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Melatonin and Magnesium Restores Neurohistopathological Changes in the Hippocampus of Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Diabetic encephalopathy and its associated end organ damage have become a major global epidemic in many patients with diabetes mellitus. These diseased conditions are complex and poorly understood, therefore the need to seek for alternative management measures to attenuate the complications associated with it. The aim of this study was to evaluate the effects of co-administration of melatonin and magnesium on the cytoarchitecture of the hippocampus of streptozotocin (STZ) induced diabetic rats. STZ was used to induce type 1 diabetes mellitus. Fifty four rats: Forty eight diabetic and six normoglycaemic rats distributed in nine groups as follow; normal control, diabetic control (DC), melatonin low dose (MLD, 10 mg/kg), magnesium low dose (MgLD, 240 mg/kg), melatonin and magnesium combined dose (MMgLD, 10 mg/kg+240 mg/kg, respectively), melatonin high dose (MHD, 20 mg/kg), magnesium high dose (MgHD, 480 mg/kg), melatonin and magnesium high dose (MMgHD, 20 mg/kg+480 mg/kg, respectively) and insulin (IN, 500 mg/kg). Melatonin and insulin were administered through intraperitoneal injections while magnesium was orally. The control groups were given placebo and all treatments were for twenty-one days. Results showed distortion of hippocampal CA1 area in the diabetic control, MgLD, MgHD, MMgHD and IN groups. MLD, MHD and MMgLD groups showed organized structures of hippocampus CA1 area with no cellular distortions, while there were less positive GFAP in the MLD, MHD and MMgLD groups. The DC, MgLD. MgHD, MMgHD and IN groups showed strong GFAP reactivity. In conclusion, MLD, MHD and MMgLD increased neuroprotection of hippocampal neurocytes.

Key words: *Diabetes; Hippocampus; Astrocytes; Melatonin; Magnesium; Neuroprotection*

INTRODUCTION

Reports published by the World Health Organization (WHO) states that diabetes mellitus (DM) is a common disease with a global prevalence estimated to be 9% among adults aged 18 years and above (Bytzer et al. 2001). Population-based studies of neuropathy (inflammation and degeneration of peripheral nerves) in persons with diabetes indicates common complications of insulin-dependent diabetes mellitus and non-insulin- dependent diabetes mellitus, with 60%-70% of patients affected with the disease (Ricci et al. 2000). Subclinical neuropathy is much more common than clinical neuropathy. Distal symmetrical polyneuropathy is the most common type of neuropathy, followed by carpal tunnel syndrome, mononeuropathies and autonomic

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neuropathy (Bytzer et al. 2001).

Behavioural and cognitive changes associated with type 1 diabetes mellitus (T1DM) has gained worldwide attention. Concerns about the deleterious effects of T1DM on the central nervous system have grown with the increasing incidence of T1DM in children (Adeghate et al. 2006). Many studies have clearly shown cognitive and behavioural changes in type 1 diabetic rats and humans, which are evident in elevated levels of anxiety, depression, and slowing of mental speed and flexibility (Ramanathan et al. 1998; Brands et al. 2005; Kuhad et al. 2007; Alvarez et al. 2009).

Diabetes-induced behavioural and cognitive changes are related to several factors. Both diabetic complications and reduced central serotonin (5-hydroxytryptamine, 5-HT) synthesis and metabolism are thought to underlie behavioural and cognitive dysfunctions in patients with T1DM (Ryan and William 1993; Thorre et al. 1997). It has become evident that insulin and C-peptide deficiencies, including perturbations of their signalling cascades, leads to cerebral dysmetabolism and interference with the regulation of neurotropic factors and their receptors. Ultimately, this cascade of events leads to neuronal loss, causing profound deficits in behavioural and cognitive functions (Sima et al. 2009). However, the specific mechanisms underlying these changes and whether they relate to the duration of diabetes are unknown. Although the magnitude of most of these cognitive decrements is relatively modest, even moderate behavioural and cognitive changes can potentially hamper the day-to-day activities of both diabetic child and adult. These cognitive decrements may present problems in more demanding situations, and can critically have a negative impact on the quality of life (Sima et al. 2009).

