The Rho Kinase Inhibitor Fasudil Mitigates Neurocognitive Deficits in D-Galactose-Induced Brain Aging in Rats: Involvement of SIRT1, BDNF and NF-κB

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Abstract

Introduction: Cognitive deficits and aging were evoked by D-galactose (D-gal). Fasudil (Fsd) slows the start and progress of neurodegenerative illnesses. **Objective:** to illustrate the neuroprotective effect of Fsd in D-gal induced cognitive deficits and the underling mechanisms involved**. Material & methods:** Thirty Wister albino male rats divided into control, D-gal, D-gal+Fsd groups. Rats were subjected to MWM, NOR and EPM tests then rats were sacrificed and MDA, catalase, TNF-α, IL-6 hippocampal gene expression of BDNF, SIRT1 and NF-kB were assessed. Histopathological and immunoreaction for caspase-3 and GFAP were done. **Results:** D-gal revealed cognitive impairment, with substantial elevation of MDA, TNF-α, IL-6 and NF-kB gene expression and significant reduction of catalase, SIRT1 and BDNF gene expression of D-gal with upregulation of caspase-3 and GFAP immunoreaction in hippocampus. Fsd dramatically improved D-gal induced changes. **Conclusion:** Fsd alleviated D-gal induced cognitive deficits by anti-oxidant, anti-inflammatory, anti-apoptotic mechanisms in addition to up-regulation of Sirt1 and BDNF gene expression and improving gliosis.

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Introduction

Aging results in increasing physiological malfunctions. its pathogenesis is well known to include oxidative stress (OS), mitochondrial malfunction, inflammation, and apoptosis. Cognitive decline is the most visible sign of aging. The most popular model is brain aging caused by D-galactose (D-gal) [1].

D-gal is broken down into hydrogen peroxide and aldose. These compounds are strong catalysts for the synthesis of free radicals produced from oxygen [2] (Gok et al., 2015) producing oxidative stress, which results in symptoms including protein loss, mitochondrial malfunction, hippocampus neural damage, and cognitive decline that are indicative of normal aging [3]

When inflammatory signaling pathways are triggered by oxidative stress, an excess of reactive oxygen species (ROS) is produced evoking apoptosis and cellular malfunction. The main target of ROS is mitochondria. The following production of ROS accelerates aging-related alterations by inducing mitochondrial malfunction and cell death [4].

Neuronal proliferation, plasticity, and synaptic transmission all depend on brain-derived neurotrophic factor (BDNF). Learning and memory are facilitated by BDNF, which increases the survival of neurons [5]. As people age, their levels of BDNF significantly drop, which can lead to cognitive problems [6].

Cell apoptosis, and metabolic processes are among the cellular processes that Sirtuin 1 (SIRT1), is thought to regulate [7]. SIRT1 prevents the aging-related brain development [8]. Down-regulating SIRT1 results in an early senescence-like phenotype, making it a potential target for postponing ageing [9].

In CNS inflammatory demyelination, degeneration, neuronal plasticity, and growth cone dynamics are all influenced by Rho-associated protein kinase (ROCK) signaling. Moreover, ROCK inhibitors enhance both neuronal survival and cognitive deterioration. As a result, several neurodegenerative illnesses have been studied as potential targets for the pharmacological suppression of ROCKs [10].

The first ROCK inhibitor to receive clinical approval was Fasudil (Fsd). Fsdmitigate the signs and development of neurodegenerative disorders [11]. By lowering OS and neuronal apoptotic process and enhancing memory, it has neuroprotective benefits [12],

While aging is acknowledged as an unavoidable process, there is evidence from a number of researches that suggests it can be altered. No research has been done in how Fsd affects memory impairment brought on by d-gal. As a result, the goal of our research was to determine if Fsd may prevent cognitive impairments caused by D-gal and to clarify the underlying processes.

Materials and methods

Animals. After receiving the required permits from the Research Ethical Committee at the Faculty of Medicine, Menoufia University, Egypt, with IRB No (10/2024Bio16-2). thirty albino male rats weighing 180–230 grams were used. ARRIVE criteria were followed throughout the experimental procedures. The rats were kept in cages (80 x 40 x 30 cm). During the research period, all animals were given unlimited access to food and water after being conditioned for two weeks under

continuous environmental conditions with a 12:12-hour light/dark cycle.

Experimental design. The animals were divided randomly into three groups (10/group):

1. Control group: For ten weeks, rats were given a daily subcutaneous (SC) injection of 0.2 ml of isotonic saline and a single daily intraperitoneal (ip) injection of the same solution.

2. D-galactose (D-gal) group: For ten weeks, rats received a daily ip injection of 1mL isotonic saline once a day along with D-gal (Sigma-Aldrich St. Louis, USA) at a dose of 150 mg/kg/day [1].

