Journal of Applied Life Sciences International
10(1): 1-15, 2017; Article no.JALSI.26546
ISSN: 2394-1103

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Lipid Peroxidation is Key to Neuronal Cell Death in the Inferior Colliculus of STZ-Induced Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author HBA designed the study, wrote the protocol and wrote the first and final drafts of the manuscript. Author PDS managed the literature searches and performed the statistical analyses. Author AKA managed the experimental process. Author TKA identified the species of the animals. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JALSI/2017/26546
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history/17414

Original Research Article

Received 22nd April 2016
Accepted 27th October 2016
Published 2nd January 2017

ABSTRACT

Neuropathy is one prominent complication yet to be separated from diabetes mellitus and various mechanisms have been identified for this complication. In this study, lipid peroxidation was investigated as a possible neuropathic agent in the inferior colliculus (IC) of Streptozotocin-induced diabetic rats.

A total of 29 adult Wistar rats of both sexes and average weight 220 ± 15.3 g were used in the study The rats were randomly divided into one control group and three experimental groups as group 1 (control – 5 rats), group 2 (P4 – 8 rats), group 3 (P7 – 8 rats) and group 4 (P10 – 8 rats). Each experimental rat was given 1ml of the equivalence of a single large dose of 70mg per kg body weight of Streptozotocin dissolved in citrate buffer (pH 4.5) by intraperitoneal injection. The control rats received 1ml of the Citrate buffer only also intraperitoneally. Enzymatic activities of LP, CAT, and SOD were estimated at post-induction periods P4, P7 and P10 in weeks. The morphology of the IC was also assessed at histological and histochemical levels.

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The major findings in this study include a sustained over four-fold elevation in blood glucose levels in experimental rats, significant body and neuronal density reductions in experimental rats compared with the control animals. Light microscopic observations revealed the dissolution of Nissl substance (chromatolysis), the swelling up of nuclei (karyolysis) and subsequent necrosis with degeneration of the neurons of IC (treated) which were more pronounced at P7 and 10 than at P4. At P10 especially, many neurophagic cells were seen in the vicinity engulfing the degenerating neurons and leaving lots of vacuolation. Significant increases were observed in Lipid Peroxidation in the Inferior Colliculus showing significantly different ($P \leq 0.05$) values between the control and treatment groups of the same period in the Inferior Colliculus. Data obtained from estimation of enzyme activities also revealed significant decreases ($P \leq 0.05$) in CAT and SOD in experimental animals when compared to those of the control animals.
In conclusion, this study offers evidence that diabetes mellitus induced by STZ is implicated in hearing impairment probably due to the destruction of membrane lipids and accumulation of the end-products of lipid peroxidation reactions which are especially dangerous for the viability of cells, in this case the neurons in the inferior colliculus.

Keywords: Diabetes mellitus; inferior colliculus; lipid peroxidation; neuropathy; oxidative stress.

1. INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by elevated blood sugar (hyperglycemia) level which results from defects in endogenous insulin secretion, or action, or both. There is a relative or absolute lack of insulin which could be due to a derangement / delay in insulin release mechanism, or circulation of biologically ineffective insulin, or the elaboration of abnormal or non-functional insulin and the presence of insulin antagonists in the circulating blood and peripheral tissues [1,2]. Due to serious derangements in carbohydrate metabolism, the primary and major source of cellular energy in the body is disrupted, so that the diabetic patient relies mainly on the metabolism of either stored or dietary lipids for energy. Reports from the world Health Organization [3] indicate that over 30% of the global population is affected by Diabetes mellitus.

Streptozotocin is primarily used in the treatment of metastasizing pancreatic islet cell tumours. It has been investigated for use in diabetes induction, since it has a specific toxic action on pancreatic β – cells [4,5]. As a diabetogenic agent, Streptozotocin (STZ) selectively destroys the insulin-producing beta cells by necrosis [6]. Necrosis entails the morphologic changes caused by the progressive degradative action of enzymes on the lethally injured cell. This selective beta cell toxicity has been attributed to the presence of the glucose moiety in the chemical structure of STZ, which enables it to enter the beta cell via the low affinity glucose transporter (GLUT2) in the plasma membrane [7]. In evaluating the carcinogenic potential of STZ, [8] discovered that both STZ and N-methylurea (NMU) caused the formation of DNA single-strand brakes. The DNA damage by STZ apparently depletes the nicotinamide adenine dinucleotide (NAD^+) of the beta cell and this in-turn inhibits insulin biosynthesis and secretion, eventually leading to beta cell death through ATP depletion. The STZ-induced diabetes is typically a type 1 (T1D) diabetes, also called the juvenile diabetes mellitus mainly because it is the type commonly found in children and young adults.

In addition to type 1 diabetes, diabetes mellitus has been categorized into types 2 (T2D) and 3 (T3D). In T2D, the pancreatic beta cells exist in their full functioning capacity but the cells of the body are not responsive to its activity thus allowing glucose to accumulate in the blood with a resultant hyperglycemia. T2D is the late onset diabetes and the major predisposing factor is lifestyle and diet. T3D describes the type of diabetes that that develops in pregnant women who originally have no history of diabetes. This type is also called gestational diabetes and predisposes the individuals to later development of T2D.

The Inferior colliculus is the main midbrain nucleus and important structure of the auditory passage. Not only does it serve as a relay center, it is also a receiver of essential information from various sources, and processes this information. Its primary roles are signal integration, frequency recognition, and pitch discrimination. It also processes sensory signals from the superior colliculi, located above it. The sensory auditory signals come as

electrochemical current from some brainstem nuclei that are more peripheral in the auditory pathway and the central auditory inputs from the auditory cortex of the cerebrum [9].

The gross structure of the inferior colliculus presents three sub structures namely: the central nucleus of Inferior colliculus, dorsal cortex of Inferior colliculus which surrounds the central nucleus and the external cortex of Inferior colliculus located laterally [10].

