AMELIORATION OF HISTOLOGICAL CHANGES OF THE HIPPOCAMPUS IN EXPERIMENTALLY-INDUCED DIABETIC RATS VIA ANTOX COMPOSITE OF MULTI-ANTIOXIDANTS

BY

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ABSTRACT

Background: Diabetes mellitus (DM) is a chronic metabolic disorder usually complicated by cognitive dysfunction owing mainly to the diabetic-associated oxidative stress. Antox, a multi-antioxidant vitamins compound, is known to exhibit anti-diabetic and neuroprotective properties. **Aim of the work**: to highlight the possible beneficial effects of Antox in mitigating the histological alterations of the hippocampus in experimentally-induced diabetic rat model as an adjuvant therapeutic preclinical strategy. **Material and Methods:** Thirty adult male Sprague Dawley rats are randomly allocated into 5 groups that assigned alphabet and treated for 4 weeks. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/Antox/Insulin). At the end of the experimental period, the rats were anesthetized, and the brain samples were excised out and the hippocampus was dissected. Serum glucose and glycosylated haemoglobin (HbA1c) were measured. Also, malondialdehyde (MDA), Superoxide dismutase (SOD), glutathione peroxidase (GPX), in addition the expression of TNF-α gene by real-time PCR were measured in hippocampal tissue. Paraffin sections of the hippocampus were processed for staining by H&E and for immunohistochemical analysis of Glial fibrillar acidic protein (GFAP) and P53 immunoreactivity. **Results:** Diabetic rats have encountered with a remarkable changes in the cytoarchitecture of the hippocampus along with increased serum glucose and HbA1c. In the hippocampal tissue, MDA and expression of GFAP, P53, and TNFα increased, however oxidative enzymes decreased. Antox partially alleviated most deteriorated measurements in the diabetic group compared to the high efficacy effect of insulin. However, Diabetic/Antox /Insulin group has a supreme effect compared with either Antox or insulin groups. **Conclusion:** Antox elicited an adjuvant role in ameliorating the metabolic and structural changes in the hippocampus via its antioxidant and anti-inflammatory effects.
Keywords: *Diabetes, Hippocampus, Antox, Rats.*

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INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disorder caused by impaired insulin secretion and/or action, resulting in disturbances in the metabolism of carbohydrates, lipids, and proteins. This disturbance can lead to the development of long-term health complications *(Dilworth et al., 2021)*. DM is a major health concern in both developing and developed countries, with global estimates predicting a 69% increase in the proportion of the adult population with diabetes by the year 2030 *(Shaw et al., 2010)*.

Increasing evidence from diverse scientific research suggests that diabetic complications are linked to oxidative stress. Persistent hyperglycemia in DM leads to the overproduction of free radicals, particularly reactive oxygen species (ROS), in all tissues due to glucose autoxidation and glycosylation of both proteins and antioxidant enzymes *(Akpoveso et al., 2023)*.

The overproduction of free radicals in DM leads to a disturbance in the balance normally present in cells between radical generation and scavenging defense mechanisms, resulting in oxidative damage and tissue injury *(Asmat et al., 2016)*. Diabetic patients and experimental hyperglycemic animals are at risk of oxidative stress complications, which may increase their requirement for antioxidants to scavenge free radicals and

prevent oxidative damage to the tissue (*Lee et al., 2002; Ahlem et al., 2009*).

There is emerging evidence that hyperglycemic-induced brain oxidative stress is a major molecular hallmark and contributing factor in the pathogenesis and progression of cognitive dysfunction in diabetic patients (*Kim, 2019; Zhou et al., 2022*). In addition, antioxidant mechanisms are diminished in these patients, which may further augment oxidative stress (*Maritim et al., 2003; Rains and Jain, 2011*). The hippocampus is one of the most vulnerable areas of the brain to oxidative stress, resulting in marked histoarchitecture insult and early functional decline (*Wang and Michaelis, 2010*).

Antioxidants can be either enzymatic, including glutathione peroxidase, catalase, and superoxide dismutase, or non-enzymatic, such as vitamins and carotenoids derived from natural sources by dietary intake (*Arulselvan and Subramanian, 2007; Gezginci-Oktayoglu et al., 2009*). Vitamins play an important role in glucose homeostasis, and their supplementation has potential utility in the prevention and management of diabetes. The majority of diabetic individuals should receive daily vitamins within the recommended values from consumption of natural food sources and/or fortified foods (*Fernández-Mejía, 2013*).

Currently, more attention is paid to multi-antioxidant vitamins, particularly A, C, E, and alpha-lipoic acid, as preventive and/or therapeutic modalities to tackle the threats posed by oxidative stress in cases of diabetes. The antioxidant role of these multivitamins emerged from their unique participant roles in the regeneration of multiple endogenous antioxidant enzymes (*Martini, 2010*).

Antox is a multivitamin compound manufactured with vitamin A, C, E, and selenium. It is available over-the-counter and can be dispensed directly to consumers without a prescription from a healthcare professional. Antox is highly effective and has a high bioavailability. It is also allergen-free, cost-effective, and has no known side effects (*Helal et al., 2011*).

AIM OF THE WORK
The aim of this study was to investigate the potential of Antox in improving the histological alterations and dysfunction of the hippocampus in a type-1 diabetic rat model.

MATERIAL AND METHODS
Chemicals:
- Streptozotocin (STZ) powder of 98% purity (Sigma-Aldrich chemical Co., St. Louis, USA).
- Antox tablets containing multi-vitamins (A, C, E, and selenium) purchased from a local pharmacy in Egypt.
- Insulin Mixtard (30/70; Novo Nordisk).
- Primers obtained from delta Company Egypt. **(Table 1)**.

Experimental Animals:
The study was conducted using 30 adult male Sprague Dawley rats aged approximately 12-16 weeks and weighing 250±25 g. The animals were acclimatized to laboratory conditions for one week without handling. They were kept at a constant temperature of 23±2°C under a natural 12-hour day/night cycle and had free access to chow and water ad libitum. The experimental protocol was reviewed and approved by the local ethical committee of the Faculty of Medicine, Zagazig University, Egypt, and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (No. ZU-IACUC/3/F/17/2018).

Experimental Design:
The rats were randomly divided into 5 groups, each containing 6 rats:
- **Group A (Control):** received a single intraperitoneal (IP) injection of sodium citrate buffer (0.1 M, pH 4.5) as a vehicle for 4 weeks.
- **Group B (Diabetic):** received a single IP injection of 50 mg/kg of STZ dissolved in citrate buffer (*Kamboj et al., 2008*).
- **Group C (Diabetic/Antox):** rendered diabetic as in group B, then supplemented orally with Antox (10 mg/kg/day) for 4 weeks (*Amal and Mona, 2009; Helal et al., 2011*).
- **Group D (Diabetic/Insulin):** rendered diabetic as in group B, then received a single subcutaneous (SC) injection of insulin 1 IU/kg/day for 4 weeks (*Qinna and Badwan, 2015*).

- **Group E (Diabetic/Antox/Insulin)**: was induced with type 1 diabetes mellitus by a single IP injection of STZ, as described in group B. After confirmation of diabetes, the rats received Antox and Insulin in the same doses as groups C and D, respectively, for four weeks.

Induction of type 1 DM was confirmed within 48-72 hours post injection when the blood obtained from the animals' tail vein had a glucose level of 200 mg/dL or higher using a glucometer (AccuChek, Germany).

