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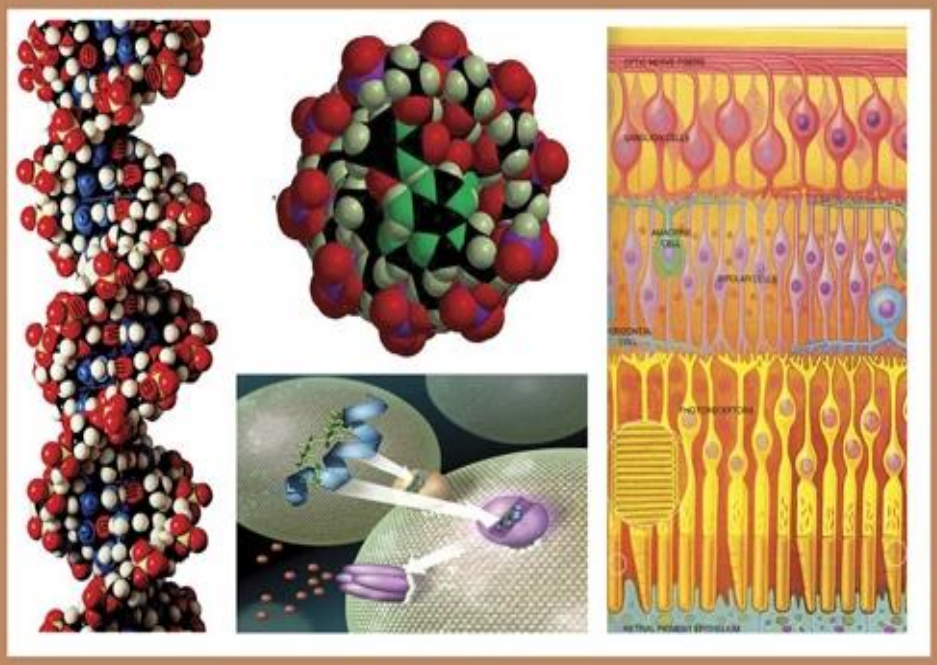

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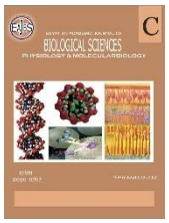

Combined Mesenchymal Stem Cell-Derived Exosomes and H_2S Ameliorated the Neurodegenerative Changes in Parkinson's Disease: Implication of PI3K/AKT Signaling Pathway

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ABSTRACT

Background: Parkinson's disease (PD) is the most common age-related motor neurodegenerative disease. Current therapeutic modalities for PD are directed at controlling the motor symptoms to slow disease progression, while no exact therapy exists to repair the immient neuronal damage. Despite the involvement of exosomes in the pathogenesis of PD, they serve as a promising therapeutic tool. Exosomes can restore the homeostasis of oxidative stress, neuro-inflammation, and cell apoptosis. Although H_2S is involved in the pathogenesis of PD, it has anti-oxidant, anti-inflammatory and anti-apoptotic neuroprotective effects, giving hope for the role of this gaseous molecule in PD therapy. **Objective**: We aimed to evaluate the therapeutic effect of exosomes and/ or H_2S-donor (NaHS) on PD, through modulation of PI3K/AKT signaling pathway, and to compare their combined effect with the standard treatment, L-DOPA. **Methods:** This study was conducted on forty-eight female white albino rats divided equally into 6 groups: control group, Parkinson group induced by 6-hydroxydopamine (6-OHDA), 3 Parkinson groups treated with either L-DOPA, exosomes, or H_2S-donor (NaHS), and Parkinson group treated with both exosomes and NaHS. The following parameters were assessed in the rat brain tissues: gene expression of Nrf2, Keap1, α-synuclein, and miRNA-141, protein levels of PI3K, AKT, Dopamine, TNF-α and caspase-3. GSH and MDA levels were also measured. In addition, behavioral function tests and histo-pathological examination of rat brain tissues were also performed. **Results:** In the Parkinson group, there was a significant deficit in behavioral functions, along with down-regulation of the Nrf2 gene and its downstream antioxidant GSH, and increased levels of the lipid peroxidation biomarker MDA. Also, there was increased neuro-inflammation as evidenced by increased levels of TNF-α with decreased levels of the neurotransmitter Dopamine. Moreover, there was increased gene expression of Keap-1, miRNA-141, and α-synuclein associated with decreased levels of AKT, PI3K and increased caspase-3 levels. On the other hand, all treated groups, especially the combined exosomes and H_2S donor-treated group, significantly reversed the deteriorating impacts of 6-OHDA on rat brains, as evidenced by the improvement of the behavioral dysfunction and the histopathological picture, which agrees with the biochemical and molecular findings of PI3K/AKT signaling pathway. **Conclusion:** These data suggest that combined exosomes and H_2S-donor could be considered as a potential and effective line for treating PD.

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It is a progressive disorder that has high rates of morbidity and mortality. Although the main diagnostic signs of Parkinson's disease are motor symptoms such as bradykinesia, rigidity, and tremor; it is also accompanied by non-motor ones as autonomic dysfunction, depression, and hallucinations making its initial diagnosis challenging (Halli-Tierney *et al*., 2020). The cause of the movement disorder is the loss of dopaminergic neurons of the substantia nigra pars compacta and the pathological hallmark is the aggregation of α-synuclein (α-syn) intracellular, in the form of both Lewy bodies as well as Lewy neurites. While the majority of PD cases are idiopathic, around 10% of the cases were found to be genetic in origin with both autosomal-dominant and recessive susceptibility genes have now been identified (Stefanis, 2012).

Apoptosis and autophagy are crucial intracellular processes that support the organism's homeostasis and promote survival. Autophagy selectively breakdown damaged cellular organelles and protein aggregates, while apoptosis eliminates damaged or aged cells. A balance maintained between both autophagy and apoptosis is critical for cell fate, particularly the long-lived cells as neurons. On the contrary, their imbalance is related to neurodegenerative conditions like PD (Liu *et al*., 2019). The phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) signaling pathway has a role in regulating signal transduction as well as several biological processes including cell proliferation, apoptosis and autophagy. Previous studies have shown that PI3K/AKT signaling pathway can regulate neurotoxicity and mediate neuronal survival through its involvement in the Nrf2-dependent transcription in many cell types as a response to ROS insults (Zhuang *et al*., 2019). Accumulating evidence indicates that some drugs can act as neuroprotective by modifying the PI3K/AKT/Nrf2 pathway and so reduces cognitive impairment and neurological dysfunction (Long *et al*., 2021).

A new approach that allows a better understanding of the cellular and molecular mechanisms of PD to identify new therapeutic strategies and targets is necessary (Vilaça-Faria *et al.*, 2019). Exosomes are considered the smallest extracellular vesicles having a diameter ranging from 50 to 200 nm. They are released by the fusion of multivesicular endosomal bodies with the plasma membrane (Le Saux *et al*., 2020; Van Niel *et al*., 2018). Because the release of exosomes is closely related to intracellular protein transport along the endosomal-lysosomal pathway, their biology may be relevant to PD (Zagrean *et al*., 2018). Studies have revealed that exosomes transport toxic α-syn across the cells and induce apoptosis that is involved in the degenerative progression of PD. On the contrary, exosomes have been found to have potential neuroprotective benefits in PD (Wu *et al*., 2017), this is attributed to their role in intercellular communication and their capability to remove misfolded proteins which hinder the formation of neural stem cells (Zagrean *et al., 2018).* Moreover, Brain neurons and glial cells are capable of getting rid of and reducing harmful metabolites and proteins (e.g., α-syn) in cells through exosome extravasation (*Ohmichi et al., 2018).*

