The role of quercetin and some antidiabetic agents in oligodendrocyte proliferation in toxin-induced demyelination in rats

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ABSTRACT

Background: Multiple sclerosis is an inflammatory disease affecting central nervous system, and its characterized by inflammation, progressive neurodegeneration , demyelination. It is the most common causes of neurological disability in middle age.

Aim: In this study we aimed to assess the possible role of quercetin and some antidiabetic agents (pioglitazone, metformin and dapagliflozin) in remyelination process by enhancing oligodendrocyte proliferation After the demyelination induced by ethidium bromide (EB).

Methods: A group of 60 male wister rats weighing between 250-300gm were included in the study. They were randomly divided into a sham-operated group and 5 demyelination groups, each group consisting of 10 rats. Demyelination was induced through intrapontine stereotaxic injection of EB (10 µl of 0.1 % EB). The rats were then furtherly subdivided into the EB control group, the quercetin-treated group (50 mg/kg/day), the pioglitazone-treated group (10 mg/kg/day), the metformin-treated group (500 mg/kg/day), and the dapagliflozin-treated group (10 mg/kg/day). Behavioral tests, including beam balance, foot fault, rotarod, and inverted screen tests, were conducted for all groups. Immunohistochemical analysis of CC1 antibody was performed on pontine tissues to visualize mature oligodendrocyte cell bodies.

Results: the study showed deterioration of motor performance in the EB control group during the behavioral tests. Examination of pontine tissue that was immunostained for CC+1 oligodendrocyte antibodies of EB subgroup revealed limited reactivity in grey matter areas. After EB demyelination, the treated groups demonstrated a significant improvement in motor performance and a substantial increase in reactivity in all examined pontine tissue samples immunostained for CC+1 oligodendrocyte antibodies. In conclusion, quercetine, metformin, dapagliflozine, and pioglitazone displayed a neuroprotective effect and enhanced remyelination.

Keywords: Remyelination, Myelin basic protein, Multiple sclerosis, Oligodendrocyte

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease affecting the central nervous system and characterized by inflammation, progressive neurodegeneration and demyelination (1). It is a widespread cause of neurological disability, with a predominant presentation in young women (2). The most common symptom experienced by patients was weakness, with a percentage of 57%. Following this, sensory symptoms were reported by 19.9% of the patients, visual symptoms by 15.9%, and ataxia by 15.8% (3).

The main pathological feature in MS is autoimmune inflammatory response that manifests through demyelination of axons resulting in loss of saltatory conduction, physical protection and metabolic support causing neurodegeneration.(4). The classic histopathological lesions of MS are demyelinated areas called plaque, which indicates loss of myelin sheaths and oligodendrocytes in white and gray matter of the brain. The presence of these lesions results from the infiltration of immune cells such as macrophages, CD8+ T cells, a reduced amount of CD4+ T cells, as well as B cells and plasma cells (5).

The ability of the adult CNS to heal the injury is limited. This limitation also applies to fully developed oligodendrocytes, which cannot make up for the loss of myelin as they deteriorate. This demyelination initiates an automatic process for repairing myelin, known as remyelination (6).

Three main goals are being pursued in MS research: preventing the development of new demyelinating lesions, protecting demyelinated axons from degeneration, and promoting remyelination (7) . The ability for remyelination is influenced by several factors including gender, genetic makeup, age, and the existence of various inhibitors that specifically limit the potential for glial regeneration (6). This prompts investigations into possible remyelination

targets for therapy that either require overcoming inhibitors of cell differentiation or stimulating the natural oligodendrocyte response.

Ethidium bromide (EB) serves as a toxin-induced model of demyelination, producing consistent and prolonged demyelination lesions. These lesions distinctly separate the demyelination and remyelination processes, making them valuable for investigating the specific functions of individual drug molecules in the repair process (8). Due to its DNA intercalating properties, EB causes toxic effects, impacting all nucleated cells and leading to the destruction of astrocytes and oligodendrocytes within the demyelinated area (9). On the other hand, Quercetin, belongs to the flavonoids family and has antioxidant capacity due to the presence of phenolic groups in its structure (10). In addition to its biological benefits; it is significantly protecting neuronal cells from oxidative stress-induced neurotoxicity (11).

In addition, the anti-inflammatory and antioxidant properties of Metformin enable it to restore endothelial dysfunction (12). Metformin operates by activating adenosine monophosphate kinase (AMPK) to carry out its mechanism of action. Research has demonstrated that AMPK activators reduce inflammation in different animal models both in vitro and in vivo (13). Also, Pioglitazone activates the selective peroxisome proliferator-activated receptor (PPAR) γ, which is a type of ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. This receptor is associated with retinoid and steroid hormone receptors (14). PPAR activation exhibits anti-inflammatory properties that are advantageous in various neurodegenerative models (15).

Moreover, Dapagliflozin has been authorized for managing Type 2 diabetes, as it acts as a selective inhibitor of sodium/glucose cotransporter-2 (SGLT2) and also triggers AMP-activated protein kinase (AMPK) (16).

MATERIALS AND METHODS

2.1. Experimental animals

The current study employed 60 mature male Wister rats that weighed between 250 and 300 g. They were housed in climate-controlled environments in cages made of stainless-steel wire mesh. The surrounding air was kept at 25 ± 2 °C, and a 12-hour light-dark cycle was recorded. The animals in the Faculty of Medicine, Alexandria University, Animal House have unrestricted access to food and water.

The experiments were conducted in accordance with the guidelines for the care and use of laboratory animals.

2.2. Chemicals & drugs

Ethidium bromide (from medico pharm company), Quercetin (10 gm., Medico pharm), Pioglitazone (30mg Amoun Pharmaceutical Company S.A.E), Metformin (500 mg Minapharm), Dapagliflozin (10mg AstraZeneca).