An increased concentration of end products of lipid peroxidation plays a very crucial role in the involvement of free radicals in human diseases. However, it is likely that increased oxidative damage occurs in most, if not all, human diseases and is implicated in the pathology many of them. For example, peroxidation appears to be important in atherosclerosis and in worsening the initial tissue injury caused by ischemic or traumatic brain damage [11,12]. One of the consequences of uncontrolled oxidative stress which is the clear imbalance between the pro-oxidant and antioxidant levels in favor of pro-oxidants is cells, tissues, and organs injury caused by oxidative damage. High levels of free radicals or reactive oxygen species (ROS) often inflict direct damage to lipids and the primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes [13] through a variety of mechanisms including enzymatic reactions and/or auto-oxidation of several compounds.

Lipid peroxidation refers to the oxidative degradation of lipids [14]. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism consisting of the three major steps of initiation, propagation and termination. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene bridges ($-CH_2-$) that possess especially reactive hydrogen [15].

Lipid peroxidation exists as a crucial step in the pathogenesis of several disease states in adult and infant patients. Interestingly, it is a process generated naturally in small amounts in the body, mainly by the effects of several reactive oxygen species, particularly the hydroxyl radical and hydrogen peroxide molecules as noted above. It can also be generated by the action of several phagocytes [16]. These reactive oxygen species readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells and even tissues. The body, however, has several enzymatic (e.g. Catalase, Superoxide dismutase and Peroxidase) and non-enzymatic (e.g. vitamins A and E) natural antioxidant defense mechanisms but, these mechanisms may be overcome, causing lipid peroxidation to take place. Since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage. Furthermore, end-products of lipid peroxidation may be mutagenic and carcinogenic [17], Like the case of the end-product malondialdehyde which reacts with deoxyadenosine and deoxyguanosine of the DNA, to form DNA adducts, primarily M_1G. In this study, lipid peroxidation was investigated as a possible neuropathic agent in the inferior colliculus (IC) of Streptozotocin-induced diabetic rats.

2. MATERIALS AND METHODS

This study was carried out under strict adherence with the recommendations of the Guide for the Care and Use of Laboratory Animals, Published by the US National Institutes of Health (NIH Publication, 8th Edition) [18], and approved by the Faculty of Basic Medical Sciences (FBMS) Animal Care Committee of our University.

2.1 Animals and Management

A total of twenty-nine (29) adult Wistar rats, of both sexes and weighing between 200g and 220 g were used in this investigation. The animals were procured from the breeding stock of the Anatomy Department of the Olabisi Onabanjo University, Remo Campus, Ikenne, Ogun State. Animals were maintained on a standard rat chow (Top feeds, Ogun State, Nigeria) and clean water was provided *ad libitum* both during the acclimatization and experimental periods with regular weight taking and physical observation.

2.2 Experimental Design

The experiment consisted of 3 treatment groups of eight (8) animals each and one (1) control group of Five (5) animals. Group 1 was the control group, group 2 was the Post-induction week 4 (P4) group; group 3 was the P7 group and group 4 was the P10 group. The basis of this

time selection was that at four (4) weeks, the duration of induced diabetes was not long enough (based on our pilot studies) to produce any significant change in the morphology of the auditory relay centers under study. Three (3) weeks were allowed for further observation and at seven (7) weeks of diabetic state, some significant effects occurred in the relay centers. Three more weeks of uninterrupted hyperglycemia were allowed to determine further extent of damage.

2.3 Drug Procurement and Administration

Streptozotocin was obtained from the Zayo-Sigma company, Lagos, a sales representative of Sigma, USA. Each experimental rat was given 1 ml of the equivalence of a single large dose of 70 mg per kg body weight of Streptozotocin dissolved in citrate buffer (pH 4.5) by intraperitoneal injection. The control rats were given 1 ml of the citrate buffer only by intraperitoneal injection. The intraperitoneal injection is best achieved by two workers thus: For the first worker with hands protected in hard gloves: hold the animal in a supine position with its dorsal neck skin and head well secured in the right hand and the tail and limbs in the left hand; second worker: swab the ventral abdominal wall with sterile cotton wool soaked in 70% alcohol or methylated spirit; next, lift up the wall around the ilio-colic region, insert the needle ensuring that it is well inside the abdominal cavity in the space between the wall superiorly and the abdominal viscera inferiorly. The needle should move freely from left to right without touching any organ in the abdomen. Then release the syringe content into that space enroute to its target site.

2.4 Confirmation and Monitoring of Induced Diabetes Mellitus

The rats fasted overnight before the blood glucose test. Blood samples were obtained by puncture of the tail vein, and blood glucose level was determined by using an automated One-Touch Basic glucometer and glucose test strips (LifeScan, Milpitas, CA) [19].

2.5 Sacrifice of Animals and Preparation for Tissue Processing

The sacrifice of animals at the end of each experimental period was by exsanguination after which the brain was excised and weighed. Blood was collected from left ventricle into EDTA and Heparinized bottles, centrifuged and preserved in the refrigerator for enzyme assay. Whole brains were first dissected out, weighed and divided into two equal halves (the right and left hemispheres). One half meant for histology was quickly was fixed in 10% Formal Calcium solution, and one for estimation of enzyme activities. With the aid of the rat brain stereotaxic map at interaural levels from 3.80 mm – 0.16 mm and bregma levels from -5.20 mm to -9.16 mm, sections of the brain enclosing the inferior colliculus were processed variously for Haematoxylin and Eosin (H&E) Staining, Special histological staining and quantitative histochemical analyses were performed as stated below. Brain parts for enzyme quantification were homogenized manually, centrifuged and supernatants collected into plain bottles for Spectrophotometric readings at wavelengths specific for each enzyme assay.