Necropsy and tissue sampling:

At the end of the experimental period, the rats in each group were anesthetized with a single intraperitoneal injection of thiopental (50 mg/kg BW). Blood samples were collected from the retro-orbital venous plexus of each rat, with 1 mL of blood collected in an EDTA tube for measuring glycosylated hemoglobin and another 2 mL of blood collected in a sodium fluoride tube for measuring glucose. The blood samples were left to clot at room temperature, and then these clotted blood samples were centrifuged at 3000 rpm for 15 minutes to obtain the serum, which was stored at -20°C until the glucose level measurement. Afterwards, the rats were immediately decapitated, and their skull vaults were removed, and the whole brains were carefully excised outside the skulls. Three brain specimens from each group were kept on plates of ice-cold saline, immediately frozen at -80°C, and processed later for dissection and homogenization of the hippocampi. The other three brain specimens from each group were kept in 10% neutral buffered formalin (NBF) for 2 hours to be hardened. Then, these specimens were divided midsagittally and immersed again in NBF for 24 hours to be fixed and later processed for staining by H&E and for immunohistochemical analysis of GFAP and P53 reactivity (*Ramos-Vara et al., 2008; Bancroft and Layton, 2018*).

Determination of serum glucose and glycosylated hemoglobin (HbA1c):

The fasting serum glucose was measured using the glucose oxidase method. Additionally, glycosylated hemoglobin (HbA1c) was measured in EDTA-blood samples using a rat HbA1c ELISA kit and expressed as a percentage of the total hemoglobin.

Homogenization of hippocampal specimens:

The hippocampal specimens were homogenized at 15% (w/v) in ice-cold potassium phosphate buffer (PBS, pH 8) using a Polytron 300 D homogenizer. The homogenate was then centrifuged at 11,000 x g for 20 minutes at 4°C, and the supernatant was stored in aliquots of 100 µL at -80°C.

Assay of lipid peroxidation and antioxidant enzyme activities in the hippocampal tissue:

The supernatant of the homogenized hippocampal tissue was analyzed for spectrophotometric evaluation of MDA, a lipid peroxidation product, using the method described by *Draper and Hadley (1990)*. Additionally, the supernatant was analyzed for SOD activity using the method described by *Spitz and Oberley (1989)* and GPX activity using the method described by *Ellman (1959)*.

Real time PCR detection of TNF-α gene in the hippocampal tissue:

The TNF-α gene in the supernatant of the homogenized hippocampal tissue was evaluated by Real-time PCR using the following primer pairs (**Table 1**). The expression level of the TNF-α gene was normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the standard control. The following primer pairs were used (**Table 1**).

Table (1): Sequences of primers used in RT-PCR reactions.

	Forward	Reverse
TNF	5'- CCACCACGCTCTTCTGTCTAC-3'	5'-ACCACCAGTTGGTTGTCTTTG-3'
GAPD	5'-AGATCCACAACGGATACATT-3'	5'-TCCCTCAAGATTGTCAGCAA-3'

Histological studies:

Hematoxylin and eosin stain (H&E):

The NBF-embedded midsagittal brain specimens were dehydrated in ascending grades of ethanol then cleaned in xylene and impregnated in paraffin wax forming paraffin blocks from which paramedian sections of 5 μm-thick were cut using a microtome, mounted on glass slides, and stained with Hematoxelin and Eosin (H&E) for routine histological examination of the hippocampus *(Bancroft and Layton, 2018)*. As the functional neuronal circuits and connections of the hippocampus is known to be mainly between CA1 and CA3 and other brain areas *(Brivanlou et al., 2004)*, therefore in this study we will focus on the histological changes that may occur in these two subregions of the hippocampus under the effect of diabetes. Histological sections were viewed under a light microscope (Leica, DM-500, Microsystems; AG, Heerbrugg, CH-9435, Switzerland) and photographed using Leica digital camera (ICC50, W camera) in the Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University.

Immunohistochemical stains of GFAP and P53:

The paraffin blocks of the brain specimens were processed for immunohistochemical detection of GFAP and P53. Paraffin sections of 4 μm thick were cut and mounted on positively charged slides, deparaffinized in xylene, hydrated in descending grades of ethanol, and placed in citrate buffer (pH 6.0). The sections were incubated in a microwave oven to unmask the antigen and treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Additionally, the slides were treated with goat serum to block nonspecific binding sites for antibodies. Rabbit anti-rat polyclonal GFAP primary antibodies, dilution 1:100 (Lab Vision Corporation, Medico Co., Egypt) and P53 primary antibodies, dilutions 1:80 (Dako, Glostrup, Denmark), were then applied. The slides were incubated with 2% biotinylated goat anti-rabbit secondary antibodies for 30 minutes, followed by the addition of a streptavidin-peroxidase labeling reagent. Diaminobenzidine (DAB) chromogen solution was added to stain the antigen-antibody reaction, and the sections were counterstained with Mayer's hematoxylin. The positive immunostaining reaction of GFAP was identified under the Leica DM-500 light microscope as brown coloration of the astrocyte cell membrane and cytoplasm. Positive immunostaining reaction of P53 was identified as brown coloration in the nuclei of the neuronal cell bodies, indicating positive apoptotic neurocytes *(Ramos-Vara et al., 2008)*.

Morphometrical study:

From each group, six non-overlapping high-power fields at a magnification of ×400 from the immunohistochemically-stained sections of GFAP and P53 were morphometrically analyzed using the Image J analyzer computer system (Wayne Rasband, NIH, Bethesda, Maryland, USA) in the Anatomy and Embryology Department, Faculty of Medicine, Zagazig University. The area % of GFAP and the number of positive immune P53 cells in the pyramidal cell layer were calculated. The obtained data from each group were quantitatively presented as Mean±SD and statistically analyzed.

STATISTICAL ANALYSIS:

The statistical analysis was performed using SPSS version 19 for Windows (SPSS Inc, Chicago, IL, USA). The numerical variables were expressed as Mean±SD and were checked for normality using the Shapiro-Wilk test. The statistical difference between groups in normally distributed variables was determined using one-way analysis of variance (ANOVA), followed by post-hoc least significant difference (LSD) test for multiple comparisons. A p-value less than 0.05 was considered statistically significant *(Petrie and Sabin, 2005)*.

RESULTS

Regarding serum glucose and glycosylated hemoglobin (HbA1c)

The mean serum glucose (mg/dl) and HbA1c (%) in Group B (Diabetic) was significantly higher compared to Group A (Control). However, they decreased significantly in group C (Diabetic/Antox), D (Diabetic/ insulin), and E (Diabetic/Antox/Insulin) compared to the group B (diabetic), however serum glucose levels and HbA1c (%) were

still significantly higher in group C and D than in the control group. The pronounced amelioration of serum glucose level and HbA1c (%) was in group E that exhibited insignificant difference with the control group **(Table 2)**.

Regarding lipid peroxidation and antioxidant enzyme activities in the hippocampal tissue

The mean MDA (nmol/mg protein) in Group B was significantly increased compared to Group A (Control). However, it decreased significantly in groups C, D and E compared to group B (Diabetic). However, MDA levels were still significantly higher in groups C and D than in the control group, while there was insignificant difference in MDA level between group E and the control group **(Table 3)**.

The mean SOD and GPx (U/mg protein) significantly decreased in group B in comparison with group A. However, they increased significantly in groups C, D, and E compared to the group B (Diabetic), however SOD and GPx levels were still significantly lower in both C and D groups compared to the control group, while an obvious improvement of SOD level and GPx was in group E that revealed insignificant difference with the control group **(Table 3)**.