Hydrogen sulfide (H_2S) is considered the third gaseous transmitter alongside nitric oxide (NO) and carbon monoxide (CO). The mammalian brain produces H_2S abundantly through four enzymatic pathways, ensuring its redundancy in the organ (Cao *et al*., 2018). Currently, it is considered an emerging neuroprotectant as well as a neuromodulator *(Panthi et al*., 2018). Its neuroprotective effects against apoptosis and degeneration may be attributed to its anti-inflammatory and anti-oxidative effects (Popov, 2013). Also, the neuroprotective function of H_2S can be mediated by controlling the intracellular pH of microglia and limiting the

damage of activated microglia in the injured site (Lu *et al*., 2010). Moreover, it modifies neurotransmission by affecting the actions of N-methyl-D-aspartate (NMDA) receptors as well as second messenger systems including intracellular Ca (2+) concentration and intracellular cAMP levels. Therefore, H_2S exhibits potential therapeutic efficacy in a number of neurological conditions, such as Alzheimer's disease, Parkinson's disease, ischemic stroke, and traumatic brain injury (Zhang and Bian, 2014). Based on the foregoing, the current study was performed to evaluate the therapeutic effect of exosomes and/ or H_2S-donor (NaHS) on PD, through modulation of PI3K/AKT signaling pathway, and also to compare their combined effect with the standard treatment of PD, L-DOPA.

MATERIALS AND METHODS

1. Experimental Animals:

All animals were obtained from the Kasr Al-Ainy hospital's animal house. The Institutional Animal Care and Use Committee (IACUC) at Cairo University gave its approval to the study protocol. The study was carried out at the Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Cairo University, Egypt.

This study included 48 female white albino rats obtained from an inbred strain of matched age and weight (160–200 g). Animals were kept in standard stainless-steel cages under standard environmental conditions with free access to water and fed ad-libitum. All procedures of the experiment were done following the international guidelines for the care and use of laboratory animals published by the U.S. National Institute of Health. They were raised in an air-conditioned animal house under specific pathogen-free conditions in accordance with IACUC's standard guidelines.

2. Experimental Design:

Animals were divided equally into 6 groups each containing 8 rats.

- Group I: Control group
- Group II: PD rats have been experimentally done through unilateral injection of 2 µL of 6-OHDA hydrochloride in the right hemisphere (Sigma-Aldrich, St. Louis, MO, USA). The neurotoxin was injected into the medial forebrain bundle (MFB) according to Barata-Antunes *et al.* (2020), under stereotaxic coordinates Paxinos and Watson (1998). Ketamine anesthesia at a dose of 75 mg/kg, i.p. was administered during the injection.
- Group III: PD rats treated with L-DOPA (10 mg/kg) with benserazide (6.5 mg/kg) twice daily i.p. for 3 weeks (Picconi *et al.,* 2004). We gave Benserazide to prevent decarboxylation of L-DOPA in the periphery.
- Group IV: PD rats treated with a weekly intraperitoneal injection of 200 µg/mL of MSC-derived exosomes in PBS (Xue *et al.*,2021).
- Group V: PD rats treated with an intraperitoneal injection of NaHS, as a donor of H_2S, at a dose of 5.6 mg/kg, daily for seven days (Reza *et al.*,2018)
- Group VI: PD rats treated with both exosomes and NaHS as mentioned before.

3. Preparation of Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs):

The isolation and culture of the BM-MSCs were done as described in previous research (Sabry *et al.*,2020), They were characterized by morphology, adherence and FACS (fluorescence-activated cell sorting) by assessing CD29 and CD 105 positivity and CD34 negativity specific to MSCs. When large colonies developed, washing of the cultures was done two times with PBS. Trypsinization of the cells was done with 0.25% trypsin in 1 mM EDTA to obtain a second passage.

4. Extraction and Characterization of Exosomes Derived From BM-MSCs:

The extraction of MSC-derived exosomes was performed as described previously (Sabry *et al.*,2020). For characterization, fixation of the purified exosomes was done with 2.5% glutaraldehyde for 2 h, following washing; ultra-centrifugation was performed for the exosomes and suspension in 100 µL human serum albumin (HSA) was done. A total of

20 µL of exosomes was loaded onto a formvar/carbon-coated grid, negatively stained with 3% aqueous phosphor-tungstic acid for one minute and observed by transmission electron microscopy. After the end of the study (six weeks), scarification of the animals was performed by cervical dislocation. Brain tissues were obtained, where the striatum and substantia nigra were fixed with 4% paraformaldehyde and frozen at -80 ℃ for further investigations.

5. Estimation of Gene Expression of Nrf2, Keap1, α-synuclein, and miRNA-141 by RT-PCR:

Total RNA extraction from rat brain tissues was done using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Quality check of RNA was done using NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Following that synthesis of complementary DNA (cDNA) was done utilizing the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Page 5/15 Scientific) following the instructions. Amplification of Nrf2, Keap1, α-synuclein and miRNA-141 genes was done by Real time-qPCR by using SYBR Premix Ex TaqTM II (Perfect Real Time, TaKaRa, Japan). The following primers were used: Nrf2 Forward primer: 5′ CACATCCAGA CAGACACCAGT 3′ Reverse primer: 5′ CTACAAATGGGAATGTCTCTGC 3′, Keap1 Forward primer: 5′GGACGGCA ACACTGATTC3′ Reverse primer: 5′ TCGTCTCGATCTGGCTCATA 3′, α-synuclein Forward primer: 5′ TCAGCCC AGAGCCTTTCAC - 3′ Reverse primer: 5′ AGCCACAACTCCCTCCTTG 3′, miRNA-141 forward primer: 5′ CGGGCTA ACACTGTCTGGTAAAG 3′ reverse primer 5′ GTGCAGGGTCCGAGGTATTC3′. The conditions of the PCR reaction were as follows: 95°C for 5 min, then 40 cycles at 95°C for 15 seconds then 60°C for 60 seconds. The 2 -ΔΔCt method was applied to quantitatively analyze the results. Normalization to β-actin and U6 snRNA was done.

6. Estimation of PI3K and AKT Protein Levels by Western Blot Technique (Using V3 Western Workflow Complete System, Bio-Rad Hercules, California):

Homogenization of 5 milligrams of the rat brain tissue was performed in RIPA buffer, following that centrifugation at 12,000 rpm for 20 min. Bradford assay was used to detect the protein concentration for each cell lysate. Equal amounts of protein (20–30 µg of total protein from cell lysate) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked in TBS buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 1 hr, and incubated with the specific primary antibodies for PI3K and AKT supplied by Thermoscientific overnight at pH 7.6 at 4 °C with gentle shaking. Following washing, the addition of peroxidase-labeled secondary antibodies was done. Incubation of the membranes was performed at 37 °C for 1 hr. Analysis of the band intensity was done by ChemiDoc imaging system with Image Lab software version 5.1 (Bio-Rad Laboratories Inc., Hercules, California). Beta-actin was utilized to normalize the protein levels.

7. Estimation of Dopamine, TNF-α and Caspase-3 Levels in Rat Brain Tissues by ELISA:

ELISA was performed according to the manufacturer's recommendations of (Elabscience Biotechnology Inc., Texas) for estimation of Dopamine, TNF-α and caspase-3.

8. Estimation of GSH and MDA Levels in Rat Brain Tissues by Routine Colorimetric Methods*:*

For measurement of MDA, we added thiobarbituric acid (TBA) in 2 Mol. sodium sulfate, 2.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% TBA to 0.5 ml of tissue homogenate. Heating of the mixture was done in a boiling water bath for 30 min. The resulting chromogen is extracted with 4 ml of N butyl alcohol. The absorbance of the organic phase is determined at the wavelength of 530 nm.