There are many studies on this subject but none has co-administered melatonin and magnesium to ameliorate hyperglycaemia, and neuropathological changes associated with streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Materials

Plastic cages, standard rat feed, organ sample containers, centrifuge, temperature controlled refrigerator, microwave oven, water bath, humidity chamber, Leica auto processor, Leica auto stainer, Leica DM750, camera ICC50 E, AmScope D200 digital camera, MRC spectrophotometer, HP 8TH gen intel@ CoreTH i5 processor. melatonin M5250-1G (Sigma Aldrich, USA), magnesium (Randox, USA), streptozotocin (SP0130, Sigma Aldrich, USA), haematoxylin and eosin (H&E), and glial fibrillary acidic protein (GFAP) antibody (Abcam, USA) were used in the study.

Source of Animals and Management

Sixty four Normoglycaemic rats were weighing 120–150 g, were obtained from the Faculty of Pharmaceutical Sciences Animal House of the Ahmadu Bello University, Zaria for the study. The rats were maintained on *ad libitum* access to food (Standard feeds, standard rat pellets) and water, with 12/12 h day and night cycle at room temperature (20-25 ℃). All experiments were performed between 08:00 and 12:00 h.

Methods

Induction of Type 1 Diabetes Mellitus

Type 1 diabetes mellitus was induced after two weeks acclimatization period; baseline blood glucose levels, and behavioural and cognitive assessments were performed for all test animals. This was done to ensure that the animals were all normoglycaemic with no anxiety, depression and had normal locomotive functions using the elevated plus maze (data not included).

Fifty eight male Wistar rats were randomly selected the sixty four rats and induced with 55 mg/kg of STZ in citrate buffer (0.1M, pH 4.5), while six normoglycaemic male Wistar rats was used as control. The solution was used within 5 min to induce diabetes in the rats after an overnight fast of 12 h (Godam et al. 2014).

Hyperglycaemia Screening and Confirmation of T1DM

Four days after STZ-induced diabetes mellitus, blood was collected from the tail vein following an overnight fast (Meiri et al. 1997; Walther et al. 1998; Kulkarni 1999). Fasting blood sugar was measured with a standard glucometer (Optimum, Germany). The day that hyperglycaemia (200 mg/dL; 11 mmol/l) was confirmed was considered to be diabetic day 1. Rats with fasting blood glucose levels lower than 200 mg/dL were excluded from the study.

Animal Grouping and Treatment Procedure

Forty eight diabetic rats and six normoglycaemic rats were randomly divided into nine groups as shown in the Table 1, and treated with melatonin and insulin intraperitoneally, and magnesium orally for twenty one days according to the bioactive compounds and drugs dosage assigned to each group. The controls were given distilled water. Melatonin and insulin were given intraperitoneally while magnesium was administered orally.

At the end of the study (24 h after last administration, day 21) animals where sacrificed humanely using ketamine as anaesthesia intraperitoneally at a dose of 0.8 mg/kg. The rat brains were carefully removed cleared of any debris, weighed and washed in normal saline. They were further fixed in buffered formalin.

Histological and Histochemical Studies

Tissue samples were harvested and fixed in 10% buffered formalin for 72 h. The samples were grossed and labelled in tissues cassettes and processed for histology using varying concentration of alcohols (70, 80, 90 and 100%) for dehydration, cleared through three changes of toluene, infiltrated, embedded in molten paraffin wax and blocked on cold ice packs. The tissues were sectioned at 10 µm using a rotary microtome (Leica, Germany) and the ribbons obtained were picked on clean grease free Leica charged slides for histological and immunohistochemical studies. The slides were drained and heat fixed on a hot plate at 70 ℃. The tissues were further dewaxed in toluene, hydrated in decreasing concentration of alcohol, and taken to water before proceeding to the staining procedures. Portions of the hippocampus were stained using H&E methods for general histological studies (Suvarna et al. 2018).