2.3. Experimental design& procedures

The rats were classified into two groups:

1. **Sham group:** included 10 rats that received a single intrapontine injection of 10 µl of saline (0.9 % NaCl).
2. **Demyelination group:** It included 50 rats that were received a single injection of 10 µl of 0.1 % Ethidium bromide (17) intra pontine. Then, they were further randomly subdivided into 5 subgroups 10 rats each.
   - **EB Control group:** Rats were treated with normal saline orally daily for 3 weeks.
   - **Quercetin treated group:** Rats were treated with quercetin at dose of (50 mg/kg/day) (18).
   - **Metformin treated group:** Rats were treated with metformin at dose of (500 mg/kg/ day) (19).
   - **Dapagliflozin treated group:** Rats were treated with dapagliflozin at dose of (10 mg/kg/day) (20).
   - **Pioglitazone treated group:** Rats were treated with pioglitazone at dose of (10 mg/kg/day) (21).

For three weeks, all medications were administered daily after being dissolved in 1% (w/v) methylcelluose (USP-grade, Sigma-Aldrich) as a vehicle. On the twenty-first day of the experiment, every rat was decapitated.

Stereotaxic Surgery

The rats were given anesthesia using a combination of ketamine and xylazine (i.e. 80–100 mg of ketamine and 10 mg of xylazine per kg body weight) administered via intraperitoneal injection. Then , each animal was secured in the Kopf stereotaxic frame from Germany, with the incisor bar positioned at -3.3 mm to ensure head alignment. To prevent corneal drying during the surgery, lubricant eye ointment was used (22). In order to induce demyelination, EB was injected intrapontically using stereotaxic methods. The specific coordinates utilized were 9.16 mm posterior, 9 mm ventral, and 1.4 mm lateral to the bregma, as outlined in the Paxinos and Watson Atlas (23). The target area's skull was drilled using a handheld drill. Using a 28-gauge Hamilton microsyringe, 10µl of 0.1% EB was injected (17). After the injection, the needle was kept in place for an additional 10 minutes to prevent potential "back-leakage". Once

the needle was removed, the skin and remaining subcutaneous tissue were sutured with a 4.0 nylon thread, and the animals were allowed to recover (24).

Neurological and Behavioral Tests

The rats were evaluated for clinical signs on the 7th and 21st days after EB injection in both the control and demyelination groups to confirm the occurrence of demyelination and analyze the treatment's impact. All behavioral measures were completed by researchers who were uninformed of the group's identities.

1- Beam balance test

The purpose of this assessment is to measure balance and precise motor coordination. A 25 mm diameter and 59 cm long horizontal beam was used, and each rat was placed on it. The evaluation focused on the thoracic and pelvic limbs of the rats, and they were given different scores. A score of 0 indicated that the foot was positioned on top of the beam without any slippage, which was considered 'normal'. If the whole foot slipped below the lower surface of the beam, it received a score of 1, which was classified as an 'error' (25).

2- Foot fault test

The experiment involves positioning the rat on a raised grid floor measuring 40×40 cm with grid openings of 3 cm2. As the rats moved along the grid, they put their paws on the wire frame. The experimenters recorded the moment when the paw dropped or slipped through the wire during each weight-bearing step. Each rat completed a total of 50 steps, and the number of foot faults was recorded during this time (26).

3- Rotarod test

Motor functions, gross coordination, and motor learning were evaluated using a rotating drum that can vary its speed from 4 to 40 revolutions per minute over 5 minutes. Rats received training on the rotarod for 5 trials per day over a period of 3 days. Each rat was placed on the drum individually, and the time taken to fall off was recorded (27).

4- Inverted screen test

The strength of the muscles was tested by using all four limbs. A wire mesh screen measuring 43 cm2 with 12 mm squares made of 1 mm diameter wire was utilized. Rats were positioned in the middle of the wire mesh screen, a stopwatch was initiated, and the screen was gradually inverted over a period of 2 seconds. The screen had to be held steady at a height of 40-50 cm above a cushioned surface. The time at which the rat fell off was documented and categorized as follows: Falling between 1-10 sec = 1, Falling between 11-25 sec = 2, Falling between 26-60 sec = 3, Falling after 60 sec = 4 (28).

Extraction of brain tissue

Following the behavioral assessment at the 21st day after the surgery, rats were euthanized with deep anesthesia using thiopental sodium. The brain was then removed and rinsed with phosphate buffered saline (PBS) solution at pH 7.4 to eliminate any red blood cells and clots. Samples of pontine tissue were then gathered.The specimens were placed in 10% neutral formalin solution. For immunohistochemical analysis the specimens were processed through ascending set of ethanol alcohols and embedded blocks cut by microtome (5 µm thick). Slides fixed in oven for 24 hours and stained by using Ventana Benchmark Gx through automated system. The CC+1 antibody titer was 1:200 for 16 minutes.

Data analysis

Data was input into the computer and analyzed with IBM SPSS software package version 20.0 by IBM Corp located in Armonk, NY. The data analysis was conducted using StepOne™ Software v2.3. The normality of distribution was verified using the Kolmogorov-Smirnov test. Quantitative data were characterized using the range (minimum and maximum), mean, standard deviation, standard error of the mean, and median. The significance of the results was assessed at the 5% level.

The F-test (ANOVA) was utilized for normally distributed quantitative variables to compare between more than two groups, and the Post Hoc test (Tukey) was employed for pairwise comparisons.