Table (2): Serum glucose and glycosylated hemoglobin (HbA1c) in all animal groups (mean±SD)

	Group A	Group B	Group C	Group D	Group E	F test	P-value
Glucose (mg/dl)	96.4±8.44	436.4±51.81*	248.8±33.87*#	138.4±17.15*#	123±13.2#	102.6	P<0.001
HbA1c (%)	2.99±0.64	10.42±1.22*	7.75±1.12*#	4.92±1.30*#	3.53±0.81#	43.21	P<0.001

Values are expressed as Mean±SD of 6 rats of each group. Statistical analysis was carried out using One-way ANOVA followed by LSD post-hoc test. Statistical significance was set at p value<0.05. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/Antox/Insulin).

* significant p value when compared with the control group   # significant p value when compared to the diabetic group.

Table (3): MDA, SOD, and GPX in the hippocampal tissue in all animal groups (mean±SD).

	Group A	Group B	Group C	Group D	Group E	F test	P-value
MDA (nmol/mg protein)	478.62±13.08	910.59±32.73*	644.06±32.29*#	657.2±13.90*#	554.62±16.13#	242.8	P<0.001
SOD (U/mg protein)	33.6±2.7	21.04±2.30*	27.77±1.80*#	27.62±3.02*#	32.18±2.50#	19.26	P<0.001
GPX (U/mg protein)	60.85±2.19	37.78±6.20*	55.48±2.28*#	55.72±2.70*#	60.21±2.03#	42.95	P<0.001

Values are expressed as Mean±SD of 6 rats of each group. Statistical analysis was carried out using One-way ANOVA followed by LSD post-hoc test. Statistical significance was set at p value<0.05. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/Antox/Insulin).

* significant p value when compared with the control group.   # significant p value when compared to the diabetic group.

Regarding TNF-α gene expression in the hippocampal tissue:

The relative TNF-α mRNA expression in the hippocampal tissue, the Diabetic group (Mean±SD=6.86±0.98) displayed a significant rise (P value<0.001) ~7 folds increase compared with the Control group (Mean ± SD=0.89±0.12). The Diabetic/Antox (Mean±SD=3.0±0.57), Diabetic/Insulin (Mean±SD=3.27±0.61), and Diabetic/Antox/Insulin (Mean±SD= 0.90± 0.08) were associated with a significant decrease (P value<0.001) of TNF-α mRNA expression (~2.5,~2,~7 folds, respectively) compared to the diabetic group. The marked improvement of the relative TNF-α mRNA expression was in Diabetic/Antox/Insulin group **(Fig. 1)**.

Results of hematoxylin and eosin stained-sections:

Examination of the rat hippocampus of the group A (Control group) revealed the hippocampus proper or Cornu Ammonis (CA) and the dentate gyrus (DG). The CA had four subregions (CA1, CA2, CA3, and CA4. In this study, CA1 and CA3 were histologically described in all groups.

Each subregion of CA1 and CA3 of the Control group (group A) revealed three layers; polymorphic layer (POL) contained glial cell nuclei, pyramidal cell layer (PCL) enclosed pyramidal cells that appeared small compacted in CA1 and large loosely packed in CA3., the pyramidal cells had scanty cytoplasm and large rounded nuclei with prominent nucleoli and molecular layer (ML) contained glial cells and the apical dendrites of the pyramidal cells. A narrow zone (stratum lucidum) was present only in CA3. **(Fig. 2)**.

CA1 and CA3 of the group B (Diabetic group) showed disruption of their three layers. POL displayed deeply stained glial cell nuclei and dilated blood vessels. PCL displayed shrunken pyramidal cells with deeply stained elongated nuclei. Many perineural glial cells can be observed around the degenerated pyramidal cells. ML had many deeply and lightly stained glial cell nuclei. In addition, CA3 displayed disrupted fibers of SL **(Fig. 3)**.

A1 and CA3 of the group C (Diabetic/Antox) exhibited deeply and lightly stained glial cell nuclei with wide pericellular halo in POL.

The PCL showed most pyramidal cells appear normal while few cells were degenerated with shrunken deeply stained elongated nuclei. ML had deeply and lightly stained glial cell nuclei with wide pericellular space **(Fig. 4)**.

CA1 and CA3 of the group D (Diabetic/Insulin) showed POL having deeply and lightly stained glial cell nuclei with wide pericellular halo alongside dilated blood vessels. PCL revealed apparently normal pyramidal cells together with few cells were degenerated. ML revealed many glial cell nuclei with wide pericellular space **(Fig. 5)**.

CA1 and CA3 of the group E (Diabetic/Antox/Insulin) displayed POL appeared normal, PCL containing apparently normal pyramidal cells with large rounded vesicular nuclei and prominent nucleoli. ML showed lightly stained, darkly stained glial cells with little pericellular space **(Fig. 6)**.

Results of immunohistochemical staining of GFAP and P53:

GFAP immunostaining:

The hippocampus was immunohisto chemically stained with the anti-GFAP antibody to clarify the reaction of astrocytes to neural degeneration in CA1 and CA3 of the different experimental groups. The control group showed few astrocytes having short thin processes, that were normally distributed among the pyramidal cells, and also few of them were seen dispersed in ML and POL layers. Group B (Diabetic group) displayed wide distribution of GFAP staining in the cytoplasm and the processes of many astrocytes that looked larger with numerous long thick processes. Group C (Diabetic/Antox) and Group D (Diabetic/Insulin) showed some astrocytes with multiple thin processes and others with thick processes among the pyramidal cells of PCL and some astrocytes are dispersed in ML and POL. Whereas, group E (Diabetic/Antox/Insulin) showed few astrocytes with thin ramified processes were among the pyramidal cells in PCL and also few astrocytes dispersed in ML and POL **(Fig. 7)**.

P53 immunostaining:

The hippocampus was immunohisto chemically stained with P53 antibody to clarify the localization of apoptotic neurons in

CA1 and CA3 regions in all groups. The positive immune reaction was located in the cytoplasm of dead neuronal cell bodies. Group A (control group) revealed a negative immunoreactivity, while group B displayed abundant P53 positive immune-stained pyramidal cells. Group C (Diabetic/Antox) and Group D (Diabetic/Insulin) exhibited moderate to mild positive reaction of the pyramidal cells. Whereas group E (Diabetic/Antox/Insulin) showed a negative immunoreactivity of the pyramidal cells **(Fig. 8)**.

Morphometrical results

GFAP immunohistochemical staining:

Statistical analysis of Area% of GFAP immunoreactivity in CA1 and CA3 regions revealed a significant increase (p <0.001) in group B compared to group A.

Group C and D exhibited a significant decrease (p <0.001) of the area% of GFAP in comparison to group B. Concomitant administration of antox with insulin in group E exhibited a significant decline (p <0.001) in the area% of GFAP immunoreactivity in comparison to group B **(Fig. 9)**.