3. D-galactose + Fasudil (D-gal+Fsd) group: Rats received a single daily ip injection of 30 mg/kg/day of fasudil (Sigma-Aldrich St. Louis, USA) and daily administration of D-gal (150 mg/kg/day) by SC injection once a day for ten weeks. [11].

All of the rats had a neurobehavioral evaluation once the 10 weeks were over. Rats were then given anesthesia and killed by dislocating and elongating their cervical vertebrae. After being removed, the brain was cleaned. After being weighed, left hemisphere was split in half, with one half being utilized for RT-PCR research and the other for biochemical examination. In order to analyze the hippocampus tissues histopathologically and immunohistochemically, right hemisphere was fixed in 10% formalin saline.

Neurobehavioral tests

Novel Object Recognition: object in a controlled setting. A three-day testing procedure that including training, testing, and habituation was applied to each rat. Rats in an open-field apparatus measuring 50 cm by 50 cm by 40 cm were given

10 minutes to acclimate to their surroundings during the habituation phase. We placed two identical things in a room for each rat and trained them for the first five minutes. During the testing phase, each rat was maintained in the chamber for five minutes after the item swap, which took place after a 24-hour gap. We made use of the stopwatch. The discriminating index may be computed as follows: [= (familial object exploration time–novel object exploration time)/total exploration time×100%][13] Elevated Plus Maze (EPM) Test: As previously described [14]in this experiment, we evaluated the rats' anxiety-like behavior by having them identify a plus sign using an equipment. Ten minutes were given to each rat to investigate the labyrinth once it was positioned in the center of the device. A security camera mounted overhead was used to track the animals' movements. The amount of time that was spent inside the open arms maze's was carefully assessed. There was inverse relationship between the length of time and the degree of anxiety-like behavior.

Morris Water Maze (MWM) Test:

MWM test was conducted on the last five days of the trial. The circular pool at MWM was divided into four equal sections. A platform with a diameter of 10 cm was positioned in one of the quadrants, one centimeter below the water's surface, during the first four days. On each day of the acquisition session (three trials per session), each rat was assigned to one of the three randomly chosen places within the pool. The rat was submerged in the pool to begin the trial. When the rat found its way onto the platform, the experiment came to a close, and the mean escape latency was determined. The exam had a maximum duration of

sixty seconds. The rat was placed gently on the platform and the timer was set to 60 seconds if it did not climb up in that time. A "probe trial" was carried out on the fifth day to gauge how well the rats could recall the location of the concealed platform in 60 seconds. During this trial, the platform was removed from the pool[15].

Biochemical analysis. Following a PBS solution perfusion, brain tissue was homogenized and centrifuged at 3000 rpm for 20 minutes, and the supernatant was then extracted and kept at − 80 °C until Malondialdehyde (MDA) and catalase assays were made using a traditional colorimetric method (Biodiagnostic, Giza, Egypt), hippocampal citrate synthase (CS) activity, Tumour Necrosis Factor (TNF-α), Interleukin 6 (IL-6), by using ELISA kit(CS Activity Assay, Catalog No : E-BC-K178-M, Elabscience, Shizishan Ave, Hongshan, China), (TNF-α: Assaypro LLC, Saint Charles, Missouri, USA.), (IL-6: ab100772;Abcam, Cambridge, UK) according to the manufacturer's instructions.

Quantitative assay of NF-kB, BDNF and SIRT1 **genes expression using reverse transcriptase polymerase chain reaction technique (RT-PCR).**

To prepare hippocampus tissues for total RNA isolation, the Qiagen RNeasy plus Universal Kit was utilized from the United States. Next, the RNA's quality and purity were confirmed. Until it was needed, RNA was stored at -80 °C. Using an Applied Biosystems 2720 heat cycler from Singapore and a Quanti Tect Reverse Transcription Kit from Qiagen, USA, for a single cycle, the first step included creating cDNA. GAPDH primers were employed as an RNA loading control in RT-PCR procedures. Next, cDNA amplification was performed using SensiFASTTMSYBR Lo-ROX Kit, USA, and the following designed primers for SYBR green-based quantitative real-time PCR for Relative Quantification (RQ) NF-kB, BDNF, and SIRT1 gene expression. (Texas, Midland): The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC). The reverse primer was (GAGCAGTCATGTCCTTGGGT). The forward primer for BDNF was GCTGCCTTGATGTTTACTTTGand reverse ATGGGATTACACTTGGTCTCGT. SIRT1, forward was (AGA AACAATTCCTCCACCTGA) and reverse was (GCTTTGGTGGTTCTGAAAGG).

Histopathological evaluation

H&E; Histopathology was done. Before embedding in paraffin blocks, hippocampus samples were treated with formal solutions, alcohol, and xylol. Tissue samples were sliced to 5 μm thickness using a microtome. HE-stained samples. These samples were histopathologically evaluated under a light microscope.