RESULTS

Effect of different treated drugs on motor performance

The injection of ethidium bromide caused a noticeable decrease in motor performance, as evidenced by a significant reduction in the average time to fall during the rotarod test in the EB control group on both the 7th and 21st days. (37.33±16.69& 46.87±10.45 compared to 144.7±7.97&146.21±6.55 for sham group on same days. In comparison to the EB control group, the groups treated with quercetin, metformin, dapagliflozin, and pioglitazone showed a statistically significant increase in the average time taken to fall on both the 7th and 21st days ($p<0.001$*) (figure 1). The foot fault test also demonstrated a notable increase in the average number of errors in the EB control group on both the 7th and 21st days (14.38±3.74 and 14.57±4.39) when compared to the sham group on the same days. (1.56±0.53& 1.22±0.44). The groups treated with quercetin, metformin, dapagliflozine, and pioglitazon exhibited a significant decrease ($p<0.001$) in the average number of errors compared to the EB control group on the 7th and 21st days of the experiment (see figure 2). In the inverted screen test, the EB control group demonstrated a noteworthy decrease in the duration rats could grasp the screen, with mean values on the 7th and 21st days at (10.78±4.89 & 18.50± 5.55) in contrast to

(47.89 ± 8.95 & 46.50± 7.78) in the sham group. Conversely, there was an enhancement in paw strength in the groups treated with quercetin, metformin, dapagliflozine, and pioglitazon, as indicated by a substantial increase in the mean duration the rats could grasp the screen ($p<0.001$) compared to the EB control group on the 7th and 21st days. (figure3). Furthermore, there was a notable decrease in motor power and fine motor coordination in the EB control group during the beam balance test. This was evidenced by an increase in foot slippage on the 7th and 21st days (0.76 ± 0.15 and 0.56 ± 0.10) as opposed to the sham group (0.16 ± 0.05 and 0.16 ± 0.07). Conversely, the groups treated with quercetin, metformin, dapagliflozine, and pioglitazone demonstrated substantial enhancement in motor performance ($p<0.001$) on the 7th and 21st days compared to the EB control group. (figure4).

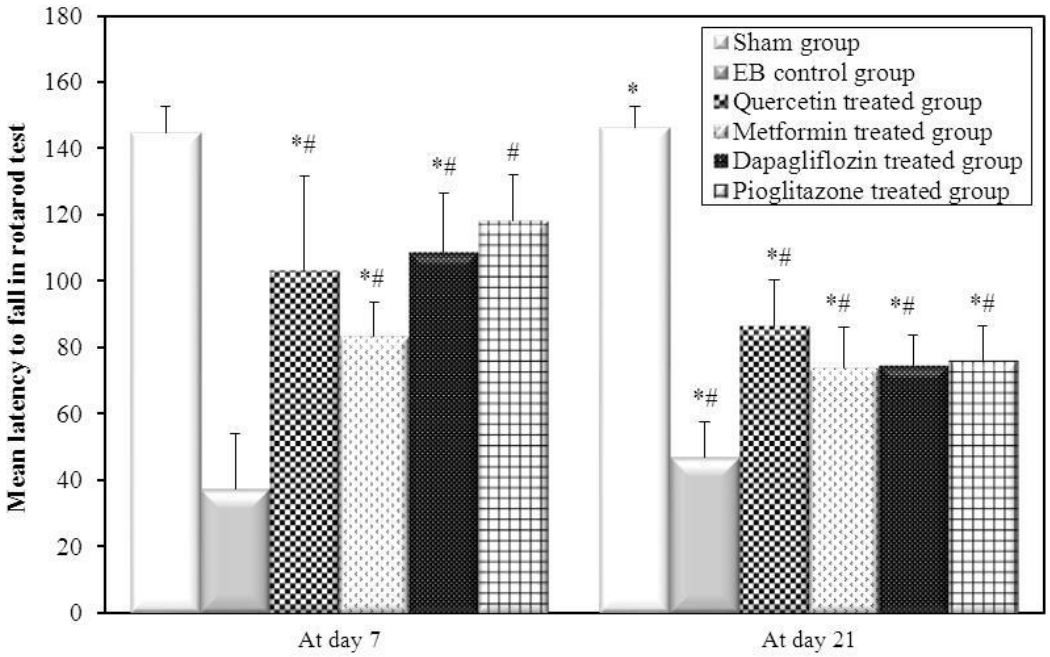

Figure 1: Mean latency to fall in rotarod test in rats of the different studied group. *: Significant with Sham ($p \leq 0.05$), #: Significant with control ($p \leq 0.05$)