As regards GSH, homogenization of

the brain tissues was done in 5–10 ml cold buffer (i,e, 50 mM potassium phosphate, pH 7.5. 1 mM EDTA) per gram tissue. Centrifugation of the homogenate was performed at 100,000×g for 15 min at 4 °C. Following that, the supernatant was removed for assay and stored on ice. Reduction of 2-nitrobenzoic acid with GSH produced a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance was measured at 405 nm.

9. Histopathological Examination of Rat Brain Tissues:

Excision of the brain tissues from the substantia nigra and striatum was done and washed with normal saline and fixed immediately in 10% formalin solution. A paraffin embedding technique was done. Sections were obtained with 5-mm thickness. A microscopical examination of the histopathological changes by H&E was done.

10. Statistical Analysis:

One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used for data analysis using SPSS statistical analysis software (version 11.5). Summarization of the data was done as mean ± *SD* (Chan, 2003). The *p*-values less than 0.05 were considered statistically significant.

RESULTS

1. Effect of L-DOPA, Exosomes and H_2S Donor on miRNA-141 Gene Expression:

Our results revealed significant upregulation in miRNA-141 expression in the PD group compared to the control group (p value < 0.05). On receiving treatment, miRNA-141 expression significantly downregulated as compared to the PD group (p value < 0 .05), with the best results seen in the group receiving combined exosomes with H_2S-donor (Fig. 1).

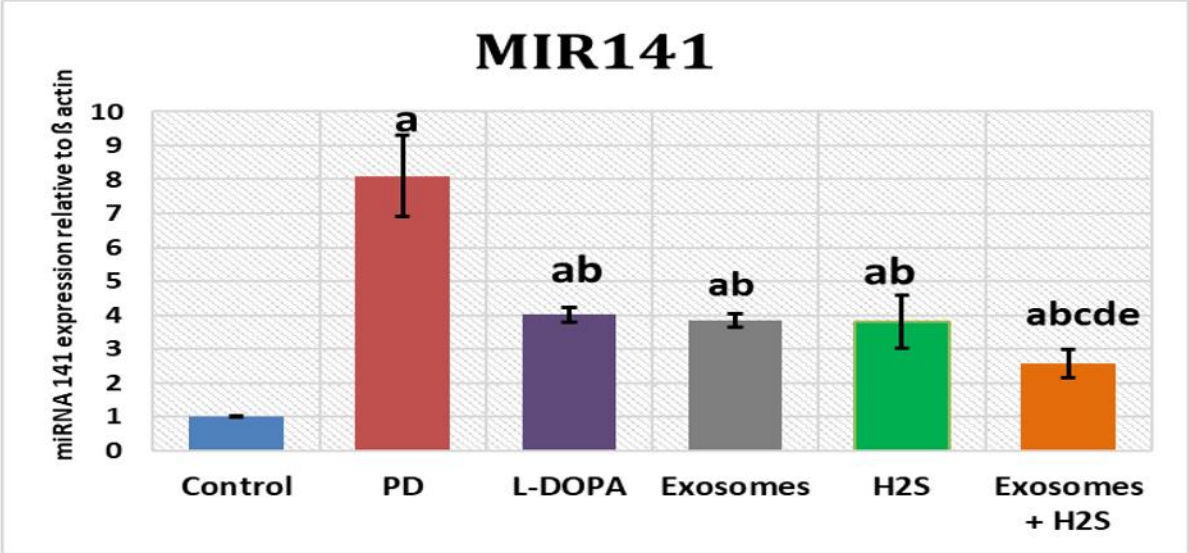


Fig. 1: Comparison between miRNA-141 gene expression in all studied groups.
Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control ([a]), PD ([b]), L-DOPA ([c]), exosomes ([d]), H_2S-donor ([e]) groups (P < 0.05).

2. Modulation of the Anti-Apoptotic PI3K/AKT Signaling Via miRNA-141 Epigenetic Regulation:

Our results concerning PI3K and AKT showed a significant decrease in the PD group as compared to the control group (p value < 0.05). On receiving treatment, the levels of PI3K and AKT significantly increased in all treated groups as compared to the PD group (p value < 0.05), with the best results seen in the group receiving combined exosomes with H_2S-donor, where there was no significant difference as compared to the normal healthy controls (Fig. 2).

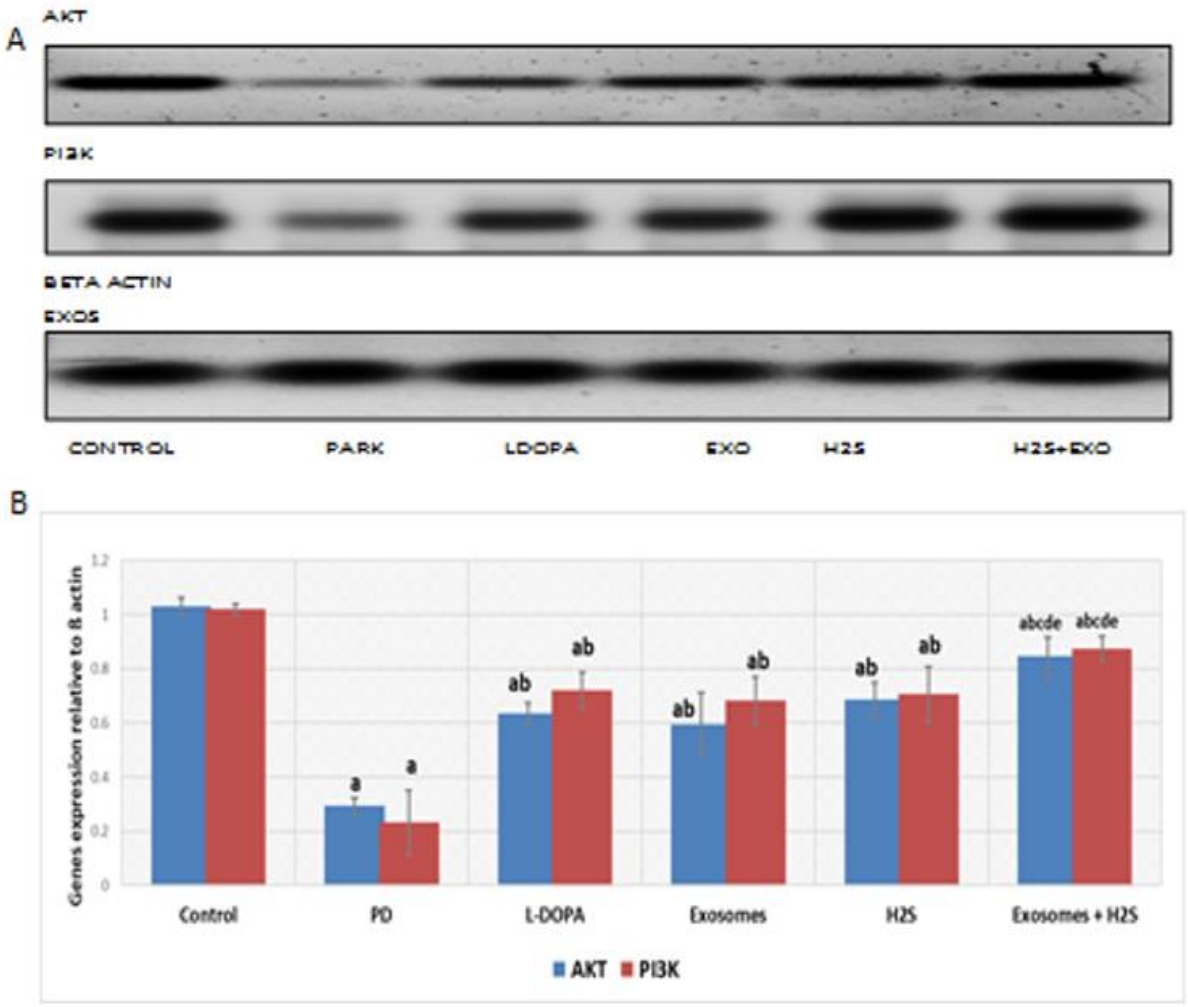


Fig. 2: Relative protein expression of PI3K and AKT in rat brain tissues among all studied groups.
Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control ([a]), PD ([b]), L-DOPA ([c]), exosomes ([d]), H_2S-donor ([e]) groups ($P < 0.05$).