P53 immunohistochemical staining:

The quantitative analysis of the number of P53 immune-stained neurons in CA1 and CA3 regions showed a significant rise (p<0.001) of the number in group B in comparison with group A. Group C and D exhibited a significant decline (p <0.001) of the number in comparison to group B. Co-administration of antox with insulin in group E exhibited a significant decline (p <0.001) in the number of P53 immune-stained neurons in comparison to group B and a non-significant difference (p >0.05) with group A **(Fig. 10)**.
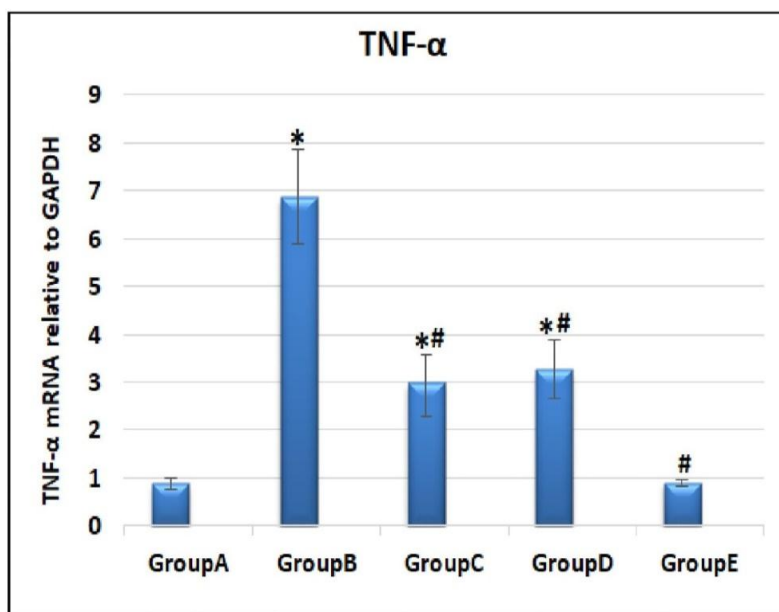

Fig. (1): Bar charts of Antox effect on relative TNF-α mRNA expression level (normalized to the corresponding GAPDH mRNA level) in the hippocampal tissue in all groups. Values are expressed as Mean±SD of 6 rats of each group. Statistical analysis was carried out using One-way ANOVA followed by LSD post-hoc test. Statistical significance was set at p value<0.05. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/Antox/Insulin).
** significant p value when compared with the control group.*
*# significant p value when compared to the diabetic group.*
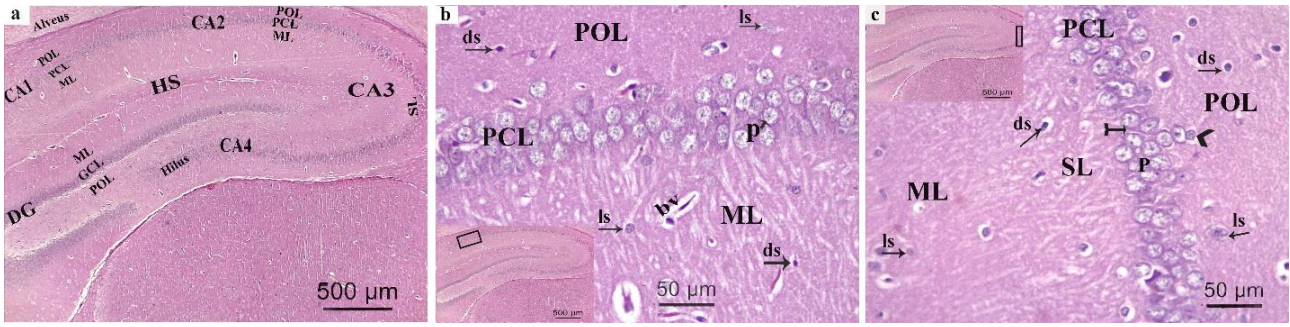
Fig. (2): Control Group (a-c): (a) *A photomicrograph of a paramedian section of a rat hippocampus showing: Cornu Ammonis with its different subregions, CA1, CA2, CA3, CA4, each subregion has polymorphic cell layer (POL), pyramidal cell layer (PCL), and Molecular cell layer (ML), in addition a stratum lucidum (SL) was present only in CA3. Dentate Gyrus (DG) has three layers; molecular layer (ML), granule cell layer (GCL) and polymorphic layer (POL). Hippocampal Sulcus (HS) separates Cornu Ammonis from DG. Alveus of the hippocampus can be seen* ***(b)*** *A higher magnification of the boxed area in the inset showing the three layers of CA1; POL that displays deeply stained (ds) and lightly stained (ls) glial nuclei, PCL displays pyramidal cells (P) with large rounded vesicular nuclei and prominent nucleoli. ML displays ds and ls glial nuclei, and blood vessel (bv)* ***(c)*** *A higher magnification of the boxed area in the inset showing the three layers of CA3. POL displays ls and ds glial cell nuclei. Stratum Lucidum layer (SL) can be observed. PCL displays pyramidal cells (P) with large rounded nuclei and scanty cytoplasm. Perineural glial cells and apical dendrite (bifid arrow) can be observed (arrowhead). Molecular layer (ML) exhibits Is and ds nuclei of glial cells.*
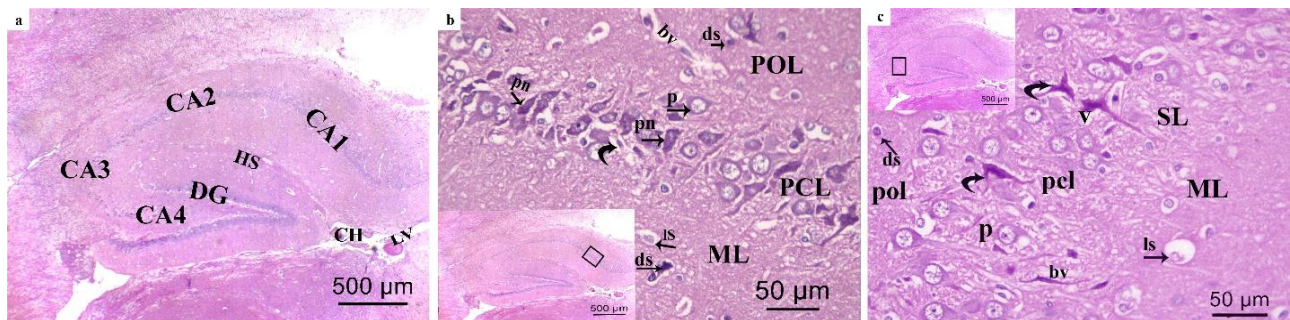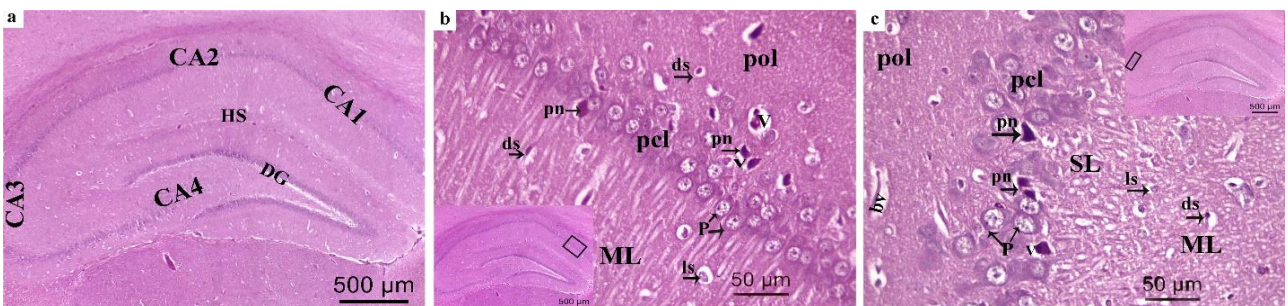