Table 1: Rats Grouping and Dosage

Group	n	Treatment (21 days)
NC	6	2.0 mL/kg distilled water
DC	6	2.0 mL/kg distilled water
MLD	6	10 mg/kg melatonin
MgLD	6	240 mg/kg magnesium
MMgLD	6	10 mg/kg melatonin+240 mg/kg magnesium
MHD	6	20 mg/kg melatonin
MgHD	6	480 mg/kg magnesium
MMgHD	6	20 mg/kg melatonin+480 mg/kg magnesium
IN	6	Insulin (500 mg/kg)

NC (control), DC (Diabetic control), MLD (Melatonin low dose), MgLD (Magnesium low dose), MMgLD (Melatonin and magnesium low dose), MgHD (Magnesium high dose), MHD (Melatonin high dose), MMGHD (Melatonin and Magnesium high dose), IN (Insulin)

The immunohistochemical marker, glial fibrillary acidic protein (GFAP) was used to study hippocampal astrocytes. The representative sections on slides was pre-treated using heat mediated antigen retrieval in citrate buffer (0.1M, pH 6.0), placed in boiling bath for 90 min. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 30 min and later blocked using serum (normal goat serum, C-0005) at 37 ℃ for 20 min.

It was further incubated with rabbits/mouse anti-GFAP, unconjugated (GF5-ab10062) at 1:400 dilutions for 45 min. It was later conjugated to the secondary antibody (SP-0023). It was later labelled with mouse/rabbit horseradish enzyme (DB Biotech, Slovakia) for 30 min, and DAB (C-0010) for 10 min. Then stained with haematoxylin for nuclear contrast, and mounted with DPX.

The photomicrographs of sections were obtained using Leica DM750 microscope with digital Camera ICC50E LAS EZ software installed in HP 8th gen Core i5 Laptop.

RESULTS

Hippocampus Histology

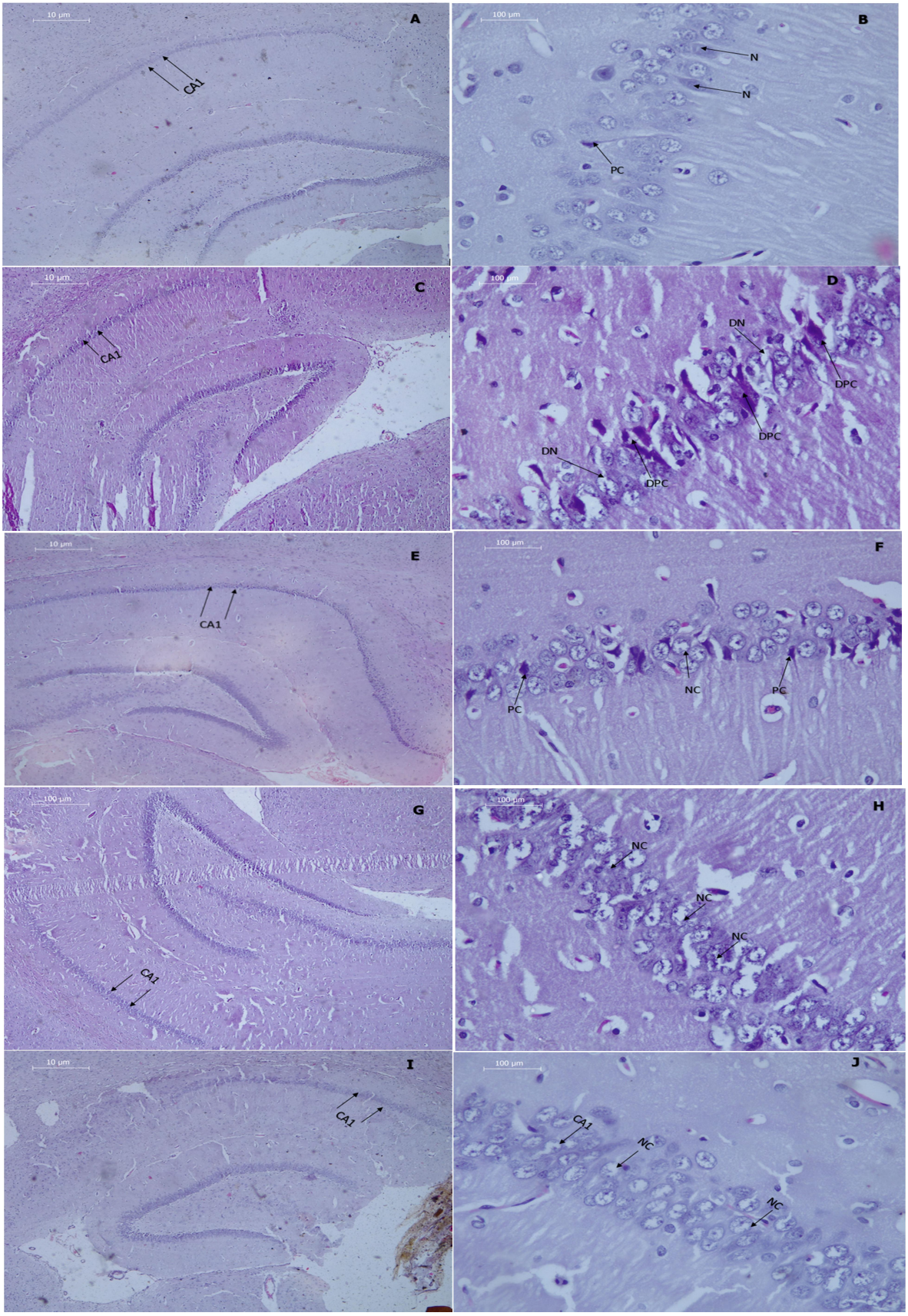
Sections of the hippocampus (CA1 area) were studied qualitatively using haematoxylin and eosin stains to characterize changes associated with STZ induced diabetes. Normal control administered distilled water showed similar normal cellular cytoarchitecture with pyramidal cells in the CA1 area of the hippocampus of the rats (Fig. 1A&B).