Table 1. Experimental animal grouping, dosages and rationale

Grouping	Animals	Dosage	Description	Rationale
Group 1	5 adult Wistar rats of both sexes	1 ml of citrate buffer (pH 4.5) intraperitoneally	Animals received vehicle given once by ip injection	Control group
Group 2	8 adult Wistar rats of both sexes	75 mg/kg body of Streptozotocin dissolved in 1 ml of citrate buffer	Animals received a single large dose of STZ intraperitoneally	Week 4 (P4) group
Group 3	8 adult Wistar rats of both sexes	75 mg/kg body of Streptozotocin dissolved in 1 ml of citrate buffer	Animals received a single large dose of STZ intraperitoneally	Week 7 (P7) group
Group 4	8 adult Wistar rats of both sexes	75 mg/kg body of Streptozotocin dissolved in 1 ml of citrate buffer	Animals received a single large dose of STZ intraperitoneally	Week 10 (P10) group

Tissue processing for haematoxylin and eosin staining (Drury and Wallngton, 1967).

2.5.1 Special staining techniques

Staining method for demonstration of nerve fibres and neurofibrils after Holmes (1943).

Staining method for demonstration of nissl substance after Fletcher (1947).

Luxol fast blue staining method for demonstration of myelin after Kluver and Barrera (1953).

2.6 Lipid Peroxidation

Lipid peroxidation (LPO) in the inferior colliculus was assayed for by the thiobarbituric acid reactive substances (TBARS) present in the test sample using the procedure of [20] and expressed as micromolar of monodialdehyde (MDA/g tissue).

2.6.1 Principle

This assay is based on the ratio of chromogenic reagent (2-thiobarbituric acid) to monodialdehyde (an end product of LPO) under acidic condition to yield a stable pink chromophore with maximum absorption at 532 nm wavelength. It is readily extractable into organic solvents like buta-1-ol.

2.6.2 Assay protocol

The aliquot of 0.2 ml of the supernatant of the blood and tissues was mixed with 1.8 ml of Tris-Kcl buffer, 0.5 ml of 30% TCA and 0.5 ml of 0.75% TBA. The reaction mixture was then placed in a water bath for 45 minutes of 80℃, cooled in ice and then centrifuged at 3500 rpm for 5 minutes. The clear supernatant was collected and the absorbance was measured against reagent blank (distilled water) at 532 nm wavelength.

2.7 Superoxide Dismutase (SOD)

The level of superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich [21].

2.7.1 Principle

The basis for this assay is the ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 Superoxide anion generated by the xanthine oxidase reaction causes the oxidation of epinephrine to adenochrome and the yield of adenochrome produced per superoxide anion introduced increases in increasing concentration of epinephrine. The enzyme activity in the supernatant solutions was determined using spectrophotometer and the absorbance readings were measured at 420 nm in the UV-Visible recording Spectrophotometer.

2.7.2 Assay protocol

0.2 ml of the sample supernatant was diluted in 0.8 ml of distilled water to make a 1 in 5 dilution. An aliquot of 0.2 ml of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) in the cuvette and allowed to equilibrate in the spectrophotometer. The reaction was started by addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion of the cuvette. The reference blank contained 2.5 ml of buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance was monitored every 30 seconds for 150 seconds.

2.8 Catalase

Catalase activity was determined according to the method of Sinha A [22].

2.8.1 Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured calorimetrically at 570-610 nm wavelength. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The Catalase preparation is allowed to split H_20_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate calorimetrically after heating the reaction mixture.

2.9 Enumeration of Cells

The packed cell population of the Inferior colliculus was determined by counting the neurons and glial cells using the method in which

a grid was fixed into the microscope and focused through stained sections of the tissues. All the cells observed were enumerated and corrections made for cells bisected by the edges of the grid. Cell population was estimated as number of cells/mm^2 of tissues. Counting was done at a magnification of X10 Objective and X10 Ocular lenses. Nerve cell diameter was measured using X40 Objective and X10 Ocular lenses. Calculations for the actual number of cells counted were made using the Abercrombie [23] mathematical correction formula

$$NV = \frac{NA\ (T+D)}{T}$$

Where

NV = Number of cells that should be counted in the sections.
NA = Actual number of cells counted in the area of the sections.
T = Thickness of sections used.
D = Average diameter of the neurons.

The results of the calculations were further analyzed by the students' T- test and ANOVA for comparison of means and expressed as Mean ± S.D.

2.10 Statistical Analysis

All values are presented as Mean ± SD of the mean. One-way analysis of variance (ANOVA) with repeated measure was used to evaluate the significance levels of animal weights, blood glucose levels, enzyme activities in blood and Inferior Colliculus, neuronal density in Inferior Colliculus.

2.11 Photomicrography

An Olympus Research Microscope (CH) with a transmitted illumination system was used for the light microscopic examination of all stained sections. The microscope was equipped with a transmitted illumination system with a standard N.A. 1.40 achromatic/aplanatic condenser well known to ensure perfect Kohler illumination ranging from large numerical aperture apochromatic objectives to standard achromatic objectives. It also has an advantage of even illumination. Standard plain achromatic 4, 10 and 40x objectives were used. Photomicrographs were taken using the Lincron digital CCD camera, and described as an HP Precision 3X optical zoom, 6.0 mm – 18 mm, 7.0 mega pixels camera (Model L2420A) with magnifying factor 0.85.

3. RESULTS

3.1 Morphological Observations

Average body weights, and average blood glucose levels of diabetic and control animals at 0, 4, 7 and 10 weeks post induction are shown in Tables 1, and 2. Briefly compared with the non-diabetic control group, the diabetic groups had significantly higher blood glucose levels, and lower body weights (P≤ 0.05).