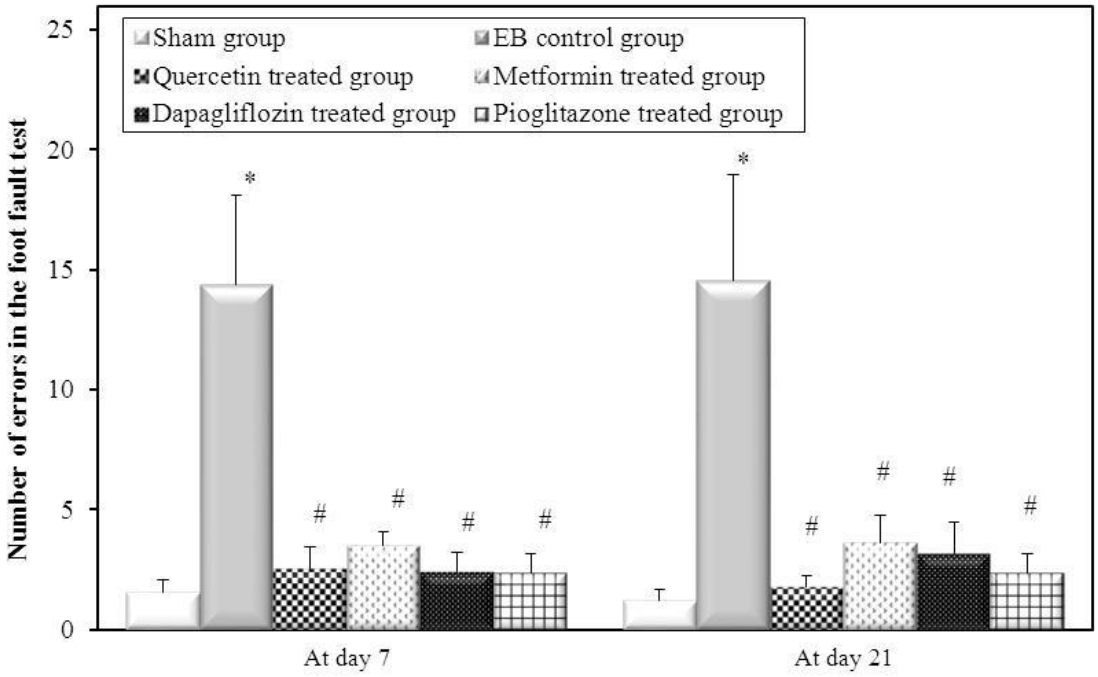

Figure 2: Number of errors in the foot fault test in rats of the different studied groups. *: Significant with Sham ($p \leq 0.05$), #: Significant with E.B ($p \leq 0.05$)

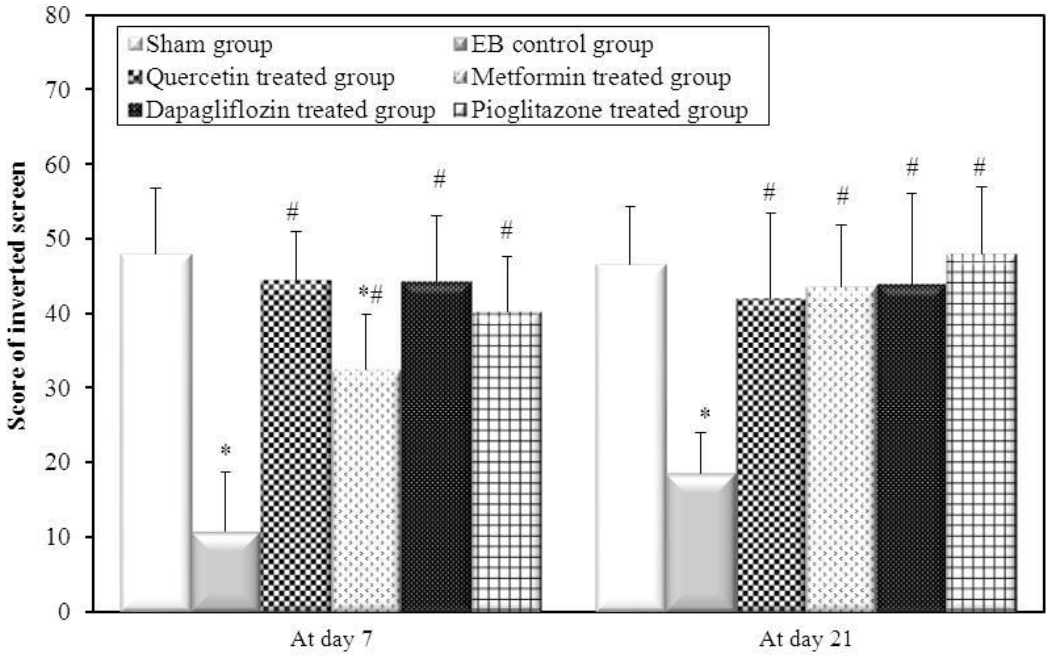

Figure 3: Score of inverted screen test in rats of the different studied groups.

*: Significant with **Sham ($p \leq 0.05$),** #: Significant with **E.B ($p \leq 0.05$)**

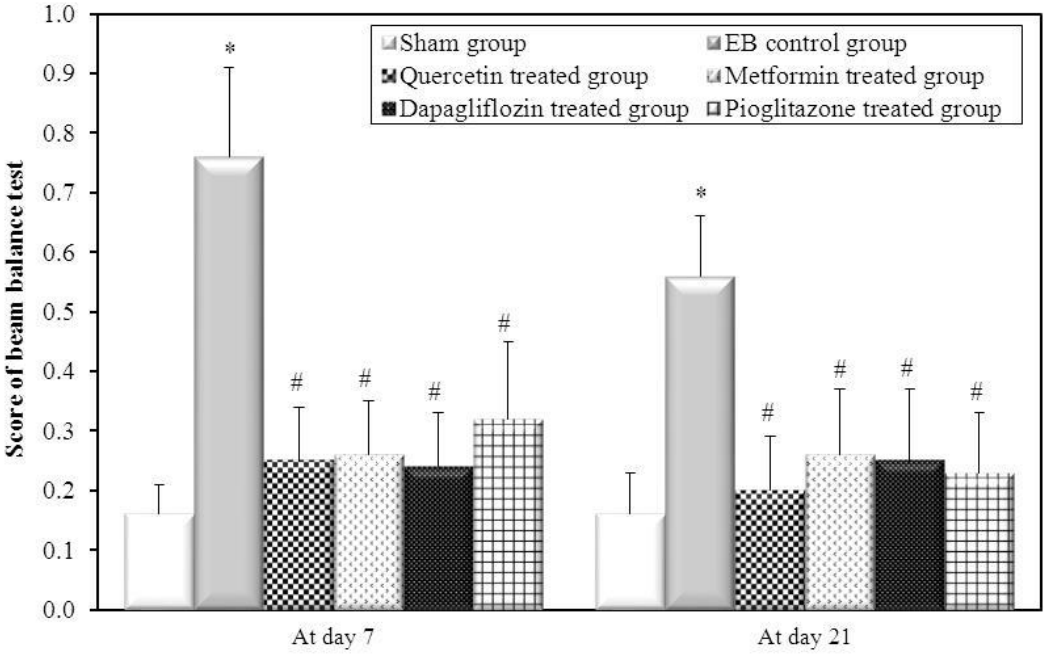

Figure 4: Score of beam balance test in rats of the different studied groups.