Fig. (3): Diabetic Group (a-c): (a) *A photomicrograph of a paramedian section of a rat hippocampus showing CA1, CA2, CA3, CA4, DG, HS and dilated lateral ventricle (LV) With choroid plexus (CH).* ***(b)*** *A higher magnification of the boxed area in the inset showing CA1 with its three layers: POL has deeply stained (ds) glial cell nuclei and dilated blood vessel (bv). Most cells of PCL have shrunken deeply stained elongated nuclei (pn); some pyramidal cells have large lightly stained normal nuclei (P) Perineural glial cells nuclei can be observed around the degenerated neurons (curved arrow). ML has deeply (ds) and lightly stained (ls) nuclei* ***(c)*** *A higher magnification of the boxed area in the inset of CA3 showing Stratum Lucidum (SL) with its disrupted fibers. PCL reveals most pyramidal cells are disturbed and degenerated with shrunken deeply stained elongated nuclei (curved arrow) with pericellular vacuolation (v). Few pyramidal cells having large lightly stained normal nuclei (p). ML have lightly stained glial nuclei (ls) with wide pericellular space. POL exhibits deeply stained nuclei (ds) of glial cells.*
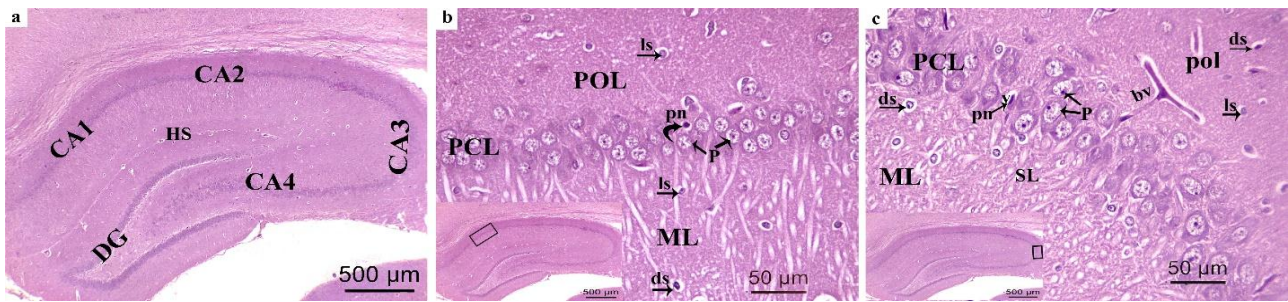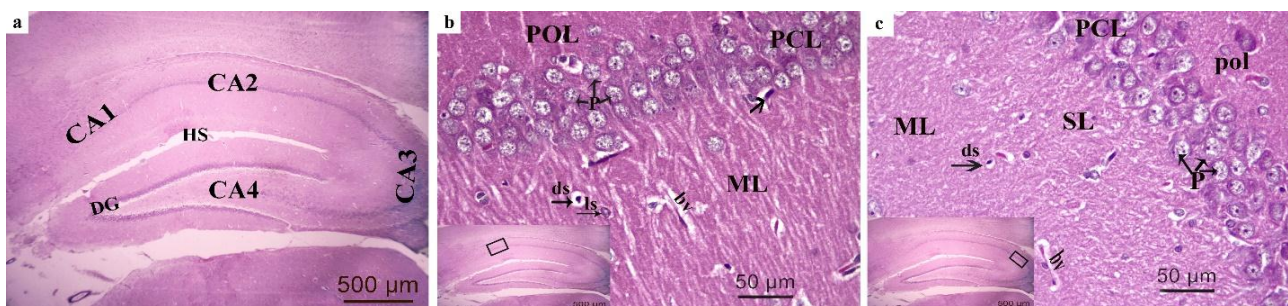
Fig. (4): Diabetic/Antox Group (a-c): (a) *A photomicrograph of a paramedian section of a rat hippocampus showing DG, CA1, CA2, CA3, CA4 and HS.* ***(b)*** *A higher magnification of the boxed area in the inset showing CA1 with its three layers POL has lightly stained nuclei (ls) with wide pericellular haloes. PCL displays apparently normal pyramidal cells (P) and few degenerated cells with shrunken deeply stained elongated and pyknotic (Pn) nuclei. ML exhibits deeply (ds) and lightly stained (ls) glial cells nuclei.* ***(c)*** *A higher magnification of the boxed area in the inset showing CA3 with its three layers, (ML) with deeply (ds) stained nuclei with wide pericellular spaces. PCL reveals most pyramidal cells (P) are apparently normal with lightly stained cytoplasm and few cells are degenerated having pyknotic nuclei (pn) with pericellular vacuolation (V). Stratum lucidum (SL) appears slightly disturbed. The polymorphic layer (POL) exhibits a slightly dilated blood vessel (bv), ds and ls glial cells nuclei.*


Fig. (5): Diabetic/insulin Group (a-c): (a) *A photomicrograph of a paramedian section of a rat hippocampus showing DG, CA1, CA2, CA3, CA4 and HS.* ***(b)*** *A higher magnification of the boxed area in the inset showing CA1 with its three layers; POL has deeply stained nuclei (ds) with wide pericellular space. PCL has apparently normal pyramidal cells (P) with few degenerated cells having shrunken deeply stained elongated pyknotic (pn) nuclei with pericellular vacuolation (V). ML has lightly stained (ls) and deeply stained (ds) glial cell nuclei.* ***(c)*** *A higher magnification of the boxed area in the inset showing CA3 with its three layers and stratum lucidum (SL). ML displays ls and ds glial cell nuclei with wide pericellular space. Most pyramidal cells in PCL appear normal (P) while few cells are degenerated having pyknotic nuclei (pn) with pericellular vacuolation (V). POL exhibits blood vessel (bv) with perivascular space.*