3. Assessment of Caspase-3 and Dopamine Levels in All Studied Groups:

As shown in Figure (3), our results showed that caspse-3 in the PD group was significantly increased than the control group (p value < 0.05). Treatment with either L-DOPA, exosomes, or H_2S-donor significantly reduced the caspse-3 levels (p value < 0.05). Moreover, on receiving combined exosomes with H_2S-donor, there was a significant decrease in caspse-3 levels compared to the L-DOPA, exosomes, and H_2S-donor groups (p value < 0.05), with no significant difference between it and the control group.

Regarding dopamine levels, our results revealed that there was a significant decrease dopamine level in the PD group as compared to the control group (p value < 0.05). Whereas all treated groups showed significantly increased dopamine levels compared to the PD group (p value < 0.05), with further significant differences between the combined treated group and the group treated with exosomes.

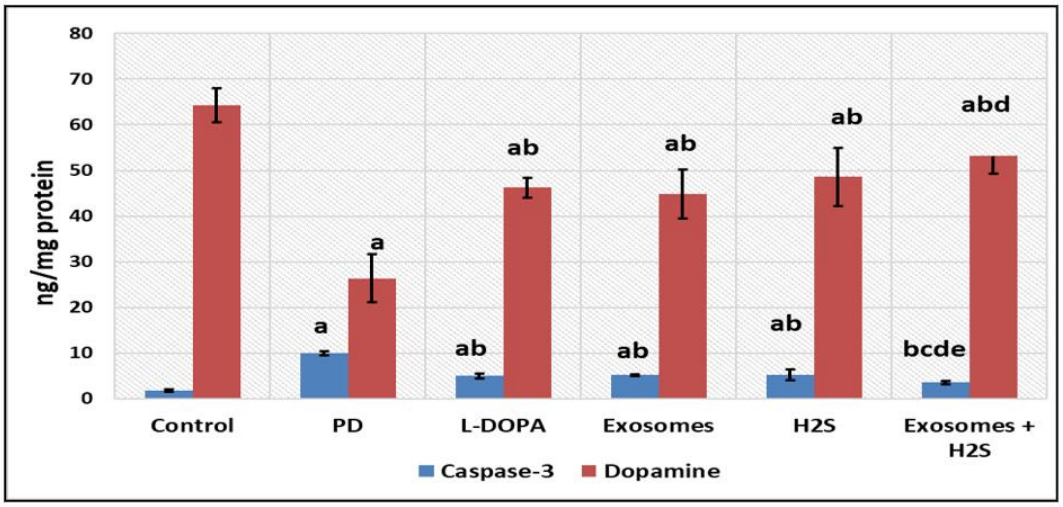


Fig. 3: Comparison of caspase-3 and dopamine levels in rat brain tissues among all studied groups.

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control ([a]), PD ([b]), L-DOPA ([c]), exosomes ([d]), H_2S-donor ([e]) groups ($P < 0.05$).

4. Effect of Treatment on Nrf2, Keap1, and α-synuclein Relative Gene Expression:

As illustrated in Figure (4), regarding Nrf2, there was a significant decrease in its expression levels in the PD group as compared to the control. Whereas all treated groups showed a significant increase in Nrf2 gene expression levels as compared to the PD group. The group that received combined exosomes and H_2S-donor showed the best results of all, where there was a significant increase in Nrf2 levels compared to all other treated groups.

However, concerning Keap1, our results showed a significant increase in the PD group as compared to the control group (p value < 0.05). On treatment, the expression level of Keap1 significantly decreased in the treated groups as compared to the PD group (p value < 0.05), with the best results seen in the groups receiving exosomes alone and combined exosomes with H_2S-donor.

Concerning α-synuclein, the current results showed a significant increase in the PD group as compared to the control group (p value < 0.05). This was significantly reversed in all treated groups, with the best results seen in the group receiving combined exosomes with H_2S-donor.

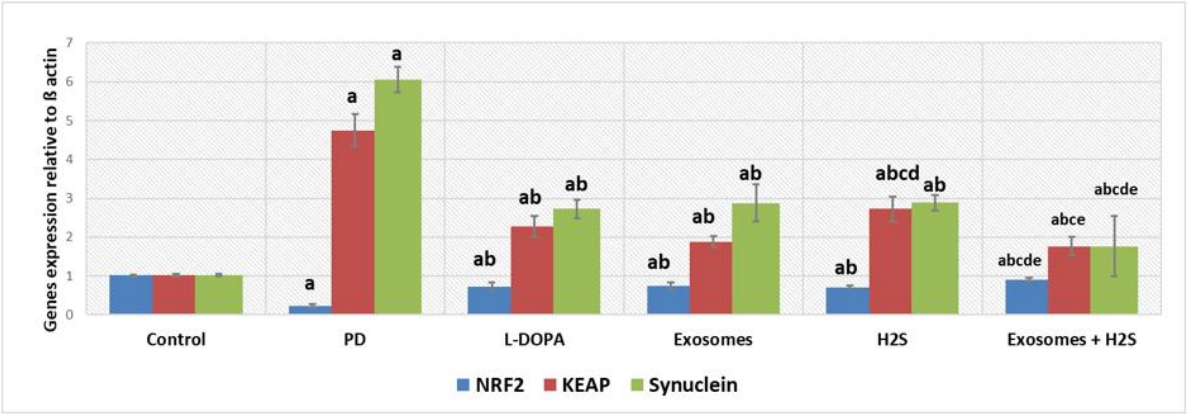


Fig. 4: Relative gene expression of Nrf2, Keap1, and α-synuclein in rat brain tissues among all studied groups.

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control ([a]), PD ([b]), L-DOPA ([c]), exosomes ([d]), H_2S-donor ([e]) groups ($P < 0.05$).

5. Assessment of Inflammatory and Oxidative Biomarkers:

As shown in Table 1, regarding the level of the inflammatory cytokine TNFα, there was a highly significant increase in the PD group compared to the control group (p value < 0.001). On receiving L- DOPA, exosomes, or H_2S-donor, each one alone, the level of TNFα significantly decreased as compared to the PD group. Furthermore, on receiving combined exosomes and H_2S-donor, there was a further significant decrease in TNFα level compared to the L-DOPA, exosomes, and H_2S-donor groups.

As we show here, there was a significant decrease in the antioxidant GSH level in PD group compared to the control group. Whereas, there was a significant increase in its levels in all treated groups as compared to PD group, with further significant increased levels in combined exosomes and H_2S-donor group compared to other treated groups with either L-DOPA, exosomes, or H_2S-donor alone.

On the other hand, the level of MDA, a marker of lipid peroxidation, in rat brain tissues showed a significant increase in PD group as compared to the control group. While there was a significant decrease in MDA level in all treated groups as compared to PD group, with a further significant decrease in combined exosomes+H_2S-donor treated group compared to other treated groups with either L-DOPA, exosomes, or H_2S-donor alone.