GFAP: The main monoclonal mouse antibody for GFAP (ab7260; Abcam, Cambridge, MA, USA 1:400 in PBS) identified astrocytes. Peroxidase-labeled streptavidin-biotin was used [16]. Later sections were counterstained with Mayer's haematoxylin.

Caspase-3: Anti-Caspase-3 (rabbit polyclonal antibody, Thermo Science, Fermont, CA 94539, USA) was utilized. The main antibody utilized for caspase-3 was ready-to-use rabbit polyclonal antibody (catalog number RB-3425-R2). Normal

lymphoid tissue was employed as a positive control for caspase-3.

Statistical Analysis

Version 22 of the Statistical Package for the Social Sciences (SPSS) program was used to tabulate the results. The data sets were analyzed for normal distribution using the Shapiro-Wilk test. The mean, plus or minus the standard deviation, was the quantitative data expression. We used a post hoc Tukey test after completing ANOVA to ascertain if the differences between the groups were statistically significant. P-values less than 0.05 are regarded as statistically significant. All of the data were displayed in graphs created with Microsoft Excel.

Results

Time in the open arms of EPM in D-gal group was dramatically lower than control (60 ± 1.51 vs 110.5 ± 2.88 sec respectively, $P< 0.05$), but D-gal +Fsd was substantially higher than D-gal (85.33 ± 0.8 Sec, $P< 0.05$) but still substantially reduced than control ($P < 0.05$). (Fig.1A)

Discrimination index of NOR Test in D-gal was substantially lower than control (16.6 ± 1.6 vs 64.6 ± 1.3 respectively, $P< 0.05$) but D-gal+Fsd was substantially higher than D-gal group (43.8 ± 1.16, $P< 0.05$) but still substantially lower than control ($P < 0.05$). (Fig.1B)

Escape latency in 1st day of MWM test in D-gal showed no substantial difference compared to control (55.3 ± 1.03 vs 55 ± 0.89 sec respectively, $P> 0.05$) and butin D-gal+Fsd showed no substantial difference compared to D-gal and control (55.5 ± 1.04 sec, $P> 0.05$). Escape latency in 2nd, 3rd, 4th, & 5th day in D-gal were substantially higher than control (56.1 ± 1.1, 55 ± 0.89, 50.8 ± 0.75, 43.8 ± 1.16 vs 44 ± 0.89, 31.5 ± 1.04, 21 ± 0.63, 9.5 ± 0.54 sec respectively, $P< 0.05$). The escape latency in 2nd, 3rd, 4th, & 5th of MWM test in D-gal+Fsd were substantially reduced than D-gal (49.6 ± 3.66, 41 ± 0.89, 31.3 ± 0.5, 21.3 ± 1.2 sec respectively, $P< 0.001$) but substantially elevated than control ($P < 0.05$). (Fig.1C)
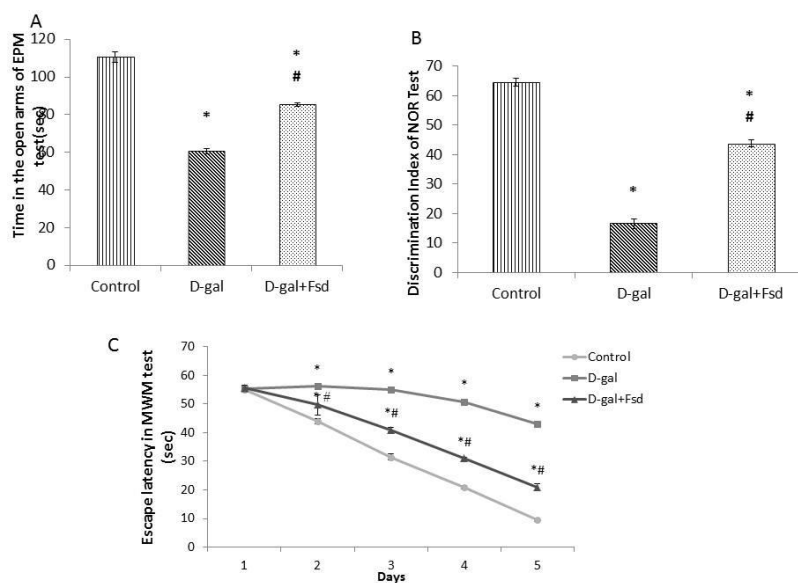

Fig 1: Fasudil impact on A) Time in the open arms of EPM (Sec) B) Discrimination Index of NOR Test C) MWM test (Sec) in all groups

Hippocampal MDA level in D-gal was substantially higher than control (31.8±1.15 vs 11.6±1.25 nmol/ gm. tissue respectively, $P< 0.05$) but in D-gal+Fsd was substantially reduced than D-gal (17.18±0.69nmol/ gm. tissue, $P< 0.05$) but still substantially elevated than control ($P < 0.05$).(Fig. 2A)