Fig. (6): Diabetic/Antox/insulin Group (a-c): (a) *A photomicrograph of a paramedian section of a rat hippocampus showing DG, CA1, CA2, CA3, CA4 and HS.* ***(b)*** *A higher magnification of the boxed area in the inset showing the three layers of CA1. PCL exhibits normal pyramidal cells (P) with large rounded vesicular nuclei and prominent nucleoli, few cells having dark shrunken nuclei (arrow) in this layer. The molecular layer (ML) exhibits lightly (ls), darkly (ds) stained glial cells nuclei with little pericellular spaces and blood vessel (bv).* ***(c)*** *A higher magnification of the boxed area in the inset showing the layers of CA3; POL, PCL displays normal pyramidal cells (P) with large rounded vesicular nuclei and prominent nucleoli, normal SL and ML having darkly stained (ds) glial nuclei with apparently normal blood vessel (bv).*
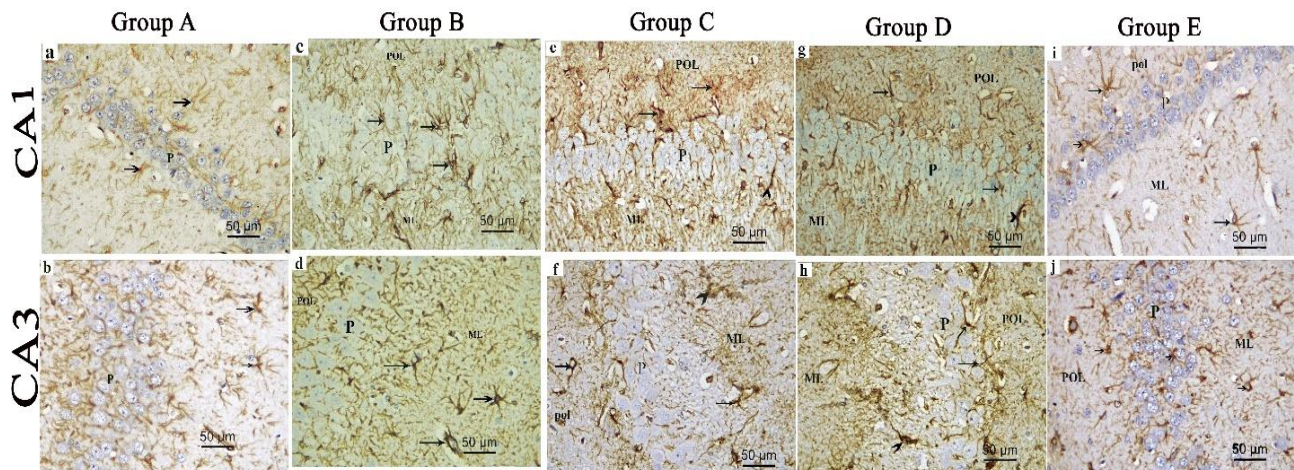
Fig.(7) (a-j): Photomicrographs of GFAP immune-stained sections of the rat hippocampus in all experimental groups. *Control group (a, b) showing astrocytes (arrow) with thin short processes among the pyramidal cells (P) in CA1 and CA3 regions,* ***Diabetic group (c, d)*** *displaying many astrocytes with multiple thick long processes (arrows) among the pyramidal cells (P) in CA1 and CA3 regions,* ***Diabetic/Antox group (e, f)*** *showing some astrocytes with thin processes (arrows) and others with thick processes (arrow head) among the pyramidal cells (P) in CA1 and CA3 regions,* ***Diabetic/Insulin group (g, h)*** *illustrating astrocytes with thin ramified processes (arrow) among the pyramidal cells (P) in CA1 and CA3 regions,* ***and Diabetic/Antox/Insulin group (I, j)*** *showing astrocytes (arrow) with thin thin short processes among the pyramidal cells (P) in CA1 and CA3.*
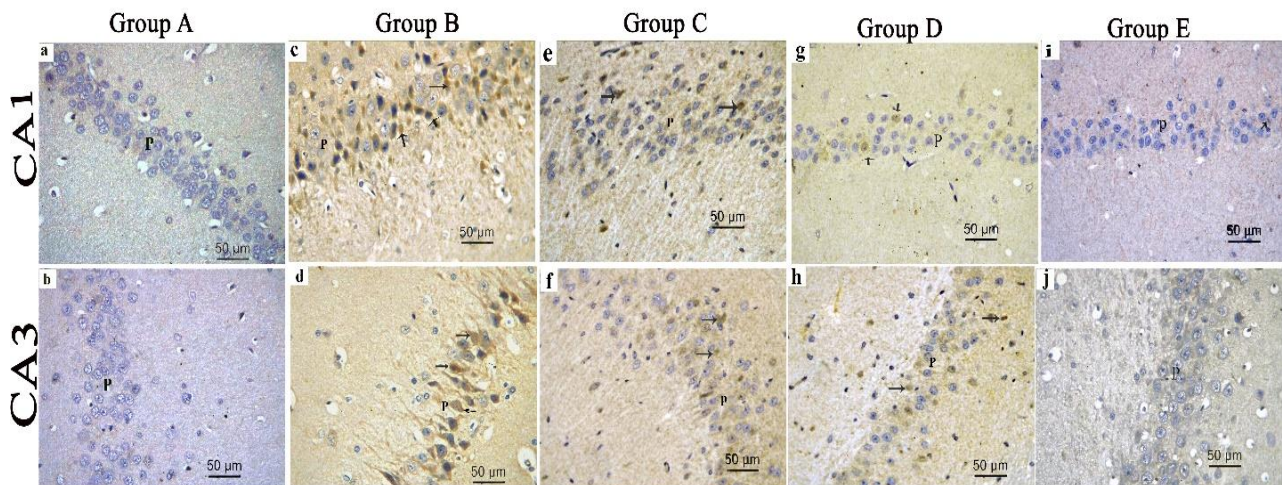
Fig. (8) (a-j): Photomicrographs of P53 immune-stained sections of the rat hippocampus in all experimental groups; *Control group (a, b) showing negative reaction of the pyramidal cells (P) in CA1 and CA3 regions,* ***Diabetic group (c, d)*** *illustrating strong positive immunoreactivity (arrow) in the cytoplasm of most pyramidal cells (P) in CA1 and CA3 regions.* ***Diabetic/Antox group (e, f)*** *demonstrating positive reaction (arrow) in the cytoplasm of some pyramidal cells in CA1 and CA3 regions,* ***Diabetic/Insulin group (g, h)*** *displaying positive immunoreactivity (arrow) in the cytoplasm of few pyramidal cells (P) in CA1 and CA3 regions, and* ***Diabetic/Antox/Insulin group (I, j)*** *showing negative reaction in most pyramidal cells (P) in CA1 and CA3 regions.*
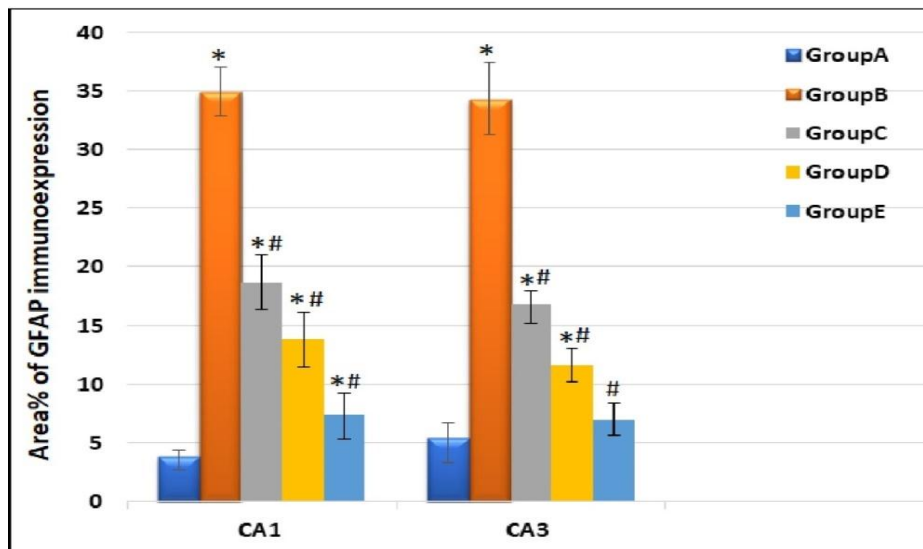
Fig. (9): Histograms demonstrating the Area % of GFAP immunoexpression in CA1, CA3 of different experimental groups. *Values are expressed as Mean±SD of 6 fields of each group. Statistical analysis was carried out using One-way ANOVA followed by LSD post-hoc test. Statistical significance was set at p value<0.05. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/ Antox/Insulin).* * *significant p value when compared with the control group.* # *significant p value when compared to the diabetic group.*
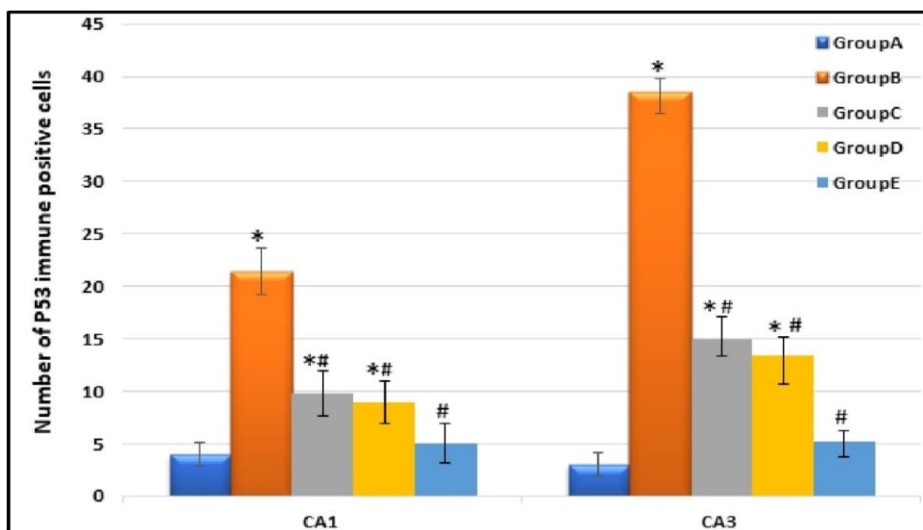
Fig. (10): Histograms showing the quantitative analysis of the number of p53 immune positive neurons in CA1 and CA3 of different experimental groups*. Values are expressed as Mean±SD of 6 fields of each group. Statistical analysis was carried out using One-way ANOVA followed by LSD post-hoc test. Statistical significance was set at p value<0.05. A (Control), B (Diabetic), C (Diabetic/Antox), D (Diabetic/Insulin), and E (Diabetic/Antox/Insulin).* * *significant p value when compared with the control group.* # *significant p value when compared to the diabetic group.*