Table (1): Comparison between TNFα, MDA, and GSH levels in all studied groups

	Control	PD	L-DOPA	Exosomes	H_2S	Exosomes + H_2S
TNFα (pg/mg tissue protein)	14.81±2.14	89.55±9.28^{a}	37.14±5.2$^{a, b}$	41.61±5.02$^{a, b}$	37.49±12.52$^{a, b}$	21.5±2.26$^{b, c, d, e}$
MDA (nmol/mg tissue protein)	18.94±2.07	120.83±6.4^{a}	65.29±2.17$^{a, b}$	69.95±11.05$^{a, b}$	63.71±10.29$^{a, b}$	27.69±4.43$^{b, c, d, e}$
GSH (nmol/mg tissue protein)	122.09±10.93	39.44±5.23^{a}	93.78±3.26$^{a, b}$	92.91±7.45$^{a, b}$	95.85±3.01$^{a, b}$	117.49±5.85$^{b, c, d, e}$

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control (^{a}), PD (^{b}), L-DOPA (^{c}), exosomes (^{d}), H_2S-donor (^{e}) groups ($P < 0.05$).

6. Behavior Study Through the Percent of Alternation and Time Consumed in the T-Maze Test:

There was a significant increase in time consumed in the T-maze test in the PD group compared to the control group. Whereas, there was a significant decrease in all treated groups compared to the PD group, with the best results seen in the group receiving combined exosomes and H_2S-donor. However, there was a significant decrease in the percent of alternation in the T-maze test in the PD group compared to the control group, while there was a significant increase in all treated groups compared to the PD group, with a more prominent effect in the group received combined treatment (Fig. 5).

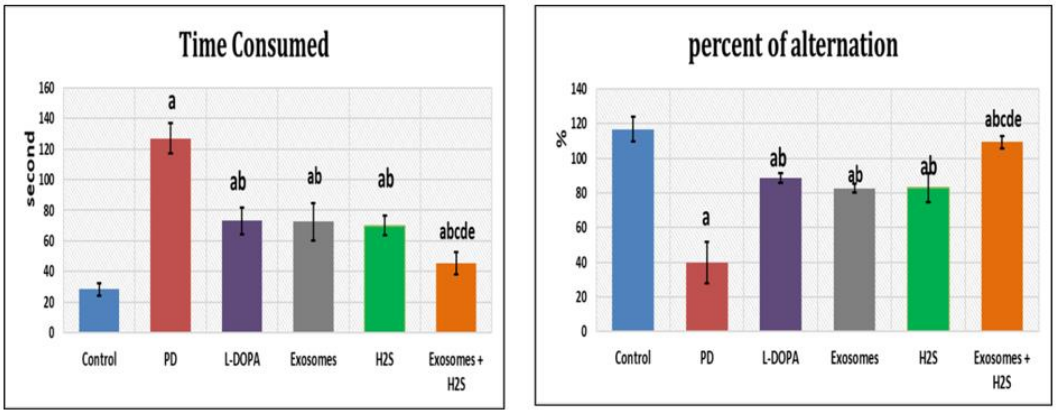
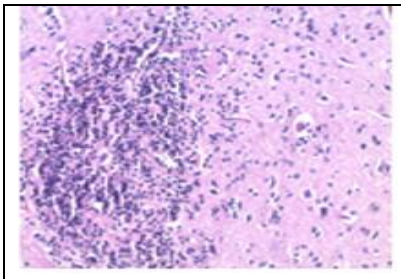


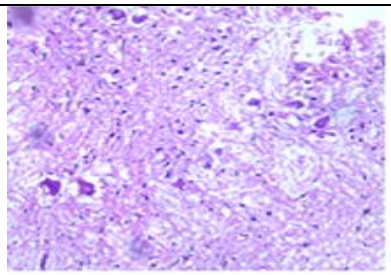
Fig. 5: Time consumed and percent of alternation in the T-maze test in all studied groups. *Results are expressed by mean ± SD. Statistically significant compared to corresponding value in: control (^{a}), PD (^{b}), L-DOPA (^{c}), exosomes (^{d}), H_2S-donor (^{e}) groups ($P < 0.05$).*

7. Histopathological Examination of The Substantia Nigra and Striatum of Rat Brain Tissues:

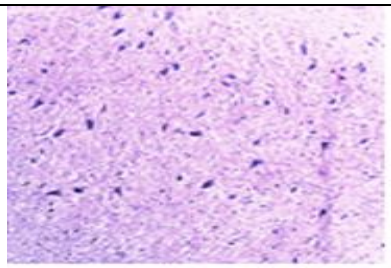
Concerning the histopathological examination of the brain tissue, the best improvement was detected in the combined group that received exosomes with H_2S where the number of ganglion cells in substantia nigra was within normal and there was a restoration of the pigmentation while less improvement was detected in each treated group alone as shown in Figure (6).



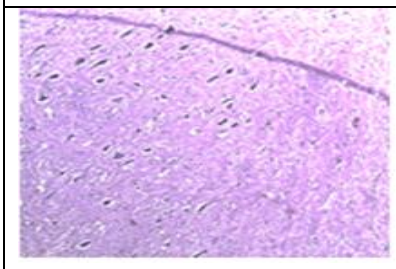
A. Section in the rat brain showing normal brain (200x, H&E).



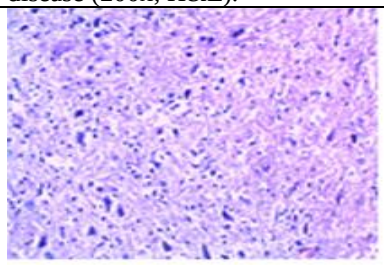
B. Section in rat brain showing loss of pigmentation in substantia nigra neurons associated with mild astrocytosis, vacuolar degeneration and edema in a case of Parkinson's disease (200x, H&E).



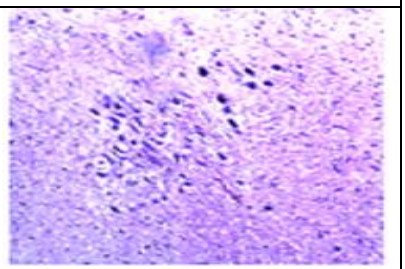
C. Section in rat brain after receiving L-DOPA showing slight improvement as regards the pigmentation of the substantia nigra ganglion cells. (200x, H&E).



D. Section in rat brain after receiving exosomes showing average number of substantia nigra ganglion cells with partial loss of pigmentation (100x, H&E).



E. Section in rat brain after receiving H_2S showing slight improvement as regards the number of the substantia nigra ganglion cells and loss of pigmentation (200x, H&E).



F. Section in rat brain after receiving combined treatment showing within normal number of ganglion cells in substantia nigra (200x, H&E).

Fig. 6: Showing the histopathological examination of the substantia nigra and striatum of rat brain tissues**.**

DISCUSSION

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease worldwide, following Alzheimer's disease (AD) (Kalia and Lang, 2015). PD has a complex and diverse etiology that involves interactions between both genetic and environmental factors. Parkinson's disease is frequently accompanied by neurotransmitters imbalance, lipid and energy metabolism disorders, and mitochondrial dysfunction (Havelund *et al.*,2017; Kori *et al.*,2016)

Exosomes, nanosized extracellular vesicles, have been shown to contribute in the pathophysiology of PD (Sun *et al.*, 2020). It has been demonstrated that exosomes can spread oligomers of α-synuclein in the brain hastening the progression of this pathology. Exosomes may exert a neuroprotective role in Parkinson's disease (PD) by limiting the toxicity of α -synuclein by removing it from cells (Biancamaria *et al.*, 2019) .

Current evidence reveals a link between H_2S with Parkinson's disease. Despite the fact that the role of endogenous H_2S is still unclear in the pathophysiology of PD yet various researches have revealed that exogenous H_2S has been shown to be protective against it.H_2S has been proved to protect against neuronal loss in a number of PD models, pointing out that this gaseous molecule may be a new hope for this devastating disease. Recent studies have found that H_2S protective effects are attributed to its anti-oxidant, anti-inflammatory, anti-apoptotic and pro-survival activity (Cao *et al*., 2018).