Hippocampal catalase in D-gal was substantially lower than control (3.06±0.08 vs 7.33±0.13 U/ g. tissue respectively, $P< 0.05$), but in D-gal+Fsd was substantially higher than D-gal (5.14±0.14 U/ g. tissue, $P< 0.05$) but substantially decreased than control ($P < 0.05$). (Fig. 2B)

Hippocampal CS activity in D-gal was substantially lower than control (4.2±0.13 vs 13.66±0.48 U/ L, $P< 0.05$) but in D-gal+Fsd was substantially higher than D-gal (8.09±0.12 U/ L, $P< 0.05$) but substantially lower than control ($P < 0.05$). (Fig. 2C)
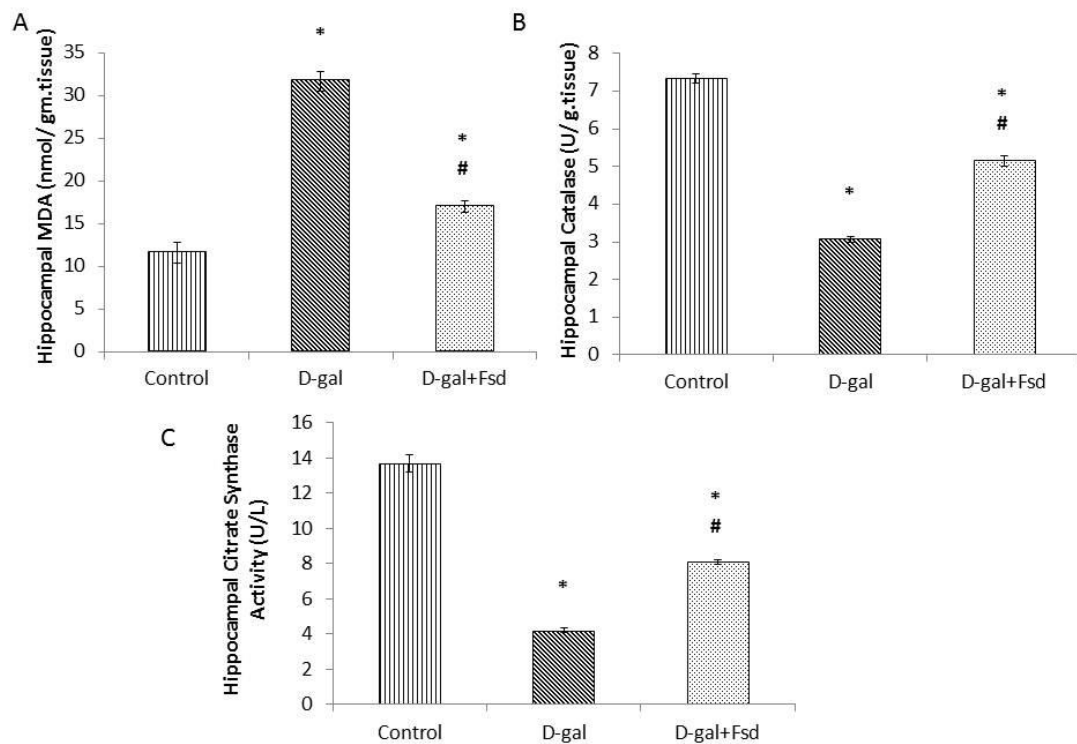

Fig 2: Fasudil impact on A) hippocampal MDA (nmol/ gm.tissue) B) hippocampal catalase (U/g.tissue) C) hippocampal citrate synthase activity (U/L) in all groups