3.1.1 Animal body weight

The body weight of the treated animals significantly decreased from 220 ± 0.15 g before induction to 166.6 ± 9.72 g (~ 24.3% decrease) at week 10. There was a 10% (P≤0.05) weight gain in the control animals whose weight increased from 220 ± 18.15 g at zero hour to 242 ± 14.14 g at 10 weeks (Table 1).

3.1.2 Blood glucose levels

The blood glucose levels in treated animals showed a statistically significant (P≤0.05) over four-fold elevation from 56.6 ± 11.58 mg/dl before induction to 304.5 ± 7.14 mg/dl (~438% increase) at week 10 (Table 2). In control animals, a fluctuating 5.93% difference was observed during the periods of experiment.

3.1.3 Lipid peroxidation in IC

The results obtained for Lipid Peroxidation in the Inferior Colliculus showed significantly different (P≤0.05) values of 36.7%, 55.1% and 67.4% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 29.0% and 27.4% respectively (P≤0.05) (Fig. 1).

3.1.4 Lipid peroxidation in the blood

The results obtained for Lipid Peroxidation in the Blood showed significantly different (P≤0.05) values of 43.9%, 52.9% and 78.7% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 16.2% and 54.7% respectively (P≤0.05) (Fig. 1).

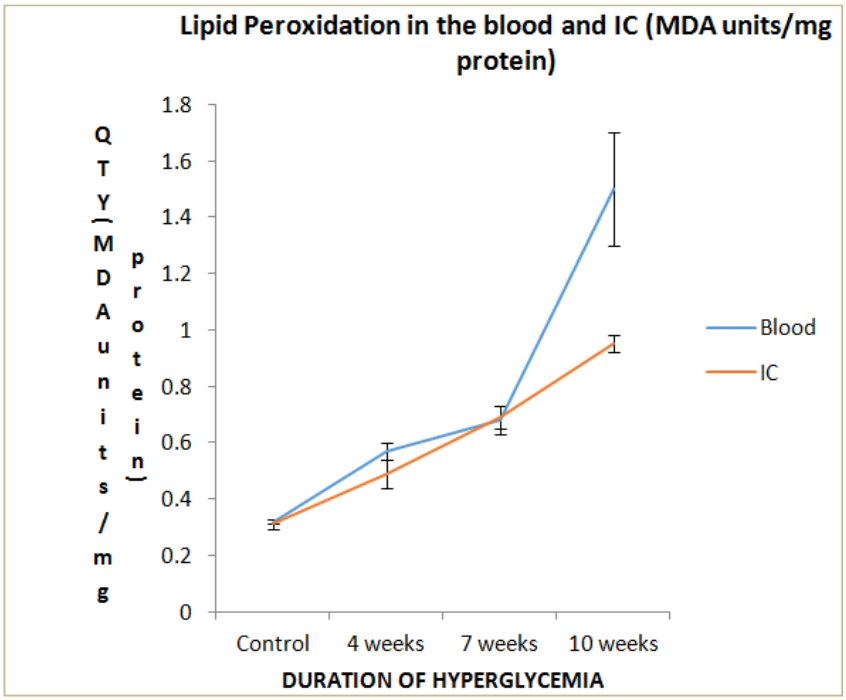

Fig. 1. Lipid peroxidation in the blood and IC (MDA units/mg protein)

3.1.5 SOD in IC

The results obtained for SOD activity in the Inferior Colliculus showed significantly decreasing (P≤0.05) values of 52.6%, 60.7% and 94.5% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 16.9% and 91.1% respectively (P≤0.05) (Fig. 2).

3.1.6 SOD in the blood

The results obtained for SOD activity in the Blood showed significantly different (P≤0.05) values of 57.1%, 74.1% and 82.9% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 39.6% and 33.9% respectively (P≤0.05) (Fig. 2).

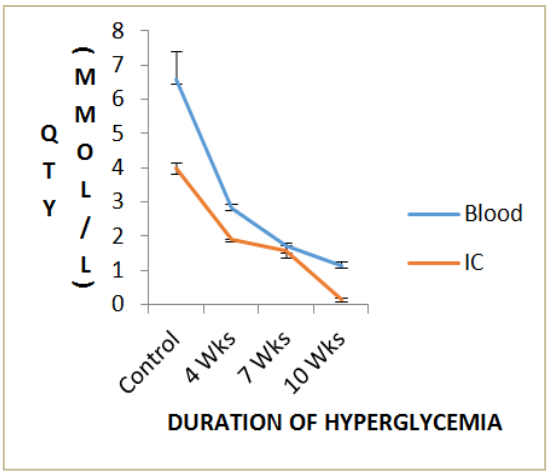

Fig. 2. SOD activity in the blood and IC (mmol/L)

3.1.7 CAT in IC

The results obtained for Catalase activity in the Inferior Colliculus showed significantly different (P≤0.05) values of 25.9%, 49.7% and 59.9% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 32.1% and 20.3% respectively (P≤0.05) (Fig. 3).

3.1.8 CAT in blood

The results obtained for Catalase activity in the Blood showed significantly different (P≤0.05) values of 9.5%, 29.6% and 31.7% at P4, P7 and P10 respectively between the control and treatment groups of the same period, while P4/P7 and P7/P10 values stood at 22.2% and 3.0% respectively (P≤0.05) (Fig. 3).

3.2 Morphometric Analysis

Results of IC neuron morphometry revealed a steady and statistically significant (P≤ 0.05) decline in the mean neuronal densities in diabetic animal inferior colliculi compared to the sections from control animals at weeks P4, P7 & P10 (Table 3). Neuron atrophy was apparent in diabetic rats (weeks P7 & P10). Sections of IC presented with a tendency (P≤0.05) towards smaller total numbers of neurons in diabetic animals compared with non-diabetics; although the differences were slight, they were statistically significant considering the periods of treatment (Table 3). At the light microscopic level, severe alterations in neuron appearance were observed in IC at 7 and 10 weeks but not at 4 weeks. Neurons appeared micro-vacuolated, shrunken and degenerated; some appeared dark forming nests of Nageotte. Some showed eccentric nuclei and central chromatolysis (Figs. 4-7).