***: Significant with Sham ($p \leq 0.05$), #: Significant with E.B ($p \leq 0.05$)**

Immunohistochemistry Results

Examination of pontine tissue immunostained for CC+1 oligodendrocyte antibodies showed a positive reaction in the form of brown pigmentation of the cell bodies as well as the process extending to the neighboring neuronal axons to form their surrounding myelin.

Examining the sham control group revealed limited positive reactions in areas of their axonal process and nearly negative reactions in areas of grey matter (Figure 5 a-b). Sections from pontine tissue of EB subgroup revealed limited reaction in areas of grey matter. The brown stained cell bodies appeared scattered in between unstained neuronal cell bodies while the reaction around their axonal process was still limited (Figure 5 c-d).

On the other hand, the administration of all tested drugs revealed markedly increased reaction in all examined pontine tissue samples in between the neuron and extending their process to cover the nearby axons. The reaction was markedly noticed in dapagliflozin (Figure 6 e-f) and quercetin treated subgroup (Figure 28 e-f) followed by pioglitazone treated subgroup (Figure 6 a-b) and mildly positive reactions in metformin treated subgroup (Figure 6 c-d).

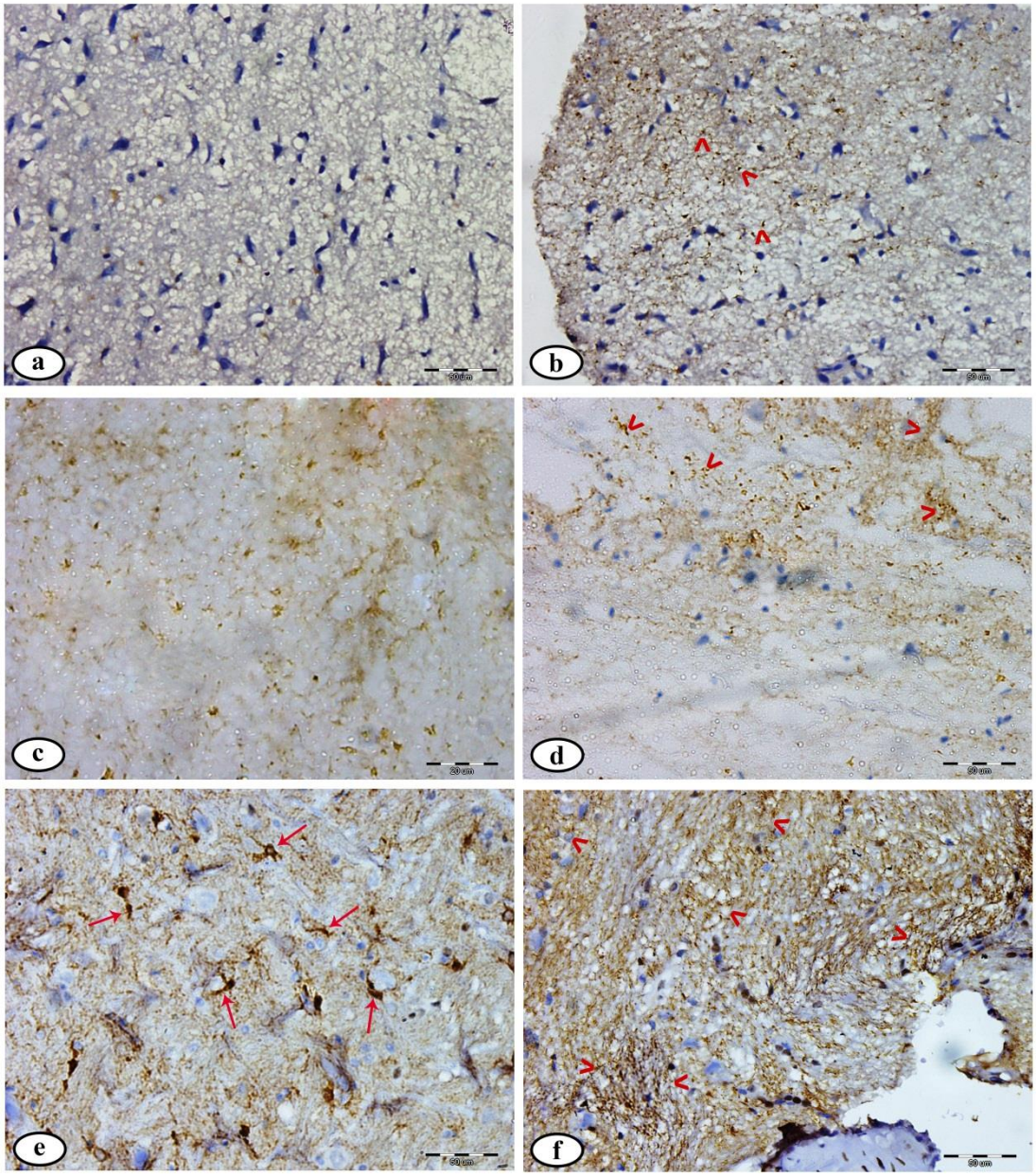

Figure 5: **immunohistochemistry staining of oligodendrocyte in pontine tissue of the rat** using CC+1 oligodendrocyte antibody (Mic Mag X 400). **The saining revealed** brown positively stained oligodendrocytes (↑) in between neuronal bodies (a-c-e) or along the neuronal axonal process (➤) (b-d-f). Both the EB-treated group (c-d) and the Sham group (a-b) show very little staining. Observe

the noticeably stronger response in Quercetin treated subgroup (e–f).