The hippocampus of the STZ-induced diabetic control showed disorganized CA1 area with degenerated pyramidal cells and shrunken darkly stained nuclei, wide pericellular spaces and pyknosis (Fig. 1C&D). Melatonin (10 mg/kg) treated rats showed restored hippocampal neurocytes with decreased pericellular spaces (Fig. 1E&F). Magnesium (240 mg/kg) treated rats showed distorted cellular cytoarchitecture with wide pericellular spaces (Fig. 1G&H).

Melatonin (10 mg/kg) + magnesium (240 mg/kg) treated rats showed restoration of neurocytes with less pericellular spaces and preservation of the cytoarchitecture (Fig. 1I&J). Melatonin (20 mg/kg) showed distortions of cellular cytoarchitecture and loss of neuronal cells (Fig. 1K&L). Magnesium (480 mg/kg) treated rats showed preservation of pyramidal cells and reduced cellular distortion with improved cytoarchitecture (Fig. 1M&N). Melatonin (20 mg/kg) + magnesium (480 mg/kg) treated rats showed distortions of cellular cytoarchitecture and loss of neurocytes (Fig. 1O&P). The insulin treated diabetic rats showing mild distortion of cellular cytoarchitecture and disorganization of neuronal cells (Fig. 1Q&R).

Hippocampus Astrocytes Study

The normal control group showed normal GFAP positivity (Fig. 2A&B). The diabetic control rats showed strong positive GFAP reaction with astrocytic inflammation (Fig. 2C&D). The melatonin (10 mg/kg) treated rats showed less positive GFAP with no astrocytic inflammation (Fig. 2E&F). The magnesium (240 mg/kg) treated rats showed strong GFAP positivity (Fig. 2G&H). The melatonin (10 mg/kg) + magnesium (240 mg/kg) treated rats showed less GFAP positivity (Fig. 2I&J). The melatonin (20 mg/kg) treated rats showed less GFAP positivity (Fig. 2K&L). The magnesium (480 mg/kg) treated rats showed mild positive astrocyte reaction (Fig. 2M&N). The melatonin (20 mg/kg) + magnesium (480 mg/kg) treated rats showed strong positive GFAP (Fig. 2O&P). The insulin (500 mg/kg) treated rats showed strong GFAP positivity (Fig. 2Q&R).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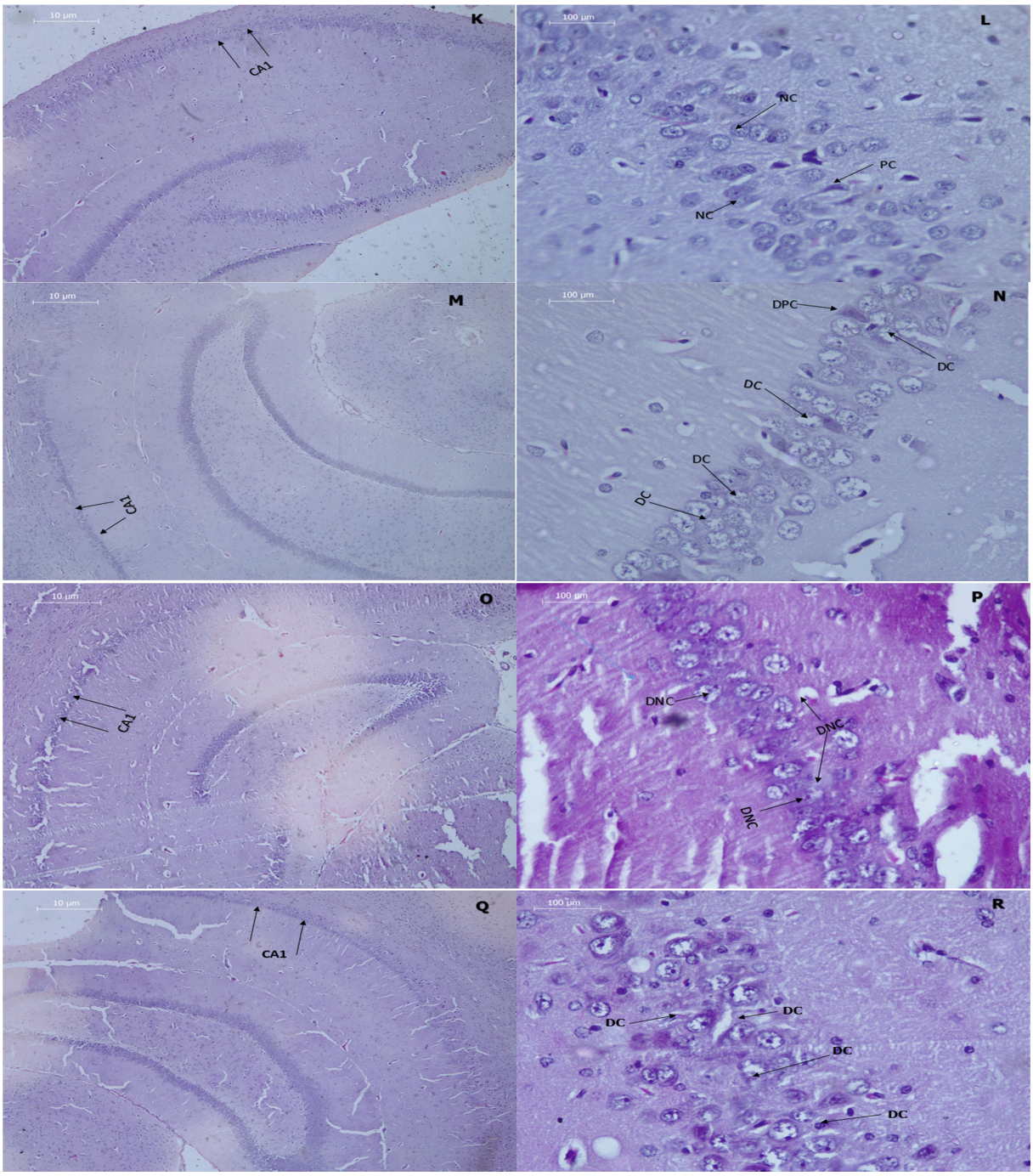