4. DISCUSSION

The first finding of the present study was that Streptozotocin successfully induced diabetes mellitus in the experimentally treated animals. This was evident by the production of and a subsequent uncontrollable rise in blood glucose level as confirmed by the data on blood sugar levels before and after the intraperitoneal administration of Streptozotocin into the experimental rats. The rise in blood sugar readings (hyperglycemia) means that insulin meant to effect the absorption of glucose into body cells was not performing its normal function and therefore, the accumulation of the glucose in the blood of the animals. Earlier investigations by Adewole et al. [24] clearly indicated that administration of a single large dose of Streptozotocin to rats successfully results in selective destruction of the β – cells of the pancreatic islets, which cells are responsible for the production of insulin.

The incidence of diabetic neuropathy has been increasing. Studies have shown that oxidative stress, probably due to increased prooxidant production and/or decreased antioxidant activity forms a critical underlying mechanism in the development of neuropathic complications during diabetes. NADPH has been found to be the principal intercellular reductant and its production is mainly dependent on glucose-6-phosphate dehydrogenase (G6-PDH) activity [25]. Zang et al. [26] reported that G6PDH activity is inhibited by high glucose (hyperglycemia) levels. This is achieved through the activation of protein kinase A (PKA) and subsequent phosphorylation of G6PDH. Inhibition of G6PDH activity further decreases the production of NADPH. A concomitant increase in lipid peroxidation forms a major feature in those conditions, with all the changes being ameliorated by insulin treatment or other measures to correct hyperglycemia.

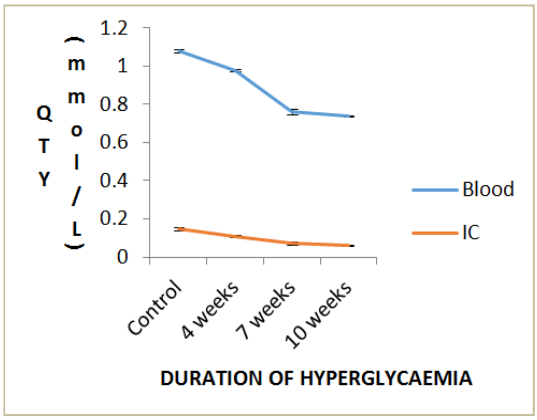

Fig. 3. Catalase activity in the blood and IC (mmol/L)

One interesting point raised by several studies is the enzyme(s) responsible for free radical production in arterial vessel. NADH oxidase has been proposed to be a major source of superoxide anion (O) in normal and diseased blood vessels [27]. To test this hypothesis, Lund et al. [28] assessed O production in vessels from normal or diabetic rabbits that were stimulated by NADH oxidase. Superoxide production in response to NADH was 2-fold greater in carotid arteries from diabetic rabbits than in normal carotids. These data suggest increased propensity to generate high superoxide levels in arterial vessels from diabetic animals. In this study, such increase could not be countered due to decreased concentration of superoxide dismutase in the IC thus leaving the neurons exposed to attack by superoxide ions.

The primary lipid peroxidation product-Lipid Hydroperoxides are produced during the propagation phase and constitute the major primary product of lipid peroxidation process. The hydroperoxide group can attach itself to various lipid structures, for example, free fatty acids, triacylglycerols, phospholipids, and sterols. Hydroperoxides can also decompose *In vivo* through one-electron reduction and take part in initiation/propagation steps [15,29,30] induce new lipid hydroperoxides, and feed the lipid

peroxidation process; all these mechanisms can contribute to peroxidative damage induction/ expansion and subsequent damage to cells and tissues. Our study revealed escalating levels of lipid peroxidation which in itself is detrimental to the survival of cells.

Histological Observations

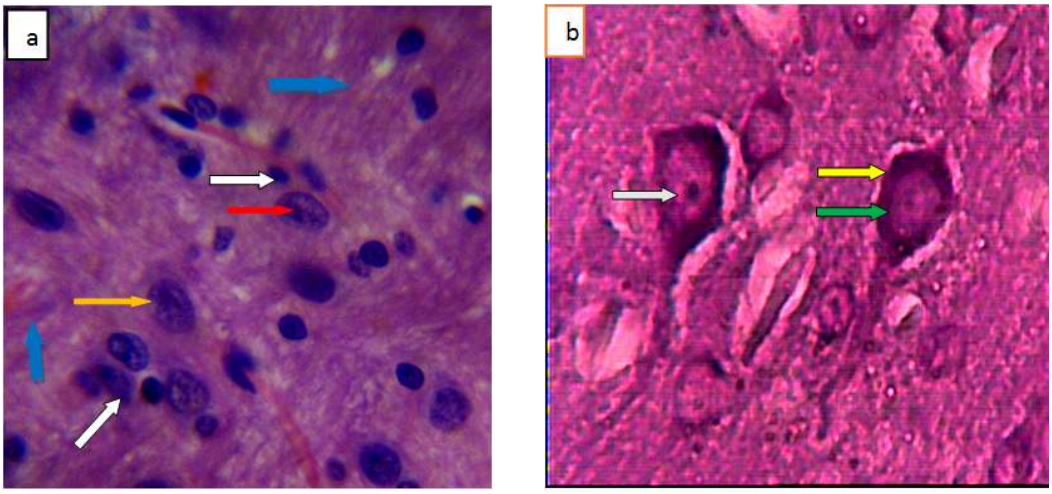

Fig. 4. Photomicrographs (a) and (b) represent control sections of the Inferior Colliculus. (a) H&E mag X400 section, note some basic parts of the neuronal field - large nucleus (yellow arrow), prominent nucleolus (red arrow) and the cytoplasm (green arrow), neuroglial cells (white arrows) and neuronal fibres (blue arrows). (b) Nissl mag X1600 stained section of control specimen showing the morphological structure of the multipolar neuron