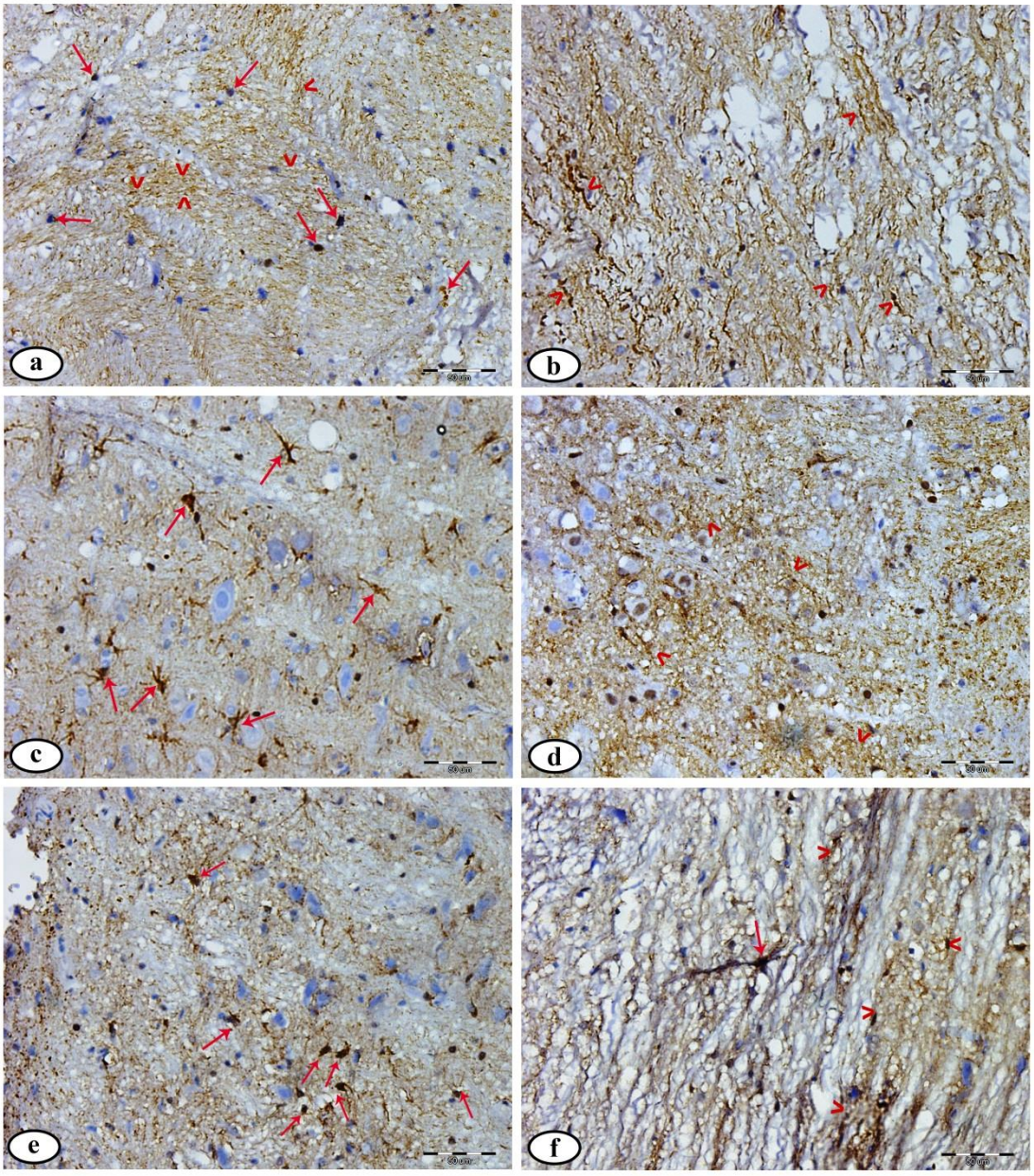

Figure 6: **immunohistochemistry staining of oligodendrocyte in pontine tissue of the rat using CC+1 oligodendrocyte antibody (Mic Mag X 400).** The saining revealed markedly increased positive reaction in stained oligodendrocytes (↑) in between neuronal cell bodies (➤) (a-c-f) as well as along axonal processes (f-b) with mild positive reaction in (d).

a-b: pioglitazone treated subgroup. c-d :Metformin treated subgroup.

e-f : Dapagliflozine treated subgroup.

Histomorphometry Results

Effect of different tested drugs on color percent area of CC+1 immunostained pontine tissues white matter

There was no significant difference in color percent area between the sham group and the EB subgroup (5.24±3.56 for the sham group and 7.38±1.56 for the EB subgroup). Only the metformin-treated subgroup showed a significant difference to the EB subgroup ($p \leq 0.05$). The drug-treated subgroups of quercetin, metformin, and dapagliflozin showed significant differences to the sham group (Figure 7)