Hippocampal TNF-α and IL-6 in D-gal were substantially higher than control (48.05±0.88 vs 23.7±0.93 ng/L, 195.2±0.77 vs 140.2±1.05pg/mL respectively, $P< 0.05$) but in D-gal+Fsd were substantially lower than D-gal (32.9±0.65 ng/L,   172±1.4 vs 11.6±1.25pg/mL$P< 0.05$) but still substantially higher than control ($P < 0.05$).(Fig. 3A,B)
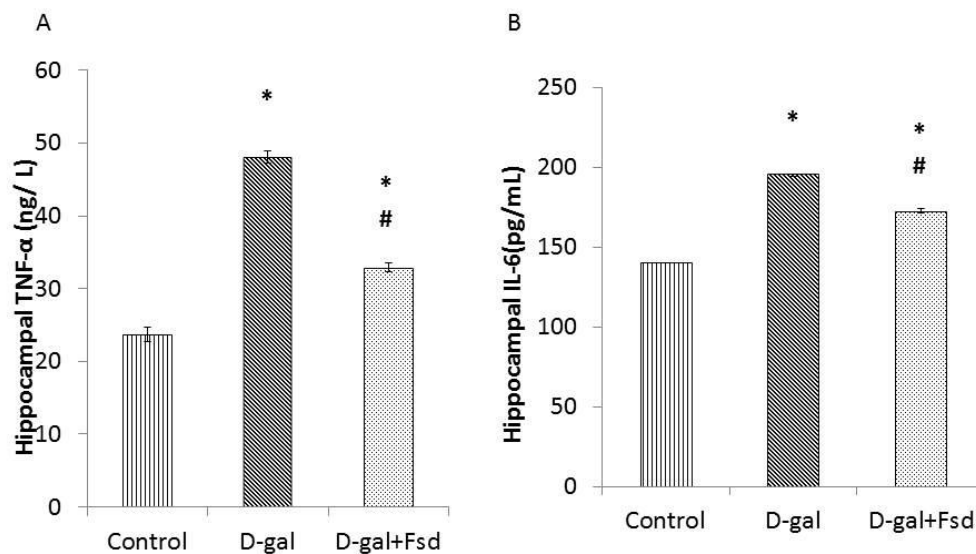
Fig 3: Fasudil impact on A) hippocampal TNF-α (ng/L) B) hippocampal IL-6 (pg/mL) in all groups

NF-kB gene expression in D-gal was substantially up-regulated compared to control (3.55±0.07, *P*< 0.05) but inD-gal+Fsd was substantially down-regulated than D-gal (2.3±0.04, *P*< 0.05) but substantially up-regulated than control (*P* < 0.05). (Fig. 4A)

Hippocampal BDNF and SIRT1 genesin D-gal were substantially down-regulated compared to control (0.42±0.013, 0.24±0.02 respectively, *P*< 0.05but inD-gal+Fsd were substantially up-regulated than D-gal (0.7±0.011 ,0.57±0.012, *P*< 0.05) but substantially down-regulated than control (*P* < 0.05). (Fig. 4 B, C)
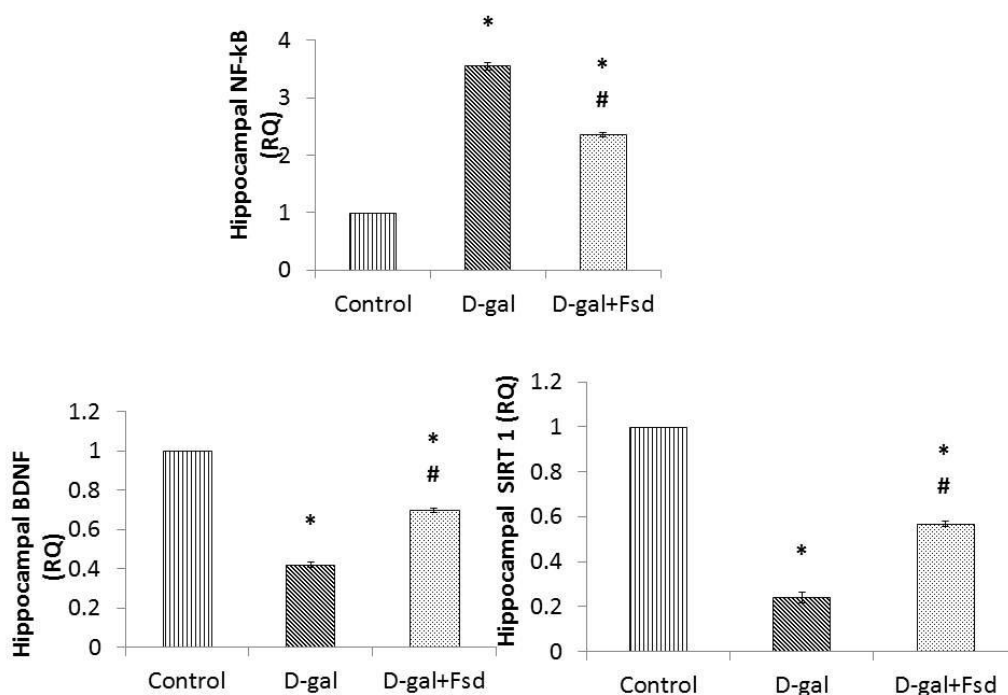

Fig 4: Fasudil impact on hippocampal gene expression of A) NF-kB, B) BDNF, C) SIRT1 in all groups

H&E Brain sections indicated a C-shaped control group hippocampus. CA1, CA2, CA3, dentate gyrus (DG), and hilum were the main sections. Hippocampal cells had external polymorphic, middle pyramidal, and inner molecular layers. The largest pyramidal neurons with rounded vesicular nuclei were in the pyramidal layer. It was packed in CA1. D-gal-treated hippocampus CA1 pyramidal cells exhibited a decline in size and a disorganized appearance. The outer polymorphic layer contained numerous pyramidal cells that exhibited apoptosis, as well as small cells with dark pyknotic nuclei. The D-gal+Fsd-treated CA1 hippocampus exhibited histological improvement. Euchromatic vesicular nuclei were present in the majority of pyramidal cells. Some pyramidal cells exhibited shrinkage, accompanied by dark, small pyknotic nuclei (Fig 5).

