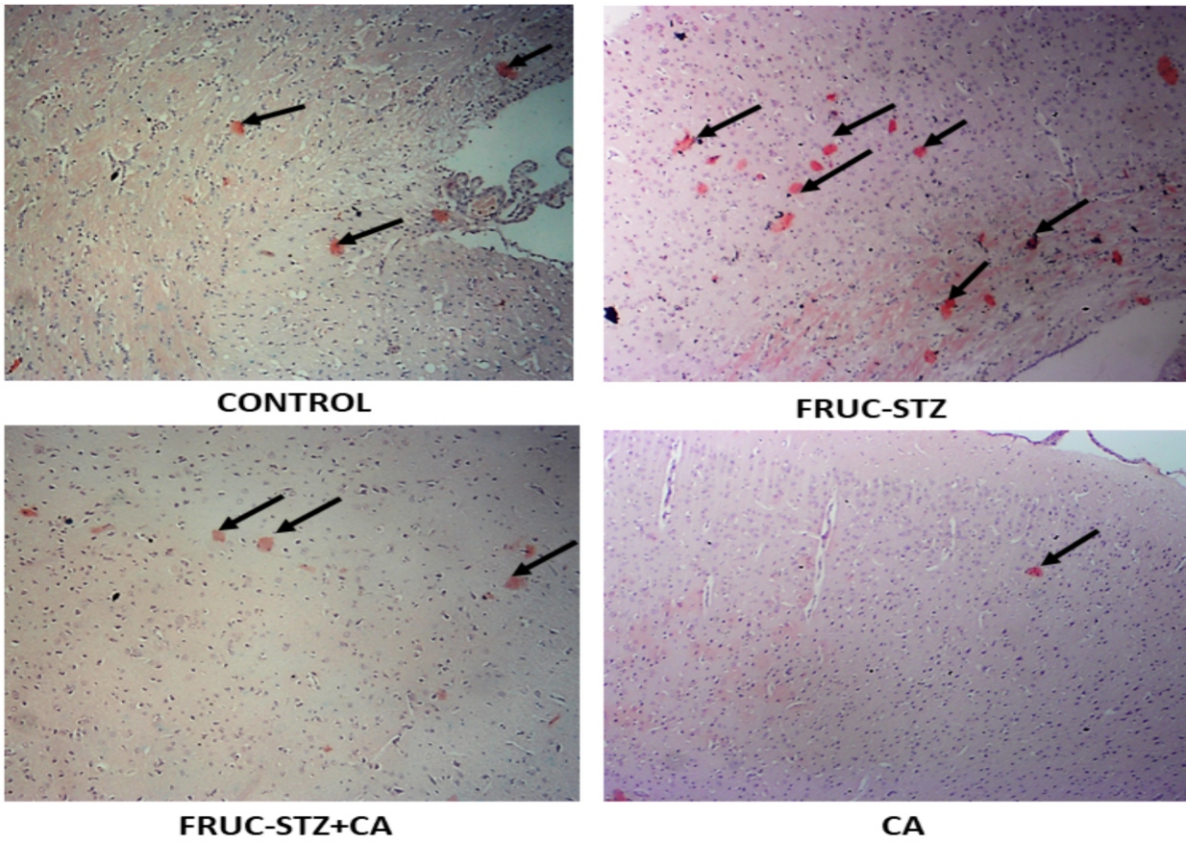

Figure 10: Representative photomicrographs of the hippocampus (Congo Red)

Cresyl Fast Violet: Figure 11-12 are representative photomicrographs of the hippocampus showing the level of cellularity in the CA3 region and pyramidal layer of the cortex. The FRUC-STZ group shows a low degree of cellularity and severe chromatolysis compared to the FRUC-STZ+CA group. There is also an increase in the density of pyramidal cells in the cortex of the therapeutic FRUC-STZ +CA group compared to diabetic control FRUC-STZ group.