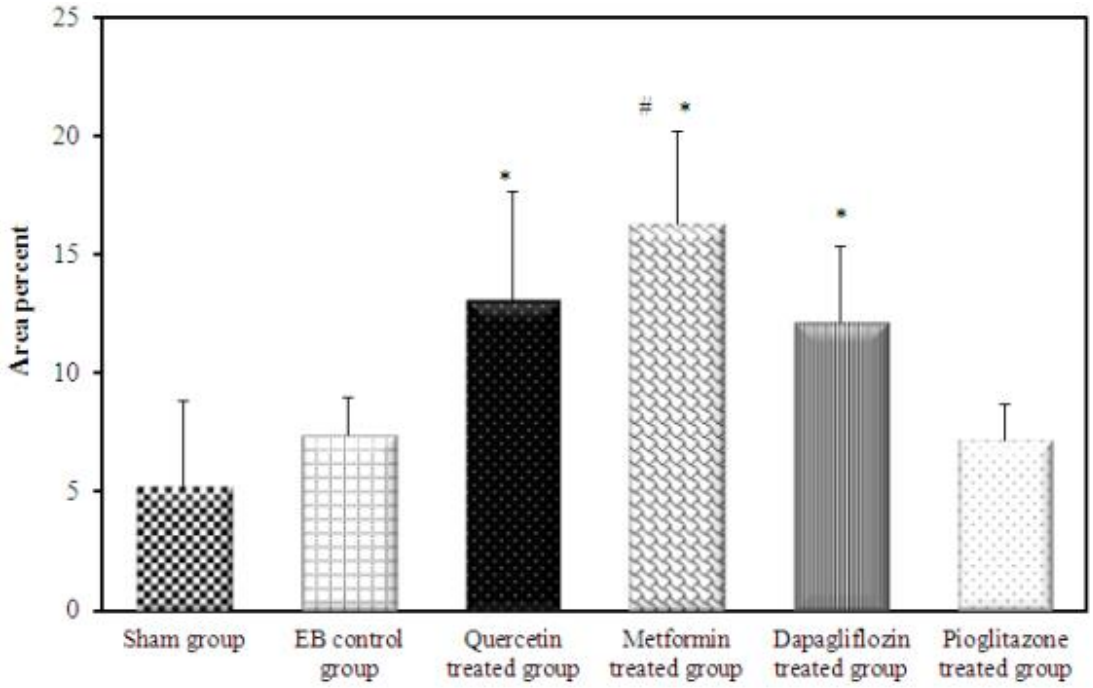

Figure 7: Effect of different tested drugs on color percent area of CC+1 immunostained pontine tissues white matter. *: Significant with **Sham** **($p \leq 0.05$),** #: Significant with **EB ($p \leq 0.05$)**

Effect of different tested drugs on color percent area of CC+1 immunostained pontine tissues grey matter

There was no significant variation in color percent area between the sham and EB subgroups. The mean result was 0.89±0.85 for the sham group and 1.68±2.25 for the EB subgroup. The drug-treated subgroups of quercetin, metformin, and dapagliflozine differed significantly from the sham group. Their mean values were (6.53± 2.75), (6.70± 3.56), and (6.32± 1.85) respectively, compared to (0.89±0.85) in the sham group (Figure 8)

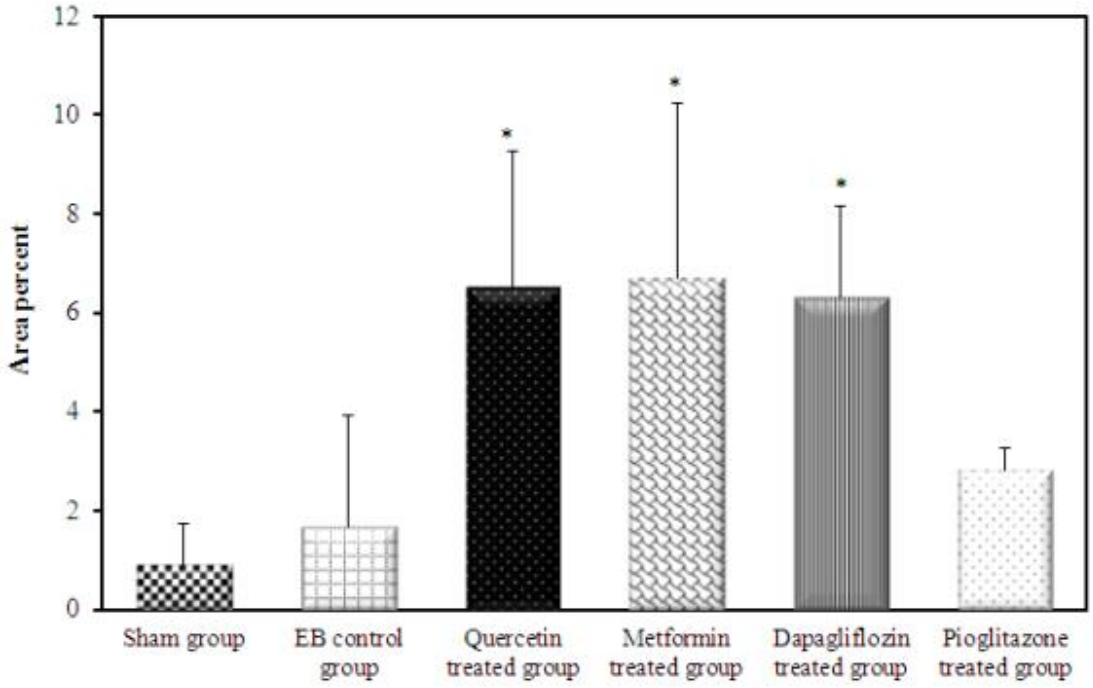

Figure 8: Effect of different tested drugs on color percent area of CC+1 immunostained pontine tissues grey matter. *: Significant with **Sham ($p \leq 0.05$),** #: Significant with **EB ($p \leq 0.05$)**

DISCUSSION

Improving the restoration of myelin sheaths in the CNS is recognized as a crucial therapeutic goal for addressing the effects of ongoing demyelination. The regeneration of myelin is facilitated by oligodendrocyte progenitor cells, which are attracted to regions of demyelination. Subsequently, these cells interact with axons and develop into myelin-producing oligodendrocytes (29).