Note the three basic parts of the neuron - large nucleus (green arrow), prominent nucleolus (white arrow) and the cytoplasm (yellow arrow)

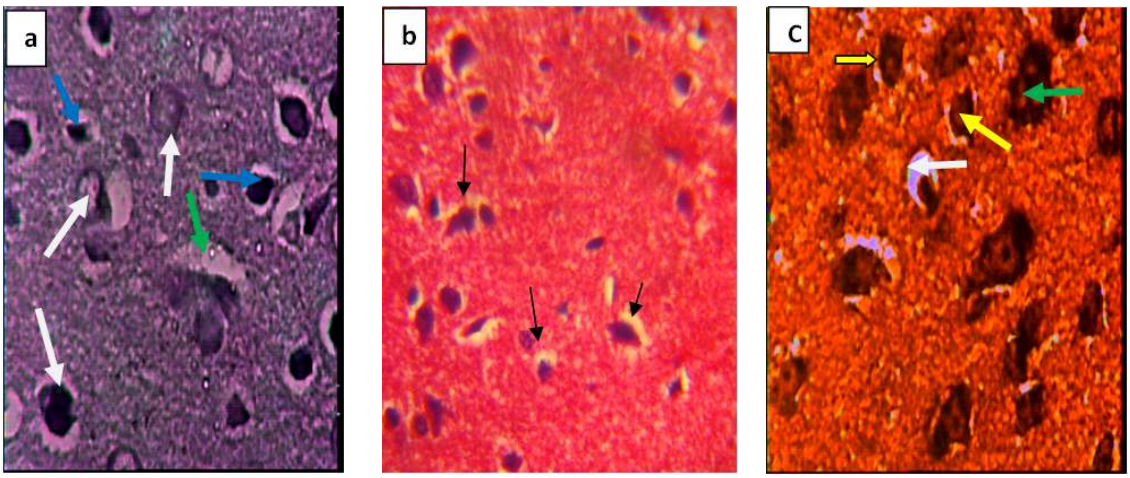

Fig. 5. Photomicrographs (a), (b) and (c) represent P4 sections of the Inferior Colliculus. (a) Nissl mag X1200 stained section of showing the effects of STZ-induced diabetes mellitus on the inferior colliculus at 4 weeks post-induction. Note: An unusually large perivascular space (green arrow), early signs of karyolysis indicated by fading and swelling (white arrows) and numerous shrinking nuclei (blue arrows). In (b) H&E mag X400 section at P4, the cytoplasm is filled with neurons showing various degrees of mild karyolitic changes (black arrows). In (c) Silver mag X1200 section at P4

Note: Presence of vacuolated lacuna (white arrow), dissolving nucleoli (Yellow arrows) and fragmented nucleus (green arrow)