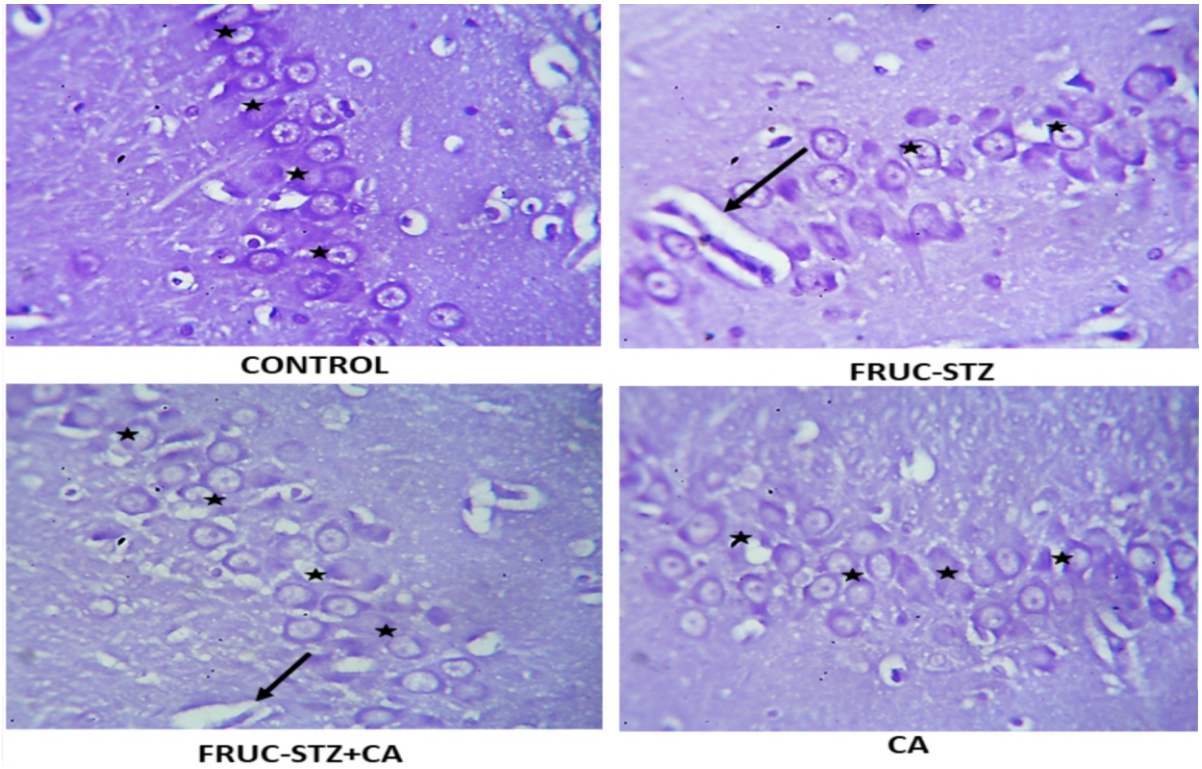

Figure 11: Representative photmicrographs of the hippocampus (Cresyl Fast Violet)

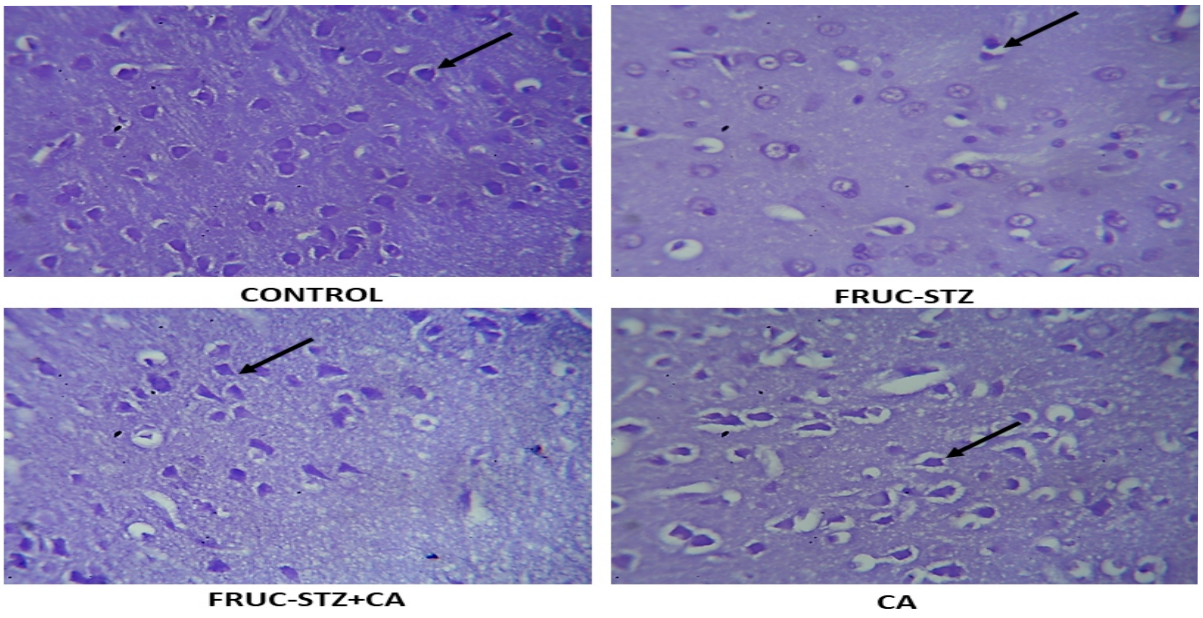

Figure 11&12: Representative photmicrographs of the hippocampus (Cresyl Fast Violet)

DISCUSSION

Diabetes mellitus has grown to become a global problem with improvement of standard of living associated with its increased prevalence. The chronic hyperglcycemia associated with diabetes mellitus has been known to result in multi-systemic complications. Amongst these complications is Diabetic neuropathy which results from a conjugate of reduced neural blood flow, increased oxidative stress and inflammation.[18]

Streptozotocin is widely used to induce hyperglycemia in in vitro animal studies. STZ acts by intermembrane transport via GLUT-2 transporter of pancreatic beta cells to cause DNA methylation and alkylation leading to beta cell death and decreased insulin secretion.[19]