The use of chemical agents to induce demyelination in experiments creates a model system for studying the cellular transformations associated with demyelinating disorders (30), the injection of EB has provided a consistent model for researching the science of remyelination and the process of repair (9). Also, studying the behavioral tests was a good decision because the delayed action of the medication allowed for the separation of the surgical consequences from the demyelination changes (31).

In this study, rats' capacity to move was reduced after being injected with EB, resulting in symptoms such as lack of coordination, muscular weakness, and paralysis..These finding were consisting with Hollis ER et al work, who reported, that animals injected with EB had the slowest recovery of function on the rotarod and beam-crossing task, Additionally, they required more time to grab the wire with their hind legs, and the duration spent gripping the wire was minimal (32). Immunohistochemistry staining of pontine tissue of EB subgroup revealed limited reaction in areas of grey matter, which may indicate loss of OPC from the area of demyelination, though CC1 is a well-known useful marker for oligodendrocytes (33).

Adenomatous polyposis coli (APC) is a protein that suppresses tumors and is found in the large intestine and CNS. Its presence in the CNS is restricted to neurons and mature oligodendrocytes (34). Jordan Lang et al (35) reported that during normal oligodendrocyte development and oligodendrocyte regeneration, APC is temporarily expressed in cells of the oligodendroglial lineage, indicating its involvement in the differentiation of oligodendroglial cells and the process of myelination.

Following EB-induced demyelination, Quercetin treatment demonstrated a strong protective effect, resulting in an improvement in rats' motor performance as well as a decrease in the number of degenerated neurons. These findings are consistent with Beckmann et al's study, suggesting that the behavioral outcomes in the beam walking test and foot fault test were similar

in the quercetin-treated group compared to the control group (17). It also resulted in significant increase in reactivity in immunostained pontine tissue samples more marked in CC1 stained white matter than in gray matter when compared to EB subgroup, which was more or less correlated with myelin expression on histomorphometric results. These finding may be explained by that OPCs in the white matter are more efficient at producing mature myelinating OLGs compared to OPCs in the grey matter, which have a slower proliferation rate and generate fewer mature cells (36).

Overall, the results are consistent with quercetin having a neuroprotective impact in neurodegenerative illnesses and playing a role in the process of remyelination. Although there isn't much research on quercetin's efficacy in demyelinating illnesses, there is a lot of data to support its involvement in neuroprotection.In a study involving perinatal cerebral hypoxia-ischemia-induced brain injury, quercetin was found to enhance both cognitive performance and myelination by stimulating the proliferation of OPCs and promoting the survival of oligodendrocytes (37). The presence of various molecular signaling cascades is known to actively prevent the differentiation of OPCs and the remyelination of MS lesions (38). Wnt pathway is one of these signaling cascades (34). The translocation of β-catenin into the nucleus impacts remyelination through Wnt signaling. This process is known as canonical Wnt signaling. Once β-catenin is in the nucleus, it attaches to transcription factor 4 (TCF4)(39), the activated *β-catenin*/ transcription factor Tcf4 complex can act as an inhibitor of OLP differentiation in the mammalian CNS (40).

Quercetin selectively inhibits canonical Wnt signaling by strongly suppressing the binding of Tcf complexes to specific DNA-binding sites and disrupting the binding of beta-catenin to TCF-4.[41]

Rats given EB injections demonstrated enhanced motor function when given metformin. Furthermore, there was a greater response in the grey and white matter stained with CC1, suggesting possible remyelination. Nevertheless, this process is still inefficient overall, indicating that OPCs exist but are prevented by several molecular pathways from developing into new cells that produce myelin (38). This finding is consistent with the findings of Qi et al., who found that metformin stimulates oligodendrocyte regeneration to lessen cognitive impairments in a prenatal hypoxia-ischemia rat model (42). The action of metformin is carried out by triggering AMPK, which has a key role in regulating energy balance within cells. Research indicates that AMPK activators reduce inflammation in various animal models (43). The signaling of AMPK promotes the survival of oligodendrocytes, leading to the restoration

of central nervous system (CNS) integrity and functions in animals with EAE who were treated with metformin (44).

Rats treated with pioglitazone following EB injection had improvements in their motor function that were nearly comparable to those of the subgroup treated with quercetin.. Also, markedly increased reaction in immune stained pontine tissue samples in immunohistochemistry with increased reaction of CC1 stained white matter more than the grey matter like other treated subgroups. This might be explained by the fact that normal appearing white matter has more oligodendrocytes and OPCs than normal appearing grey matter (45).

The neuroprotective effect of pioglitazone may be explained by its targeting of PPAR. Several studies have shown that PPAR stimulation produces anti-inflammatory effects, which are advantageous in a variety of mouse models of neurodegenerative diseases, including experimental autoimmune encephalomyelitis, Huntington's disease, and amyotrophic lateral sclerosis (46). PPARγ activators can stimulate the expression of astrocyte and oligodendrocyte progenitor cell differentiation genes in neural stem cells (47). Moreover, PPARγ activators can help OPCs mature into myelin-forming oligodendrocytes (48).

Treatment with dapagliflozin produced a significant improvement in behavioral tests as well as a significant increase in reactivity in immune-stained pontine tissue samples between the neurons. This outcome is consistent with the findings of Abdelsameea and Kabil (49), who reported that rats treated with SGLT2 inhibitors in addition to cisplatin showed enhanced performance on the rotarod apparatus and protection against sensory and motor neuropathy. The combined treatment also prevented neuronal degeneration and atrophy caused by cisplatin, as well as increased the expression of MBP.

Conclusion

From the result of this study it could be concluded that quercetin, metformin, pioglitazone and dapagliflozine might have beneficial role in treatment of neurodegenerative disorders as they ameliorated the degenerative effect of MS to variable degrees with decreased degenerated neurons and reestablishment cerebral neuronal architecture.

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