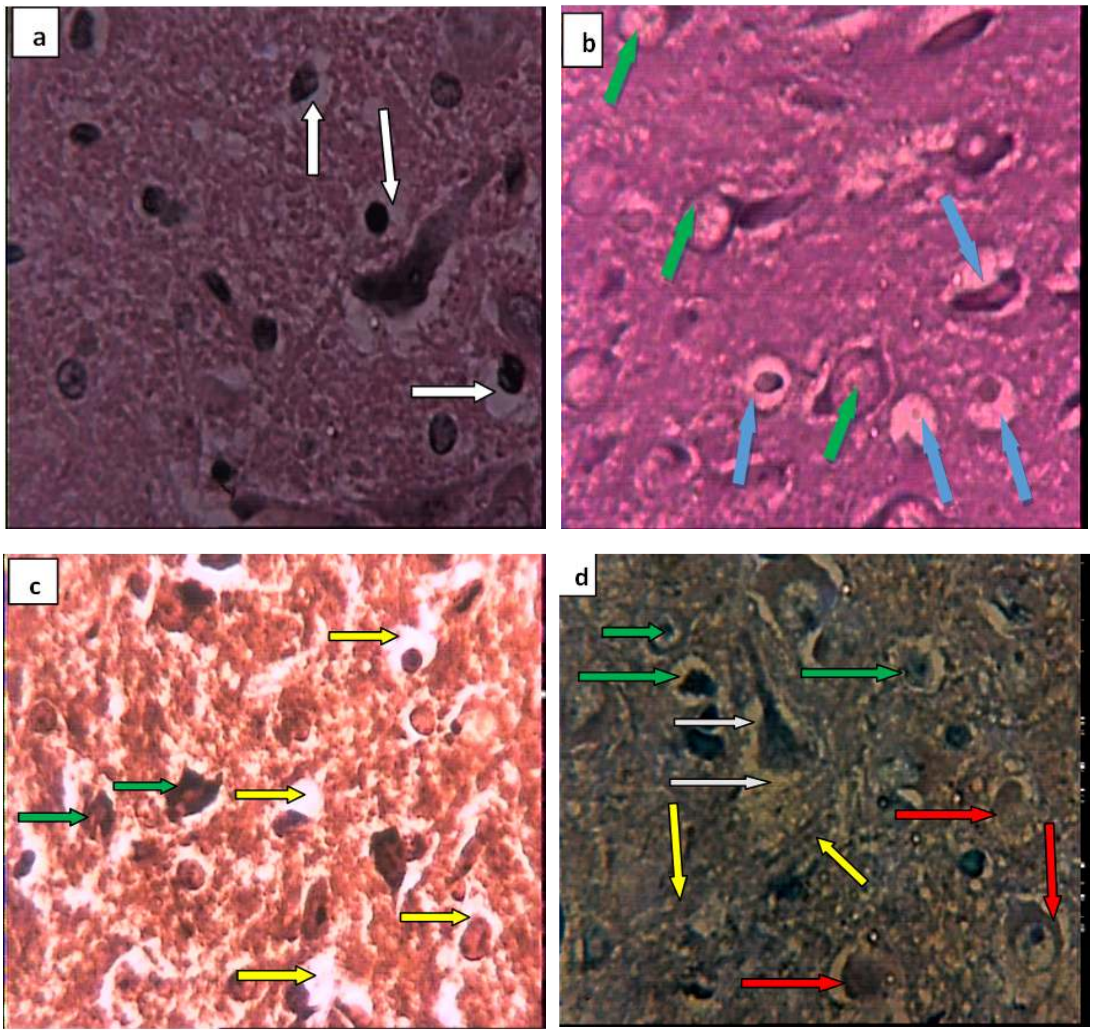

Fig. 6. Photomicrographs (a H&E mag X1000), (b Nissl mag X1200), (c silver mag X1200) and (d LFB mag X1200) represent P7 sections of the inferior colliculus: (a) shows the effects of STZ-induced diabetes mellitus on the inferior colliculus at 7 weeks post-induction): Note: Presence of vacuolated lacunae (white arrows) and general paucity of neurons on the field. (b) Nissl mag X1200 Shows the effects of STZ-induced diabetes mellitus on the inferior colliculus at 7 weeks post-induction Note: Distorted Neurons (Green arrows) and Vacuolated lacunae (Blue arrows). (c) Silver magX1200. Note: Presence of vacuolated lacunae (yellow arrows), and neurons with fragmented nuclei - karyorrexis (green arrows). (d) Presence of vacuolated lacunae (white arrows), gross demyelination (yellow arrows), neuronal shrinkage (green arrows), and distorted neurons (Red Arrows)

The intracellular concentration of ROS depends on the production and/or removal by antioxidant system. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox sensitive pathways. Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for all life in all oxygen metabolizing cells are Superoxide Dismutase (SOD), Catalase and the substrate specific peroxidases – Lipid peroxidase (LP). The SODs convert superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), while the catalase and peroxidases convert hydrogen peroxide into water and in the case of catalase to oxygen and water. The net result is that two potentially harmful species, superoxide and hydrogen peroxide are converted to water.

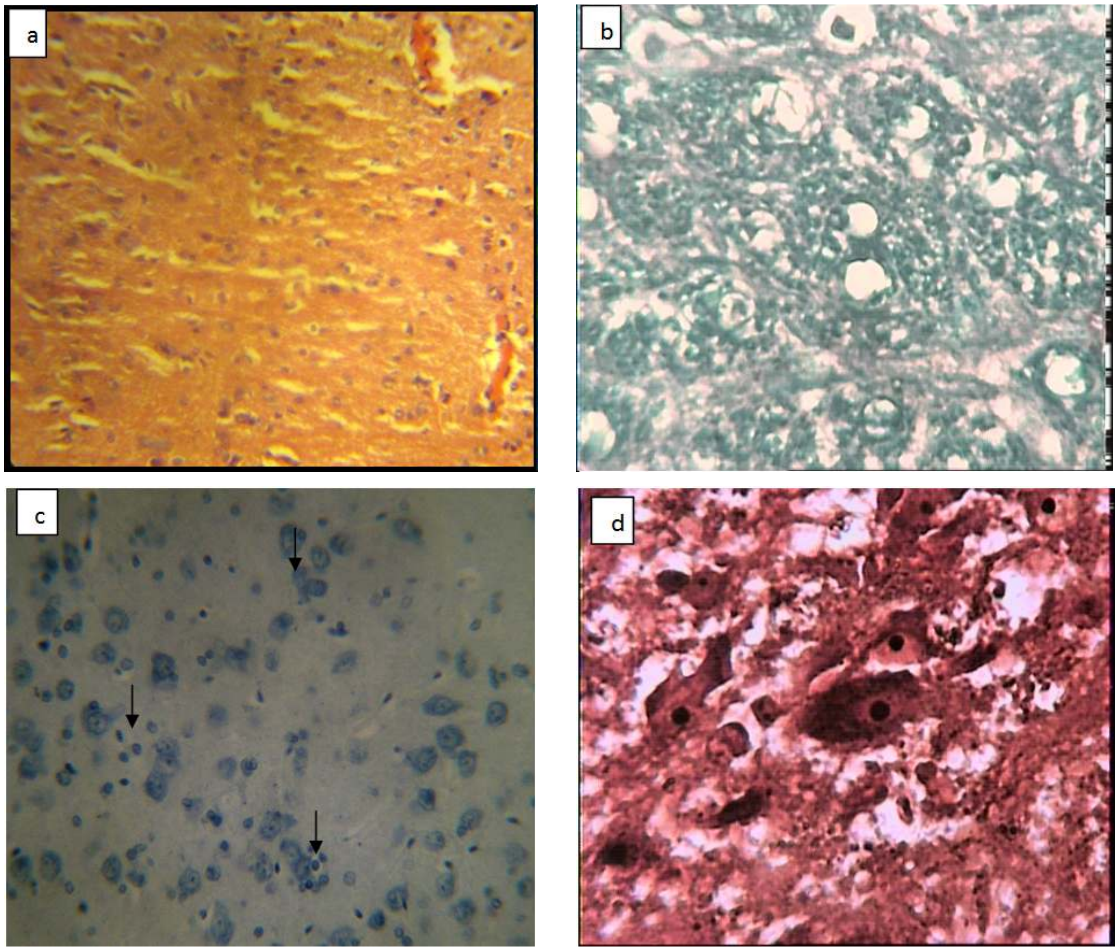

Fig. 7. Photomicrographs (a H&E mag X40), (b LFB mag X1200) (c Nissl mag X100) and (d Silver mag X1200) represent P10 sections of the inferior colliculus: all indicating the effect of STZ-induced diabetes mellitus on the Inferior Colliculus at 10 weeks post-induction. Note that there is a general breakdown of the tissue interstitium in sections a, b and d