In our study, we aimed to evaluate the therapeutic effect of exosomes and/ or H2S-donor (NaHS) on PD, through modulation of PI3K/AKT signaling pathway, and to compare their combined effect with the standard treatment, L-DOPA.

Our results confirmed that treatment of Parkinson's disease in rats with combined exosomes and H_2S has improved the condition as evidenced by the histopathological examination and the different biochemical markers assessed in this study which play an important role in the pathogenesis of PD.

Oxidative stress has been shown to have a significant role in dopaminergic neuronal cell death in PD. The nuclear factor E2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) signaling pathway is considered to provide the primary defense mechanism against this oxidative stress through the induction of the expression of antioxidant enzyme genes (Kim,2020).

Nrf2 is a basic region leucine-zipper transcription factor that has a crucial role in the coordinated gene expression of antioxidant and detoxifying enzymes, allowing for the survival of the cell under unfavorable environmental or metabolic conditions (Zgorzynska *et al.*,2021). Nrf2 transcription factor is under tight control by Keap1, a cytoplasmic repressor protein, that plays a pivotal role in Nrf2 degradation through the ubiquitin-proteasome pathway (Bryan *et al.*, 2013). As a response to oxidative stress, Nrf2 dissociates from Keap1, moves to the nucleus and upregulates a number of cytoprotective antioxidant genes (Taguchi *et al.*, 2011). Petrillo *et al.*, 2020 hypothesized a connection between the Nrf2 pathway and the disease duration, pointing to a compensatory attempt during the progression of the neurodegenerative process.

The role of Nrf2 in neuroprotection is illustrated by the fact that its deficiency increases alpha-synuclein aggregation and chronic inflammation participating in DA neuron degeneration in PD (Lastres-Becker *et al.*, 2012).

Similarly, in the present study, Nrf-2 showed a significant decrease and Keap1showed a significant increase in the PD group as compared to the control. Studies on the pathophysiology of Parkinson's disease concurred with us in demonstrating that Nrf2 expression was significantly reduced in nigral dopaminergic neurons of PD patients (Schipperab *et al.*, 1998). More

significantly, in Nrf2 (−/−) knockout mice, this decrease in dopaminergic neurons and inflammation-mediated microglia activation were increased (Rojo *et al.,* 2010) According to studies, aging and NDs may have an impact on the Nrf2/ARE pathway (Yamazaki *et al.,* 2015). For instance, a meta-analysis of AD and PD gene expression in different tissues revealed that from 54 affected genes, 31 were downregulated genes containing ARE (antioxidant response elements) (Wang *et al.,* 2017). Nrf2 deficiency also increased the sensitivity of mice to MPTP-induced behavior and biochemical changes. In the MPTP mouse model of PD, the overexpression of Nrf2 in astrocytes was sufficient to provide neuroprotection (Chen *et al.,* 2009)

On receiving treatment, the treated groups showed a significant increase in Nrf-2 levels and a significant decrease in keap1 levels as compared to the PD group with the best results seen in the groups which received H_2S and combined exosomes and H2S

Our findings can be explained by li *et al.,* 2018 who stated that exosomes stimulate wound healing because they overexpress Nrf2. Similarly, Shen *et al.,* 2021 demonstrated that ADSCs exosomes enhanced the expression and nucleus translocation of Nrf2, but downregulated its negative mediator Keap1.

Concerning H_2S and its effect on NRF2, it was shown that it enhances Nrf2 functions through the activation of downstream enzymes like HO-1 and SOD1, and through decreasing intracellular ROS levels (Benedetti *et al.,* 2017).

Coinciding with us, ADSCs exosomes enhanced expression and nucleus translocation of Nrf2, while downregulated its negative mediator Keap1 (Shen *et al.,* 2021). The activation of Nrf2 by Keap1 S-sulfhydration at Cys151, which is regarded to be a posttranslational modification of Keap1, is one interpretation of H_2S protective effects (Xie *et al.,* 2016).

The results of our study revealed that there is an alteration in the oxidative profile in PD as we found a significant increase in levels of the oxidative stress biomarker MDA and a significant decrease in levels of GSH as compared to normal controls.

These results were consistent with Javed *et al.* (2016) whose study was to find out the neuroprotective effects of nerolidol, which has antioxidant and anti-inflammatory properties, on a rotenone-induced experimental model of PD. ROT administration significantly decreased glutathione (GSH) antioxidant levels as well as increased the levels of the lipid peroxidation product malondialdehyde (MDA) (Javed *et al.,* 2016). Nerolidol supplementation, however, significantly increased the level of GSH and decreased MDA in rats that had received ROT injections (Javed *et al.,* 2016). Nerolidol's anti-oxidant and anti-inflammatory properties were responsible for its neuroprotective effects, which strongly suggests that it has therapeutic promise in the treatment of Parkinson's disease (Javed *et al.,* 2016).

After treatment, our study confirms that the best results for minimizing the oxidative stress in PD were attributed to the role of combined exosomes and H_2S as evidenced by the significant decrease in the levels of MDA and the significant increase in the levels of GSH as compared to Parkinson group, L-DOPA group, exosomes alone or H2S alone. No significant difference between this combined group and the control group confirms the role of combined exosomes and H2S in treating Parkinson's disease.

These results were consistent with Sun *et al.* (2020) who stated that blood exosomes have neuroprotective effects in a 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine hydrochloride- (MPTP-) induced PD mice. According to his findings, MPTP treatment increased the level of MDA significantly in comparison with the controls, and after exosome treatment, this change was markedly reduced. This indicates that exosome treatment can restore oxidative stress homeostasis and is considered to be a

promising target for Parkinson's disease treatment (Sun *et al.,* 2020). In agreement with us, exosomes derived from human umbilical cord mesenchymal stem cells (hucMSC-ex) were discovered to protect against cisplatin-induced renal oxidative stress and apoptosis both in vivo and in vitro. There was a marked reduction in the GSH levels whereas the MDA level was significantly increased in the cisplatin-induced acute kidney injury in comparison with the normal group. When comparing the hucMSC-ex-treated group with the PBS and cisplatin groups, the levels of GSH were clearly increased, while the MDA levels decreased (Zhou *et al.,* 2013). In agreement with our results Liu *et al.* (2020), and his colleagues discovered that in a Parkinson's disease cell model,1-methyl-4-phenylpyridinium ion (MPP+)-induced oxidative stress injury is reduced by H_2S. He found that in the control group, GSH content was 17.45 nmoL/mg protein, and decreased to 4.77 nmoL/mg protein after MPP+ induction. Pretreatment with 200 μmoL/L NaHS obviously reduced GSH depletion caused by MPP+ induction. MDA levels in SH-SY5Y cells were greatly raised by MPP+ induction, while they were significantly reduced by NaHS pretreatment (Liu *et al.,* 2020).

Tumor necrosis factor-alpha (TNF-), a pro-inflammatory cytokine, mediates neuroinflammatory processes that result in progressive neurodegeneration of dopaminergic neurons and are a key element in the pathophysiology of Parkinson's disease (PD) (Çomoğlu *et al.,* 2013). Numerous inflammatory mediators or pro-inflammatory cytokines, such as TNF-, which can cause neuronal death, are produced by overactive glial cells including microglia and astrocytes. These conditions can lead to the continual degeneration or death of dopaminergic neurons (Blaylock *et al.*, 2017). The binding of TNF- α to TNFR1 can trigger cell apoptosis via the activation of mitogen-activated protein kinase (MAPK), caspases, as well as transcription via NF-kB (Nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, that is responsible for cell death and promote inflammatory conditions (Sedger *et al.*,2014). These receptors together with TNF- α are highly expressed in brain tissues (Maddahi *et al.*,2011).