Caspase-3 of the control rats' hippocampus demonstrated a negative immune response. D-gal group substantially increased the mean area percentage of caspase-3 positive immunoreaction ($P<0.05$) compared to the control group. D-gal+Fsd treated group had a moderate positive immunoreaction and a significant decline ($P<0.05$) compared to the D-gal treated groups. Still a significant rise as compared to control. (Fig 6,7)

D-gal improved the quantity of GFAP-positive astrocytes in the hippocampus compared to the control, indicating active gliosis. In the D-gal+Fsd there was a decline in GFAP immunoreactivity compared to D-gal, as indicated (Fig 6,7)
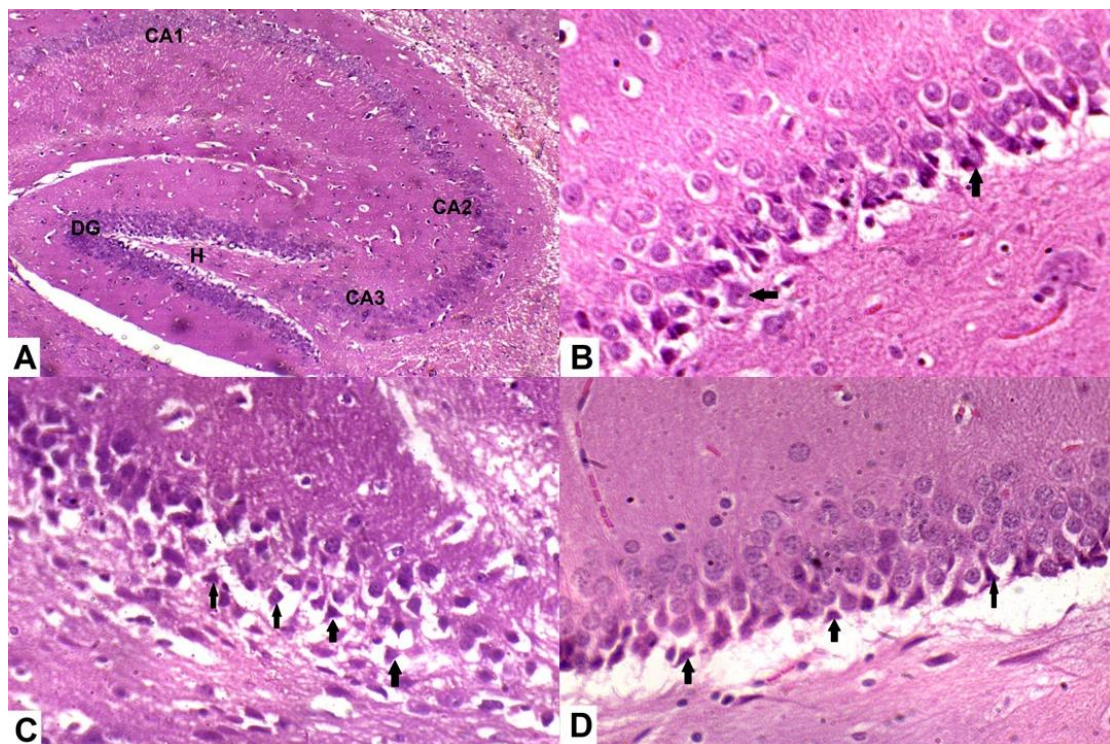