Diabetes was confirmed by Analysis of blood sugar levels of the experimental groups administered Streptozotocin. Both FRUC-STZ and the therapeutic group showed average glucose levels well above the diabetes cut-off at 306.33 and 340.33 respectively after induction. In diabetic states, the body tissues are starved and compensatory mechanisms are activated to avail the body energy for daily functioning. These mechanisms include, lipolysis and glycogenolysis which leads to respective loss in fat and muscle composition, reducing the total body weight. This was evidenced by acute difference between the initial body weight (206g) and final body weight (180g) of the FRUC-STZ group. The level of insulin in FRUC-STZ did not show significant decrease compared to other groups. This can be attributed to hyperinsulinemia which is a usual acute response of the endocrine pancreas to damage.

Caffeic acid was employed as treatment for this experiment. Caffeic acid is the bioactive compound in propolis found in honey beehives and other food items like coffee, tea and vegetables.[20] Many studies have been conducted on its anti-carcinogenic, antiviral and anti-inflammatory properties.[21] Hyperglycemia activates mechanisms of inflammation and oxidative stress by promoting formation of advanced glycation end products (AGEs) and reactive oxygen species. When AGEs bind with their Receptor (RAGE), a sequence of events follow that leads to the activation of transcription factor nuclear factor kappa beta (NF-κB) and generation of reactive oxygen species (ROS).[22] Caffeic acid has been used in experimental studies to ameliorate the oxidative stress and inflammation caused by hyperglycemia. For instance, in a lipopolysaccharide induced neuro inflammatory study by Mallik,[23] reported the up-regulatory effect of caffeic acid on GSH and its attenuating effects on TNF-α, IL-6 activity and lipid peroxidation. Wang et al.[24] also employed CA as treatment for an Alzheimer's disease model and found that it improved cognitive function and attenuated cerebral damage compared with the untreated group. They showed that caffeic acid promoted the activities of catalase and glutathione (GSH) which are important antioxidant agents, and inhibited Interleukin 6 (IL-6) and TNF-α activity and NF Kappa B (NF-κB) expression in the brain of rats.

This is in corroboration with our results which showed decreased levels of TNF- α in the FRUC-STZ+CA group compared to the untreated FRUC-STZ group. The CA group also showed the lowest levels of TNF- α. This is consistent with Zaitone,[12] who also noted that CA exhibited neuroprotective and anti-inflammatory effect by decreasing microglia expression and inflammatory mediators in a rotenone-induced neurodegeneration study.

Protein thiols are markers for free radical generation via their ability to inform the level of AGEs formation, hence protein thiols show an inverse relationship with blood glucose.[25] Our results also support this by an observable increase in protein thiol close to the control group in the CA-treated diabetic group compared to the diabetic control. This could be due to the CA-induced decrease in blood sugar or its documented free radical scavenging properties.[11] From existing research, the exact mechanism by which CA exerts its hypoglycemic effects has been debated, but from our results, the increased production of Insulin in the FRUC-STZ+CA group shows a regenerative effect on the Islet beta cells.

Nitric oxide is a compound with established vasodilatory abilities. Our results showed decreased levels of NO in the diabetic control group compared to the other groups. This is in consonance with Lin,[26] who investigated the levels of NO in a hyperglycemic state. Majorly 2 mechanisms cause NO deficiency, which are oxidative degeneration of NO and decreased synthesis of NO caused by competitive inhibition of Nitric Oxide Synthetase (NOS) by asymmetric dimethylarginine (ADMA). ADMA is significantly increased in states of high intracellular oxidative stress such as hypertension, hyperglycemia and hypercholesterolemia.[26] This elevation stems from oxidative stress induced inhibition of dimethylaminohydrolase (DDAH), the enzyme responsible for the hydrolysis of ADMA. This accumulation of ADMA inhibits NOS which further increases oxidative stress due to vasoconstriction, eventually leading to increased oxidative degradation of NO. This decrease in NO promotes atherogenesis which increases vascular resistance and causes the common cardiovascular complications of diabetes.

Histo-architectural changes also support the biochemical evidence of the anti-neurodegenerative effects of CA. Using Congo red stain which stains for beta amyloid deposits, it was observed that the FRUC-STZ+CA group showed significantly lesser numbers of these deposits compared to the FRUC-STZ group. The CA group had even lesser numbers of beta amyloid deposits compared to the control group.

CONCLUSION

In conclusion, we demonstrated that caffeic acid indeed reduces oxidative and inflammatory damage by

upregulating both antioxidants and anti-inflammatory cytokines in the hippocampus of rats treated for 14 days. Caffeic acid also improved vascular health by increasing the production of endothelial Nitric Oxide in treated rats. Caffeic acid also showed ameliorative effects on amyloid deposition, a classical sign of neurodegeneration.

ACKNOWLEDGEMENT

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DECLARATION OF INTEREST STATEMENT

We declare that there is no conflict of interest with respect to the authorship and/or publication of this paper.

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