In the present study, the level of TNF- α in the rat brain tissue showed a significant increase in the Parkinson's group compared to the control group.

It was discovered, in agreement with us, that higher levels of TNF- α were found in the tears of PD patients, indicating that neuroinflammation and TNF- α play a role in the pathophysiology of PD (Çomoğlu *et al.*,2013).

After treatment, we observed that the best results were found after using combined exosomes and H_2S as evidenced by the significant decrease in the level of TNF-α as compared to the Parkinson group, L-DOPA group, exosomes alone, or H_2S alone. No significant difference between this combined group and the control group confirms the role of combined exosomes and H2S in treating Parkinson's disease.

Similar to our findings, it was reported that injecting huc-MSC exosomes alleviated neuroinflammation in Alzheimer's disease by lowering pro-inflammatory cytokine levels and up-regulating anti-inflammatory cytokines; however, the mechanism underlying this inflammatory response is still not clear (Ding *et al.,* 2018).

Regarding H_2S's protective role, numerous in vitro and in vivo PD models have shown it to be effective, indicating that it may offer new hope for the treatment of PD. Studies have demonstrated that the anti-oxidation, anti-inflammation, anti-apoptosis, and pro-survival activity mediated by H_2S are responsible for its protective effect (Cao *et al.,* 2018). Sun *et al.* (2020) stated that H_2S's protective effect is mediated via the negative regulation of epigenetic histone acetylation in Parkinson's disease. In his research, 6-hydroxydopamine (6-OHDA)-induced PD rats showed a substantial rise in the levels of cytokines such TNF- α, IL-2, IL-17A, IL-6 and IL-1 in comparison with

the controls. Following treatment with NaHS and tubastatin, the levels of these inflammatory cytokines were decreased demonstrating that histone deacetylase (HDAC) inhibition reduces PD signaling progression. Thus, the findings of his research showed that the H_2S protective effect can be mediated through the inhibition of HDAC, contributing to the development of new strategies in PD treatment (Sun *et al.*, 2020)

PI3Ks, a family of intracellular lipid kinases, are essential elements in the upstream of the PI3K/AKT signaling pathway (Thorpe *et al.*, 2015). PI3K p110α has an important protective effect in the process of oxidative stress-induced apoptosis (Matheny and Adamo, 2009). In a study by Huang et al., (2019), he tried to find out if resveratrol (Res) can delay the progression of 6-OHDA-induced apoptosis through the activation of the PI3K/ Akt signaling pathway. His results showed that PI3K-110α was decreased in the model group compared with the sham group (Huang *et al.*, 2019). This is in agreement with our results as we found that PI3K was decreased in the PD group as compared to the control group. AKT, also known as Protein kinase B (PKB), is a serine/threonine kinase, that is considered one of the most crucial effector kinases downstream of PI3K as well as being the core of PI3K/AKT signal pathway (Hanada *et al.*, 2004).

The loss of dopaminergic neurons in the SN and striatum caused by apoptosis is an important cause of PD. Researchers have found that AKT is significantly decreased in the substantia nigra compacta (SNpc) of PD patients (Luo *et al.*, 2019). This goes with our results concerning AKT which showed a significant decrease in the PD group as compared to the control group (p value < 0.05). The PI3K/AKT signaling pathway controls signal transduction as well as biological processes like cell proliferation, apoptosis and metabolism (Long et al., 2021). It has been found that the PI3K/AKT signal pathway plays a significant role in the central nervous system. (Matsuda *et al.*, 2019). Its role in the central nervous system's physiological processes like cell survival, autophagy, neurogenesis, neuronal proliferation and differentiation, and synaptic plasticity has been widely investigated. Recently, an increasing number of studies have discovered that many natural products based on PI3K/AKT signal pathway protect dopaminergic neurons, hippocampal neurons, and cortical neurons, and inhibit microglia activation, so they can contribute to the prevention and treatment of AD and PD. Studies reported that it can regulate neurotoxicity as well as mediate the survival of neurons through various substrates (Long *et al.*, 2021).

Caspase-3 activity is a significant hallmark of Parkinson's disease. It can cause neuronal death through apoptosis as well as microglia activation inflammation (Liu *et al.*,2013). This agrees with our results that show that there is a significant increase in the level of caspase- 3 in the Parkinson's group compared to the control group.

On receiving treatment, we found that the best results were obtained when using combined exosomes and H_2S as evidenced by the significant decrease in the level of caspase3 as compared to the Parkinson group, L-DOPA group, exosomes alone, or H2S alone. No significant difference between this combined group and the control group revealing of his the role of combined exosomes and H2S in treating Parkinson's disease.

Similar to our results, Lee et al. (2018) investigated the possible therapeutic effects of exosomes derived from ADSCs (ADSC-Exo) in preventing AD phenotypes that are caused by Aβ cascade in an in vitro model of AD. There was an elevation in the apoptotic molecules including p53, Bax and caspase-3 whereas the anti-apoptotic molecule, Bcl2, was decreased in AD cells compared with wild-type littermate cells. The ADSC-Exo therapy restored the levels of Bcl-2 protein, p53, Bax, pro- and cleaved-caspase-3. According to flow cytometry analysis, ADSC-Exo decreased the enhanced cell apoptosis of AD neuronal cells. These

results suggest that ADSC-Exo has disease-modulating properties in the transgenic mice-derived AD in an in vitro model and that ADSC-Exo may be used therapeutically to slow the progression of Aβ-induced neuronal death and AD (Lee *et al.,* 2018).

In agreement with our results, Liu *et al.* (2020) and his colleagues found that H_2S reduces cell apoptosis caused by MPP+ in Parkinson's disease cell model. In comparison to the control group, Caspase 3 activity was elevated by more than three times following the treatment with 500 μmoL/L MPP+. Caspase 3 activity elicited by MPP+ was significantly lowered after pretreatment with 200 mmol/L NaHS (Liu *et al.,*2020). His research demonstrated that hydrogen sulfide protected SH-SY5Y cells from cell apoptosis and oxidative stress injury caused by MPP+ in PD cell model through the suppression of the ROS-NO signaling pathway (Liu *et al.,* 2020).

The main pathological hallmark of PD is a marked loss of dopamine-producing neurons in the substantia nigra pars compacta, which causes severe depletion of dopamine in the striatum, where these neurons project (Lotharius *et al.,* 2002). Motor deficits and potentially the cognitive deficit seen in some PD patients are caused by dopamine depletion in the PD brain. PD is hardly diagnosed in its early stage due to the long latency between the initial damage to dopaminergic cells and the manifestations of clinical symptoms (Emamzadeh *et al.,* 2018). This is in agreement with our results that showed a significant decrease in dopamine levels in the Parkinson's group compared to the control group.

On receiving treatment, we found that the best results were observed when using combined exosomes and H_2S as evidenced by the significant increase in the level of dopamine as compared to the Parkinson group or to the exosomes group alone.

In agreement with our results, Chen *et al.* (2020) utilizing high-performance liquid chromatography-mass spectrometric (HPLC–MS) discovered that exosomes upregulated dopamine and its metabolites levels in the striatum of PD rats. The levels of DA, Dihydroxy Phenyl Acetic Acid (DOPAC), and Homovanillic Acid (HVA) were significantly decreased in the 6-OHDA+group compared to the control group. Following exosome treatment, DA, DOPAC, and HVA levels showed a significant increase revealing that exosomes enhance the levels of DA and its metabolites in the striatum. His findings showed that hucMSCs-Exos are capable of treating Parkinson's disease (PD) and can cross the blood-brain barrier (BBB), suggesting their possibilities for the successful treatment of PD (Chen *et al.,* 2020).