DISCUSSION

Much evidence suggests that hippocampal injury with cognitive decline constituents an emerging complication of DM. Patients suffering from diabetes are at two times higher risk of developing cognitive dysfunction as compared with normal individuals *(Jash et al.,2020 ; Lin et al.,2022)*.

Neurodegeneration and synaptic loss in the hippocampus, which are correlated with cognitive impairment. Immunohistochemical analysis of GFAP and P53 showed increased expression of GFAP, a marker of astrocyte activation, and P53, a marker of apoptosis, in the hippocampus of the diabetic group compared to the control group. These findings suggest that diabetes may induce astrocyte activation and neuronal apoptosis in the hippocampus. Biochemical analysis revealed increased levels of MDA and decreased levels of SOD and GPX activities in the hippocampal tissue of the diabetic group, indicating oxidative stress-induced damage. Molecular analysis showed upregulation of TNF-α gene expression in the diabetic group compared to the control group, indicating inflammation-induced damage. Overall, these results suggest that diabetes induces hippocampal injury through multiple mechanisms, including oxidative stress, inflammation, astrocyte activation, and neuronal apoptosis, leading to cognitive dysfunction. Buthippocampal neuronal cell loss of the hippocampus which could be a vital contributor to memory and learning problems *(Li et al., 2002)*.

The hippocampus is highly vulnerable to elevated blood sugar levels, leading to structural and functional changes in individuals with T1DM *(Pamidi, 2012; Foghi and Ahmadpour, 2013; Sadeghi, 2016)*. Research has shown that hyperglycemia impairs neurogenesis in the CA1, CA3, and DG regions of the hippocampus, disturbs cell proliferation, and induces neuronal necrosis and apoptosis in the CA3 and DG regions *(Ahmadpour et al., 2010; Choi, 2009; Zhang et al., 2008)*.

Additionally, electrophysiological studies have demonstrated that hyperglycemia decreases synaptic plasticity in hippocampal neurons in cases of DM *(Trudeau et al., 2004)*. Therefore, the chronic hyperglycemia caused by our experimentally induced T1DM rat model is believed to be the proposed mechanism behind the histoarchitecture changes in the hippocampus.

Our study found that insulin and/or Antox significantly improved glycemic control in diabetic rats, leading to the normalization of serum glucose levels and HbA1c. This improvement in glycemic control may have an indirect role in halting diabetic-associated cognitive deficits. Hyperglycemia has been found to cause the formation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other oxidative stress markers *(Cheong et al., 2020)*. Increased oxidative stress has been linked to the development of cognitive deficits and Alzheimer's disease-like states in both humans and experimental animals *(Fukui et al., 2002)*. Therefore, the ingoing hippocampal structural changes in diabetic rats may be caused by a concomitant redox imbalance, with a significant increase in the oxidative stress biomarker MDA and a decrease in the oxidative enzymes SOD and GPX in the hippocampal tissue homogenate.

The current study found that supplementation with Antox (a combination of vitamins A, C, E, and selenium) mitigated oxidative stress in diabetic rats, resulting in a significant decrease in MDA levels and a decrease in SOD and GPX in the hippocampal tissue. The beneficial antioxidant effect of Antox may be the cause of the improvement in the cytoarchitecture changes of the hippocampi observed in the diabetic group.

The primary role of multivitamins is to address nutritional deficiencies and ensure that diabetic individuals meet their daily requirements for essential nutrients. In addition, these multivitamins may have antioxidant properties that help to scavenge free radicals and reduce oxidative stress, which could have a potential impact on diabetic outcomes *(Rosyid, 2022)*. Vitamin deficiencies may be caused by low dietary intake or malabsorption due to diabetic enteropathy, which is a common complication of diabetes *(Yahaya et al., 2021)*.

Diabetic patients require a range of vitamins and minerals to support normal physiological functions. These include vitamins A, C, D, E, and B complex vitamins such as thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, cyanocobalamin, and folic acid. Additionally, some minerals are essential for diabetic patients, including selenium, chromium, vanadium, zinc, magnesium, and manganese *(Yahaya et al., 2021; Rosyid, 2022)*. Supplementation with these vitamins and minerals, either through a multivitamin or individual supplements, may help to address nutrient deficiencies and potentially improve diabetic outcomes.

Antox is a multivitamin compound containing vitamin A, C, E, and selenium. Each of these vitamins and minerals play an important role in supporting normal physiological function and potentially improving diabetic outcomes. Vitamin A has been shown to enhance beta-cell regeneration and improve glucose metabolism. B-vitamins, including thiamin, riboflavin, niacin, pyridoxine, cobalamin, and folic acid, may lower homocysteine levels, thereby preventing oxidative stress, endothelial dysfunction, β-cell dysfunction, and insulin resistance. Vitamin C functions as an antioxidant that reduces free radicals and enhances the generation of SOD and glutathione. It is also suspected that vitamins can inhibit glycosylated proteins, potentially preventing diabetes-related complications. Vitamin E is a fat-soluble vitamin that functions as an antioxidant, reducing free radicals, preventing lipid peroxidation, and improving insulin function *(Yahaya et al., 2021)*.

Selenium is a trace mineral that is a component of enzymes catalyzing redox reactions, such as GPX, thioredoxin reductases, and iodothyronine deiodinases. Selenium also acts as an antioxidant in the form of selenoproteins. Research has shown that selenium can elicit antioxidant and anti-inflammatory effects in patients with insulin resistance and diabetes *(Navarro-Alarcon and Cabrera-Vique, 2008)*. In addition, selenium has been found to protect against diabetes-induced oxidative stress in tissues in T1DM *(Can et al., 2005)*. Overall, Antox's combination of vitamins and minerals may

have a beneficial effect on diabetic outcomes by addressing nutrient deficiencies, providing antioxidant support, and potentially reducing oxidative stress and inflammation.