Note: Infiltration with inflammatory cells (black arrows in c) and numerous necrotic cells and increased vacuolation prominent on the fields

Association between diabetes mellitus and the auditory system has been debated since it was first reported by Jordao [31]. Many different types of hearing impairments have been reported in diabetic patients such as progressive, gradual bilateral sensorineural loss affecting especially the elderly and high frequencies of other age groups. In their studies, Strauss et al. [32], Miller et al. [33], Dalton et al. [34], Gratton & Vazquez [35], Gates & Mills [36], found no direct link DM and hearing loss. In other studies, each of Friedman et al. [37], Taylor & Irwin [38], Cullen & Cinamond [39], Tay et al. [40], Fukushima et al. [41], Maia & Compos, [42], Frisina et al. [43], Vasilyeva et al. [44], Malucelli et al. [45], and Taylor et al. [46] described various forms of association between DM and hearing loss. In this study, there was a significant reduction in the neuronal population in the IC and this is a positive pointer to impaired ability to function for this relay center. This finding is in agreement with the papers in which the association between DM and hearing loss was described.

Also, in this study, the plasma and tissue levels of SOD, Catalase and Lipid Peroxidase were severely compromised and this was evidently shown in the morphology of the neurons. At four weeks of untreated diabetic condition in the experimental rats, when the concentrations of ROS were still under control of intracellular antioxidant enzymes, neurons in the inferior colliculus maintained appreciably intact morphology when compared with the controls

Table 2. Effects of STZ-induced diabetes mellitus on the body weight of animals body weight (g)

Time	Control (5)	Treated (24)	P - value
0 hour	220 ± 18.15	220 ± 9.01	*0.081
3 hours	220 ± 14.15	194 ± 7.01	*0.066
24 hours	213 ± 17.18	193 ± 7.96	†0.051
2 weeks	225 ± 18.71	173 ± 7.92	†0.050
3 weeks	228 ± 20.49	192 ± 7.86	†0.042
4 weeks	233 ± 22.36	190 ± 7.95	†0.041
5 weeks	235 ± 22.63	183 ± 5.90	††0.022
6 weeks	235 ± 9.71	173 ± 8.84	††0.021
7 weeks	237 ± 11.67	170 ± 10.13	††0.015
8 weeks	239 ± 18.76	168 ± 9.58	††0.011
9 weeks	240 ± 6.58	167 ± 9.46	††0.000
10 weeks	242 ± 14.40	166.6 ± 9.72	††0.000

*Data are expressed as means ± SD (P≤0.05). † Significant. †† Highly Significant. *Insignificant*

Table 3. Effects of STZ-induced diabetes mellitus on the blood glucose levels of animals blood glucose levels (mg/dl)

Time	Control (5)	Treated (24)	P-value
0 hour	64.1 ± 5.22	56.6 ± 11.58	*0.0522
3 hours	65.4 ± 5.21	125.3 ±16.06	†0.0012
24 hours	66.3 ± 5.10	99.9 ± 6.83	†0.0038
2 weeks	66.0 ± 4.44	135.8 ± 15.85	†0.0017
3 weeks	65.2 ± 5.33	158.9 ± 17.87	††0.0015
4 weeks	67.2 ± 5.24	184.1 ± 15.04	††0.0014
5 weeks	67.4 ± 4.60	220.3 ± 18.85	††0.0004
6 weeks	68.1 ± 3.35	243.6 ± 17.91	††0.0004
7 weeks	67.8 ± 4.24	260.2 ± 13.87	††0.0003
8 weeks	68.6 ± 3.75	271.9 ± 10.83	††0.0001
9 weeks	68.6 ± 3.24	284.4 ± 10.03	††0.0001
10 weeks	67.9 ± 3.54	304.5 ± 7.14	††0.0001

*Data are expressed as means ± SD (P≤0.05). ††Highly significant. †Significant. *Insignificant*

Table 4. Average neuronal density in inferior colliculus (neurons/mm^2)

Time (Weeks)	Control (n=5)	Treated (n=24)	P - value
Week 4	184.6 ± 6.18	155.8 ± 6.34	0.069
Week 7	184.9 ± 4.22	118.8 ± 4.70	0.058
Week 10	184 7 ± 6.43	98.9 ± 4.78	*0.041

*Data are presented as mean ± SD of values for 24 treated and 5 control rats (P≤0.05). *= Significant*

though with mild karyolitic changes. However, at seven and ten weeks, it became obvious that the strength of the intracellular antioxidant enzymes was superseded by excess accumulation of ROS in the uninterrupted hyperglycemic state. Therefore, the neurons, under the influence of oxidative stress and excessive lipid peroxidation lost their nuclei, Nissl substances, became shrunken and pyknotic, laid in large, clear perinuclear cytoplasmic vacuoles, all being varying stages of karyolysis. The sections also presented numerous neurophagic cells ready to phagocytose the dying neurons.

5. CONCLUSION

In conclusion, this study offers evidence that diabetes mellitus induced by STZ is implicated in hearing impairment probably due to the destruction of membrane lipids and accumulation

of the end-products of lipid peroxidation reactions which are especially dangerous for the viability of cells, in this case the neurons. Natural antioxidant defense mechanisms were probably overcome as evident from reductions in enzymatic activities of Catalase and Superoxide dismutase with resultant elevation of lipid peroxidation. Since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage as indicated by reduction in neuronal density and hypotrophy in the inferior colliculus.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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