Fig-5 A): Hematoxylin and eosin-stained sections. A) Control hippocampus with areas CA1, CA2, CA3, DG, and DG's hilum(H) (H & E X 100). B): Control hippocampus with normal neurons (arrow) in CA1. C) D-gal treated group with degenerated neurons (arrow). D) The D-gal+Fsd treated group exhibited some degenerated neurons (arrow). (H&Ex400).
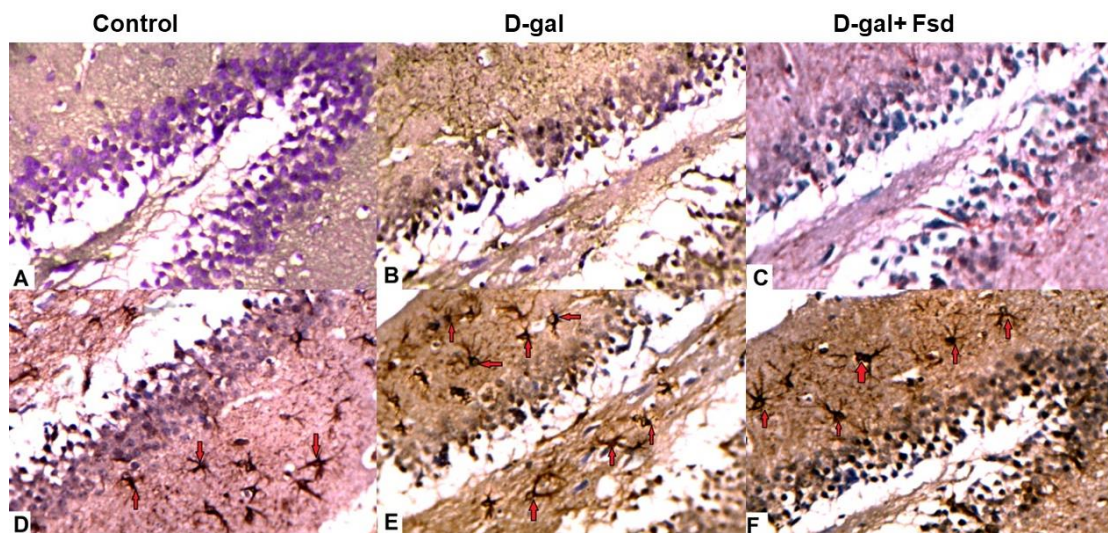
Fig-6 A). The hippocampi of control rats were stained with Caspase-3 immunostaining. B): The hippocampi of D-gal-treated rats showed high immune responsiveness compared to Control rats. C): D-gal+Fsd rats' hippocampi demonstrate reduced caspase-3 immuno-reactivity compared to D-gal rats. D): Control hippocampus stained with GFAP. E): D-gal-treated rats' hippocampi demonstrate increased GFAP compared to controls. F): D-gal+Fsd -treated rats hippocampi exhibited reduced GFAP than D-gal rats.
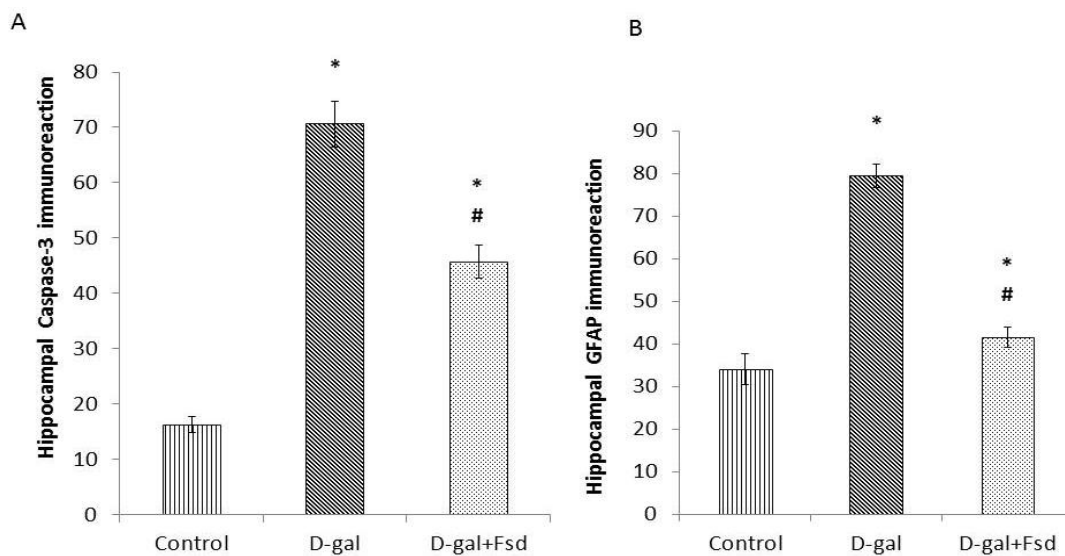

Fig-7 Fasudilimpact on A) Mean color intensity of Caspase 3 immune-staining, B) A) Mean color intensity of GFAP immune-staining

Discussion

Aging is accompanied by several disorders such as anxiety and memory impairment. When animals receive D-gal for six to ten weeks, their brains can age in a way that is strikingly comparable to that of human brain aging[1]. Our findings demonstrated distinct cognitive impairments between the D-gal and control, as shown by the interpretation of the MWM, EPM, and NOR tests. This aligns with the findings of Younis et al. [1]. D-gal produces aging-like symptoms, forms advanced glycation end products (AGES) when it combines to amines in amino[17]. In the D-gal+Fsd group, Fsd supplementation alleviated the

cognitive impairments brought on by D-gal. Numerous researches have demonstrated the neuroprotective impact of Fsd[18,19].