Fig. 1: Histological sections of the hippocampus (CA1) of rats (H&E, ×400). A&B: The control group with normal cytoarchitecture with pyramidal cells (black arrows). C&D: Diabetic control rats showing distorted cytoarchitecture with disorganized neurocytes, degenerated pyramidal cells and shrunken darkly stained nuclei (black arrows). E&F: Melatonin (10 mg/kg) treated rats showing restored pyramidal cells with decreased peri-cellular spaces. G&H: Magnesium (240 mg/kg) treated rats showing distorted cellular cytoarchitecture with wide peri-cellular spaces. I&J: Melatonin (10 mg/kg) + Magnesium (240 mg/kg) treated rats showing restoration of neurocytes, pyramidal cells with less peri-cellular spaces with preservation of the cytoarchitecture. K&L: Melatonin (20 mg/kg) showing distortions of cellular cytoarchitecture and loss of neuronal cells (black arrows). M&N: Magnesium (480 mg/kg) treated rats showing preservation of pyramidal cells and reduced cellular distortion with improved cytoarchitecture. O&P: Melatonin (20 mg/kg) + magnesium (480 mg/kg) treated rats showing distortions of cellular cytoarchitecture and loss of neurocytes. Q&R: Insulin (500 mg/kg) treated rats showing mild cytoarchitecture distortions and disorganizations of neuronal cells.

DISCUSSION

The hippocampus is an area of the brain that is involved in short- and long- term memory (Eichenbaum 2004; Staresina and Davachi 2009; Fuentemilla et al. 2010; Poch et al. 2011). In rats, amnesia can be caused by hippocampal dysfunction (Lyeth et al. 1990), whereas in higher animals including primate's memory loss can occur due to dysfunctions in both the hippocampus and amygdala (Mishkin et al. 1978). Diabetes has been linked to memory impairment in rats (Sacai et al. 2014) and in humans (Gravlee et al. 2011). Hypoglycaemia on the other hand can also cause hippocampal injury which could result to anterograde amnesia. In this study, STZ was used to induced diabetes (sustained hyperglycaemia) which has been proven to induce oxidative stress in the hippocampus (Lee et al (2014) resulting in apoptosis of the hippocampal synapses (Zhang et al. 2013). Hippocampal oxidative stress is associated with increased level of lipid peroxidation products (Mushtag et al. 2014) and decreased activity levels of endogenous intracellular antioxidant in diabetic rats (Moghaddam et al. 2014). Hence, in this study, light microscopic results from the control group showed normal CA1 area cytoarchitecture with pyramidal cells, which the diabetic rats administered melatonin (20 mg/kg) and melatonin (10 mg/kg) + magnesium (240 mg/kg) showed similar organized and restored cellular cytoarchitecture and pyramidal cells. However, the diabetic control rats showed distorted cytoarchitecture with disorganized cells, degenerated pyramidal cells and shrunken darkly stained nuclei which the groups administered respectively, magnesium (240 mg/kg and 480 mg/kg), melatonin (20 mg/kg) + magnesium (480 mg/kg), and insulin (500 mg/kg) were similar, with mild cytoarchitecture distortions and disorganizations of neuronal cells. Diabetes is associated with several adverse effects on the brain, some of which may result primarily from direct consequences of chronic hyperglycaemia (Amin et al. 2013).

Immunohistochemically, it was observed that the diabetic control group showed increased GFAP expression (strong positive expression), which showed neuroinflammation of astrocytes due to untreated diabetic hyperglycaemia. These findings are similar to the report by Sacli et al. (2015) which states that *in vivo* studies of STZ-induced diabetes increased blood-brain-barrier permeability with melatonin preventing the increase in blood brain barrier permeability. Melatonin inhibits matrix metalloproteinase-9 expression and also protects against the loss of tight junction proteins and blood brain barrier disruptions by providing anti-inflammatory and antioxidant mediators leading to axonal growth and regeneration. Astrocytes are proving critical for the survival of neurons in the central nervous system (CNS), playing a role in energy metabolism, maintenance of the blood-brain barrier, vascular reactivity, regulation of extracellular glutamate levels and protection from reactive oxygen species. This protect CNS cells through the uptake of excitotoxic glutamate, the production of the anti-oxidant glutathione and the neuroprotective adenosine (Swanson et al. 20004; Vargas at al. 2008; Chen et al. 2008). Alterations in astrocytes activity were associated with diabetes-related disturbances in the brain and levels of GFAP have been under debate (Baydas et al. 2003; Voskuhl et al. 2008).