The current study found not only an increase in oxidative stress markers but also an upregulation of TNF-(mRNA expression in diabetic rats. TNF-(upregulation is known to be regulated by NF-kB, which is a modulator of reactive oxygen species (ROS) *(Kuhad et al., 2009)*. Research has shown that upregulation of pro-inflammatory cytokines in the brain of diabetic individuals plays a crucial role in cognitive decline *(Gaspar et al., 2016)*. Activation of inflammatory pathways in the hippocampus of these individuals increases their susceptibility to neurodegeneration and further cognitive decline. The literature suggests an intimate relationship between inflammation and memory impairment in diabetes *(Sharma et al., 2020; Damanik and Yunir, 2021)*. These findings highlight the importance of addressing both oxidative stress and inflammation in the management of cognitive impairment in individuals with diabetes.

Studies in diabetic rodents have shown that cognitive and spatial recognition memory decline is associated with hippocampal inflammation and higher levels of pro-inflammatory cytokines, including IL-1, TNF, and IL-6 *(Dinel et al., 2011)*. The main source of these cytokines is brain microglia and astrocytes, which are the primary immune cells in the brain *(Vargas-Soria et al., 2023)*. These findings suggest that inflammation in the brain, specifically in the hippocampus, may play a key role in cognitive impairment in diabetes. Therefore, reducing inflammation and pro-inflammatory cytokine levels in the brain may represent a potential therapeutic approach to mitigate cognitive impairment in individuals with diabetes.

Obviously, over expression of GFAP-immunostained astrocytes with an increase in their area percentage were observed in the hippocampal tissue of the examined diabetic sections of this study. We have observed ubiquitous GFAP-positive astrocytes that appeared multiple with numerous long thick processes in CA1, CA3, diabetic rats. Biologically, GFAP is an astrocyte-specific

protein which belongs to the class-III superfamily intermediate filaments that form a part of the cytoskeleton of the mature astrocytes and provides a structural support to these cells *(Mandour et al., 2021)*. Actually, astrocytes were attracted together with microglia by the aggregated Aβ plaques *(Fakhoury, 2018)*. Once recruited, astrocytes started their unique response "reactive astrocytosis" which is a non-specific central inflammatory process, where the astrocytes become activated and proliferated as a response to the insult involving the astrocytes-nearby neurons *(Li et al., 2019)*. In this process, the reactive astrocytes respond detrimentally by releasing pro-inflammatory cytokines (including IL-1β, TNF-α and NO) and neurotoxins such as Lipocalin-2 (Lcn2) *(Liddelow and Barres, 2017; Li et al., 2019)*.

The current study found that insulin and/or Antox administration resulted in a significant decrease in GFAP immunoreactivity and TNF-(mRNA expression in diabetic rats, indicating a decline in the number of astrocytes and a decrease in astrocytosis-induced inflammation. Interestingly, Antox had a synergistic and adjuvant role with insulin in mitigating inflammation due to its multi-antioxidant capabilities. The coadministration of Antox with insulin had a superior effect over Antox or insulin alone, indicating the potential collaboration of antioxidant vitamins with antidiabetic therapy in mitigating the cognitive deficit associated with diabetes. These findings suggest that the combination of insulin and Antox may be a promising therapeutic approach for addressing the inflammation and cognitive impairment associated with diabetes.

Despite the brain isn't an insulin-dependent organ, insulin can cross the blood-brain barrier (BBB) and binds to receptors on glial cells and neurons. Strong evidence has demonstrated an important role of insulin in feeding and cognitive behavior *(Banks et al., 2012)*. In relation to learning, memory and cognition, insulin causes increased glucose uptake in the neurons of the hippocampus and frontal lobes, two of the most important regions involved in memory regulation *(Arnold et al., 2018)*. Additionally, insulin decreased Aβ40 and Aβ42 levels 'two hallmarks of Alzheimer's

disease" in the plasma and brain of mice *(Swaminathan et al., 2018)*.

In this study, the hippocampal tissue of diabetic rats not only displayed oxidative stress and proinflammatory cytokines, but also showed a significant increase in the immunohistochemical reaction of P53 protein and an increase in positive cells tagged with P53 in the PCL of the hippocampus. The p53 gene encodes for the p53 protein, which is a tumor-suppressor protein that plays a crucial role in DNA repair, cell-cycle arrest, senescence, cell death, and apoptosis. P53 has the ability to limit cellular proliferation and suppress abnormally proliferating cells from becoming malignant by increasing the transcription of various target genes involved in the induction of cell cycle arrest and/or apoptosis. Therefore, the p53 gene is considered the most significant gene for tumor suppression, and it is famously termed 'The Guardian of the Genome' *(Shen and White, 2001; Maximov and Maximov, 2008)*.

The mechanism by which p53 exerts its effects appears to be multifactorial and incompletely understood. However, apoptosis of cells exposed to environmental or oncogenic stress constitutes the major mechanism whereby p53 exerts its tumor suppressor function. Under normal conditions, p53 is maintained at a low level, but it is activated in response to various cellular stresses, including DNA damage, oxidative stress, hypoxia, and mitogenic oncogenes *(Shen and White, 2001; Maximov and Maximov, 2008)*. Once activated, p53 induces the activation of the intrinsic pathway of apoptosis via activation of proapoptotic proteins, including BAX, BID, and BAX, and inhibition of antiapoptotic proteins, such as Bcl-2 and Bcl-XL *(Hockenbery et al., 1990)*. The p53 protein also activates the extrinsic pathway of apoptosis via activating cell-surface death receptors, such as Fas (CD95), a member of the TNF-R family of receptors, and directly activates caspase 8 *(Pelengaris and Khan, 2006)*. These findings suggest that the increase in P53 protein and positive cells in the hippocampus of diabetic rats may reflect a response to DNA damage caused by oxidative stress and inflammation, potentially contributing to the cognitive decline associated with diabetes.

The upregulation of p53 expression observed by immunohistochemical studies in the hippocampus of diabetic rats may serve as a prognostic indicator of diabetic-induced oxidative stress in the hippocampal tissue of these rats, as oxidative stress is a major cellular stressor that can elicit p53 activation. Furthermore, Antox, being a multi-antioxidant, displayed a downregulating effect on the p53 protein in the hippocampal tissue of diabetic rats, which is an astonishing effect that may reflect an anti-apoptotic effect of this compound. These findings suggest that Antox may have potential therapeutic benefits in mitigating the cognitive decline associated with diabetes by reducing oxidative stress and inhibiting p53-mediated apoptotic pathways in the hippocampus. Further research is needed to fully understand the mechanisms underlying the effects of Antox on p53 expression and its potential therapeutic benefits.

CONCLUSION

The results of the study suggest that Antox may improve the structural and functional alterations of the hippocampus in cases of diabetes. As a multivitamin antioxidant, Antox may have potential as an adjuvant supplementary compound in the therapeutic strategy for cognitive impairment comorbidity in diabetes. However, further studies are needed to fully evaluate the potential clinical use of Antox in targeting cognitive impairment in diabetic patients. Additional research should investigate the optimal dosage and duration of treatment with Antox, as well as potential side effects or interactions with other medications. Nonetheless, the findings from this study suggest that Antox may be a promising therapeutic approach for addressing the cognitive decline associated with diabetes, and further research is warranted.

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