As demonstrated by our study's higher MDA level and lower D-gal catalase compared to control, OS plays a vital role in aging. This in line with those of Atef et al. [3]. D-gal causes oxidative damage to cells by increasing galactitol production and aldose reductase activity, which results in the creation of AGEs [20], causing an excess of ROS to be produced and a decrease in antioxidant capability. ROS induces oxidative damage to DNA, lipids, and proteins within cells, leading to apoptosis and cellular malfunction. The main target of ROS is mitochondria. According to Younis et al., [1], a marked indicator of mitochondrial function, CS activity, significantly decreased in the D-gal group as compared to the control, demonstrating impaired mitochondrial function in our investigation. The subsequent release of ROS evokes mitochondria dysfunction and triggers cell death [4].

In D-gal+Fsd, Fsd significantly enhanced oxidative stress indicators. According to Guo et al. [19] Fsd has the ability to up-regulate antioxidant enzymes, and its anti-oxidant effect may associate with enhanced OS by up-regulation of NAD(P)H oxidase [19]. Yang & Wang [21] found that the modulation of PPARα gene expression mediates the neuroprotective action of Fsd. Fsd's capacity to reduce OS in the D-gal+Fsd group results in improved mitochondrial activity, which is consistent with Guo et al. [19] findings.

By activating NF-kB, a redox-sensitive transcription factor, ROS can cause inflammatory reactions by promoting the release of several inflammatory mediators including TNF-α and IL-6. Furthermore, persistent inflammation probably set off a vicious loop of increasing ROS production [22].

This explains the d-gal-induced inflammatory response shown in our investigation, which is consistent with Atef et al.,[3], and is demonstrated by the considerable up-regulation of NF-kB gene expression in the hippocampal regions of the D-gal group together with a significant rise in inflammatory mediators.

Fsd dramatically reduced the inflammatory state brought on by D-gal, which agreed with Kumar et al. [1]. In the STZ-ICV model, Fsd restored PI3-kinase-eNOS signaling, which decreased the amount of TNF-α in the brain. Rats' brain NF-κB expression was pathologically increased by STZ, but this increase was mitigated when Fsd was administered [23].

A key neurotrophic factor in the regulation of several neurocognitive processes is BDNF. Moreover, BDNF regulates the inflammatory state by controlling the NF-kB signaling cascade. Accordingly, BDNF plays a significant role as a mediator between neuroinflammation and cognitive decline [3]. Our results stated that D-gal markedly down-regulated BDNF level, which had been markedly up-regulated following treatment with Fsd. These findings concur with past findings by [3].

SIRT1 provides cellular defense against OS and is implicated in memory, learning, cognitive function, and brain development [24]. It inhibits of NF-κB, which adds to its anti-inflammatory profile [8], and it regulates the expression of PPAR-γ, the primary regulator of mitochondrial activity. This

was further supported by our findings, which showed that D-gal down-regulated SIRT1 gene expression. These findings were consistent with Younis et al. [1], who stated a favorable correlation between SIRT1 relative expression and hippocampus levels of BDNF and mitochondrial CS activity. According to Yan et al. [25], Fsd significantly increased the expression of the SIRT1 gene, suggesting that SIRT1 up-regulation mediates Fsd neuroprotection.

D-gal induced disorderly pyramidal cell death in the hippocampus CA1. Our findings were corroborated by earlier findings that demonstrated that D-gal caused neurodegenerative alterations [26]. Fsd dramatically improved these changes.

There was dramatic elevation in caspase-3 immunoreactivity in the hippocampus region of the D-gal. D-gal enhances apoptosis and caspase-3, p53, and p21 overexpression in rats' hippocampus through increasing ROS levels, which trigger cellular death [17]. Data suggests that mitochondrial malfunction, OS, and inflammation all contribute to neuronal cell death by apoptosis[27]. Our study showed that D-gal+Fsd group had decreased caspase 3 immunoreaction in the hippocampus.The Fsd ROCK inhibitor regulates apoptosis and other cellular functions. Fsd has been found to inhibit apoptosis and caspase-3 activation and alleviate neurological impairment in brain ischemia injury via the ROCK/Akt pathway [28].

GFAP is a key marker for activated astrocytes. Our results demonstrated that D-gal boosted the number and activity of GFAP-positive astrocytes in the rat hippocampus. Oxidative stress may activate NF-kβ, leading to this effect [29]. We discovered that Fsd significantly decreased GFAP immunoreactivity of D-gal+Fsd, improving gliosis, which may be associated to fsd's anti-inflammatory influence, as confirmed by lower proinflammatory transcription factor NF-κB protein expression levels [30].

Conclusion

By increasing the expression of the genes SIRT1 and BDNF, improving gliosis, and reducing inflammation and apoptosis, Fsd significantly improved the cognitive deficits caused by D-gal. These actions make Fsd a promising target for modifying the aging process.

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