Results from the normal control group in our study showed that there was normal GFAP expression in normal astrocytes. This result was similar to the melatonin treatment at 10 mg/kg, 20 mg/kg and when combined with magnesium (240 mg/kg) where we observed significant decrease in GFAP positive expression. The magnesium treated groups at 240 mg/kg, 480 mg/kg and at high dose with 20 mg/kg melatonin showed positive expression of GFAP for astrocytes. The insulin treated group showed positive GFAP expression indicating astrocytic inflammation

A report by Zhang et al. (2013) collaborates with our present findings; they demonstrated that diabetes cause debilitating changes in the hippocampus and that these changes contributed to neuronal loss. Histological findings by Lee et al. (2014), Duarte et al. (2009) and Giribabu et al. (2014) confirms our reports and previous reports of neurodegenerative changes in the hippocampus of diabetic rats. They observed histopathological necrotic changes in the Cornu Ammonis (CA) and subiculum areas of hippocampus with severe loss of pyramidal neurons and glial cells. It also collaborates with earlier results by Li et al. (2002) who reported that diabetic neuronal degeneration was due to apoptosis induced by oxidative changes due to hyperglycaemia. In our present findings, melatonin and magnesium at low dose reverses this neuronal degeneration and decreased GFAP expression with normal glial cells population in the hippocampus. There was no evidence of significant astrogliosis in melatonin and melatonin and magnesium co-treated diabetic groups.

Our report supports findings by Baydas et al. (2003) who reported that GFAP was significantly elevated in the diabetic control group compared to control group. These findings showed that untreated type 1 diabetes induced glial hyperactivity with increased GFAP in the rats' hippocampus. Baydas and colleagues attributed this to the effect of reactive oxygen species especially because oxidative stress occurs earlier in type 1 diabetes (Hoeldtke et al. 2003).

Moreover, Lebed et al. (2008) assessed GFAP for three and seven days after STZ injection using immunohistochemistry, and demonstrated that the reduced GFAP-positive cell count was found on day 3 when these cells were significantly smaller and less arborized with respect to the control. This tendency

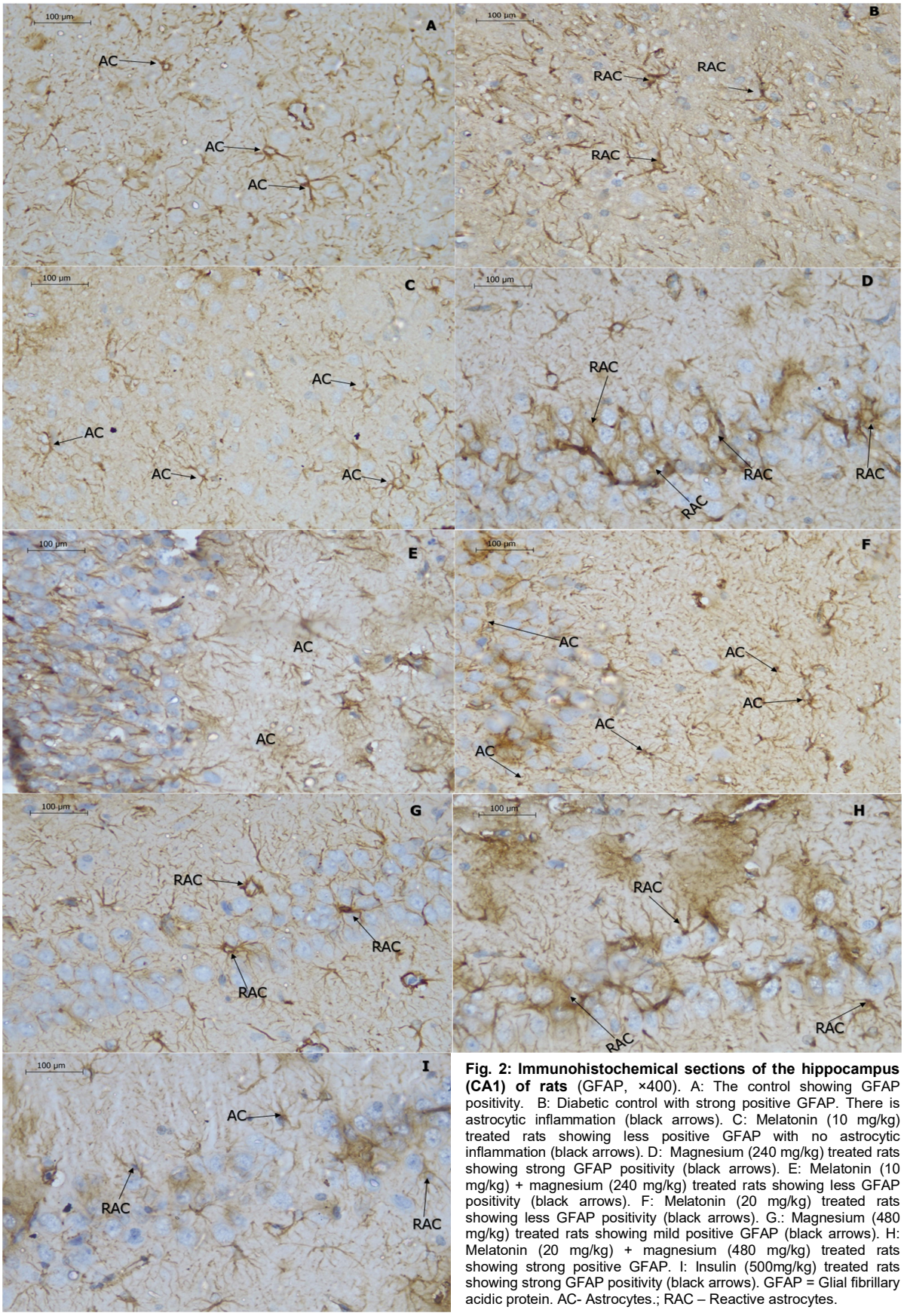

Fig. 2: Immunohistochemical sections of the hippocampus (CA1) of rats (GFAP, ×400). A: The control showing GFAP positivity. B: Diabetic control with strong positive GFAP. There is astrocytic inflammation (black arrows). C: Melatonin (10 mg/kg) treated rats showing less positive GFAP with no astrocytic inflammation (black arrows). D: Magnesium (240 mg/kg) treated rats showing strong GFAP positivity (black arrows). E: Melatonin (10 mg/kg) + magnesium (240 mg/kg) treated rats showing less GFAP positivity (black arrows). F: Melatonin (20 mg/kg) treated rats showing less GFAP positivity (black arrows). G.: Magnesium (480 mg/kg) treated rats showing mild positive GFAP (black arrows). H: Melatonin (20 mg/kg) + magnesium (480 mg/kg) treated rats showing strong positive GFAP. I: Insulin (500mg/kg) treated rats showing strong GFAP positivity (black arrows). GFAP = Glial fibrillary acidic protein. AC- Astrocytes.; RAC – Reactive astrocytes.

reversed on day 7 when more numerous GFAP-positive cells grew in size and became more ramified. Insulin in the type 1 group significantly decreased hippocampal GFAP compared to the diabetic and normal control groups. These results agree with that of Lechuga-Sancho et al. (2006), but contradict that of Coleman et al. (2010). At the end of our study (21 days of melatonin and low dose magnesium treatment) we found out that melatonin reduces astrogliosis and neuroinflammation caused by hyperglycaemia induced type 1 diabetes mellitus, improving memory and cognition in diabetic rats.

Conclusion

Melatonin administration at 10 mg/kg, 20 mg/kg and when 10 mg/kg melatonin was co-administration with 240 mg/kg magnesium ameliorates and restores hippocampal astrocytic cells inflammations; preventing diabetic encephalopathy in STZ-induced diabetic rats.

Conflict of Interest

None declared

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