Similar to our results, in the study of Sun *et al.,* (2020) who evaluated the involvement of the epigenetic mediated mechanism in the protective effect exerted by H_2S, the protective role of NaHS and TSA on the neurochemical levels were assessed in the PD rat model. They assessed the contents of DA and DOPAC (3, 4-dihydroxyphenylacetic acid) in the striatal region (HPLC). Dopamine and DOPAC levels in the 6-OHDA group were significantly decreased in compassion to the controls. On the other side, their levels were significantly raised to levels equivalent to the controls in both NaHS and TSA-administered groups (Sun *et al.,* 2020).

Alpha-synuclein (αS) is the major constituent of Lewy bodies and a pathogenic hallmark of all synucleinopathies, such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) (Meade *et al.,*2019). (Bougea *et al.,*2019) found that the level of α-Syn was increased significantly in PD patients when compared to the healthy controls. Similarly, our results showed a significant increase in the PD group as compared to the control group (p value < 0.05).

Coinciding with our results, It has been revealed that the misfolded and aggregated α-Syn is the major component of Lewy bodies and axons in inherited and sporadic forms of PD (Leggio *et al.,* 2017; Tofaris, 2017).Exosomes derived from mesenchymal

stem cells (MSCs) have been shown to be a beneficial and successful therapeutic strategy for a variety of pathological conditions such as osteoarthritis (Mianehsaz *et al.,* 2019), multiple sclerosis (Li et al., 2019), and PD. In 6-OHDA mice models of PD, MSC-derived exosomes were found to restore dopaminergic neurons, offering a possible therapy for PD (Vilaca-Faria *et al.,* 2019).Such findings could be evidenced by our results, where the level of synuclein significantly decreased in the treated groups as compared to the PD group (p value < 0.05) with the best results seen in the group receiving combined exosomes with H_2S where there is a significant decrease in synuclein levels in the group receiving combined exosomes with H_2S as compared to the groups receiving L-dopa, H_2S or exosomes alone suggesting the possible role of exosomes and H_2S in lowering alpha-synuclein levels which is the main pathological hallmark of parkinsonism.

MicroRNAs are noncoding RNAs whose dysregulation contributes to neurodegenerative diseases by influencing the majority of the mechanisms responsible for them (Delavar *et al.,* 2018). They are being studied as possible diagnostic and prognostic biomarkers, and potential therapeutic targets.The members of the miR-200 family are miR-200a, -200b, -200c, -141, and -429. According to a number of studies, members of the miR-200 family are related to the pathogenesis of neurodegenerative diseases. Owing to a number of reports, the miR-200 family, which is involved in a number of cellular processes such as beta-amyloid (A) formation, alpha-synuclein aggregation, and DNA repair, is aberrantly expressed in many neurodegenerative diseases (Fu *et al.,* 2019). In an in vitro model, miRNA-141 overexpression was proved to induce nerve cell apoptosis, suppress proliferation, promote the protein expression of caspase-3/9, Bax and p53, as well as reduce silent information regulator 1 (SIRT1) protein expression (Liu *et al.,* 2019).

In the present study, miRNA141expression showed a significant increase in the Parkinson's group compared to the control group.

Similarly, miR-141 has been shown to be upregulated in an in vitro Parkinson's disease model and it may target SIRT1 correlating with the pathogenic process that causes PD (Delavar *et al.,* 2018). Zheng *et al.,* (2020) examined the activities of sirtuin1 (SIRT1) and miR-141-3p in a PC12-cell model of Parkinson's disease caused by 1-methyl-4-phenylpyridinium (MPP+). According to his findings, when MPP+ was applied to PC12 cells, there was an upregulation of miR-141-3p while there was a downregulation of SIRT1. He came to the conclusion that elevated miR-141-3p caused apoptosis, oxidative stress, as well as mitochondrial dysfunction in MPP+-treated PC12 cells by targeting the SIRT1 expression. His research suggested a promising PD treatment strategy (Zheng *et al.* 2020).

On receiving treatment, we found that the best results were observed when using combined exosomes and H_2S as evidenced by the significant decrease in the level of mi-141 as compared to the Parkinson group, L-DOPA group, exosomes alone or H2S alone revealing the role of combined exosomes and H_2S in treating Parkinson disease.

In conclusion, combined exosomes and H_2S can be considered as a potential, effective and novel line for treating Parkinson's disease as evidenced by the improved histopathological picture of the brains of the Parkinson disease rats and the studied biochemical markers.

Conflicts of Interest: The authors declare no conflict of interest.

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ARABIC SUMMARY

الجمع فى استخدام exosomesالمشتقة من الخلايا الجذعية الوسيطة مع ال H_2S أدى الى تحسين التغيرات العصبية التنكسية فى مرض باركنسون: تأثير مسارPI3K/AKT

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يعد مرض باركنسون (PD) هو أكثر أمراض التنكس العصبي الحركي المرتبطة بالعمر شيوعًا. يتم توجيه الأساليب العلاجية الحالية لمرض باركنسون في السيطرة على الأعراض الحركية لإبطاء تقدم المرض، بينما لا يوجد علاج دقيق لإصلاح الضرر العصبي الوشيك. على الرغم من تورط exosomes في التسبب في مرض باركنسون، إلا أنها تعمل كأداة علاجية واعدة. يمكن أن تعيد Exosomes التوازن من الإجهاد التأكسدي، والتهاب الأعصاب، وموت الخلايا المبرمج.

على الرغم من أن H2S متورط في التسبب في مرض باركنسون ، إلا أنه يحتوي على تأثيرات وقائية عصبية مضادة للأكسدة ومضادة للالتهابات ومضادة للاستماتة ، مما يعطي الأمل في دور هذا الجزيء الغازي في علاج PD.

الطريقة :أجريت هذه الدراسة على 48 أنثى من الجرذان البيضاء البيضاء مقسمة بالتساوي إلى 6 مجموعات: مجموعة التحكم ، مجموعة باركنسون التي يسببها 6-هيدروكسيدوبامين ,(6-OHDA)، 3 مجموعات باركنسون عولجت إما بـ L-DOPA، exosomes ، أو H2S- المتبرع (NaHS) ومجموعة باركنسون عولجت بكل من exosomes و NaHS. تم تقييم المعلمات التالية في أنسجة دماغ الفئران: التعبير الجيني عن Nrf2 و Keap1 و α-synuclein و miRNA-141 ، ومستويات البروتين من PI3K و AKT و Dopamine و TNF-α و caspase-3. كما تم قياس مستويات GSH و MDA

بالإضافة إلى ذلك، تم إجراء اختبارات الوظيفة السلوكية والفحص النسيجي المرضي لأنسجة دماغ الفئران.

النتائج: في مجموعة باركنسون ، كان هناك عجز كبير في الوظائف السلوكية ، جنبًا إلى جنب مع انخفاض المستوى التعبيرى الجينى لجين Nrf2 ومضاد الأكسدة GSH ، وزيادة مستويات المرقم الحيوي لبيروكسيد الدهون MDA. أيضًا، كان هناك زيادة في الالتهاب العصبي كما يتضح من زيادة مستويات TNF-α مع انخفاض مستويات الناقل العصبي الدوبامين. علاوة على ذلك، كان هناك زيادة في التعبير الجيني لـ Keap-1 و miRNA-141 و α-synuclein المرتبط بانخفاض مستويات AKT و PI3K وزيادة مستويات caspase-3 من ناحية أخرى، عكست جميع المجموعات المعالجة، وخاصةً exosomes مجتمعة والمجموعة المعالجة ب H2S ، بشكل كبير الآثار المتدهورة لـ OHDA-6 على أدمغة الفئران ، كما يتضح من تحسين الخلل الوظيفي السلوكي والصورة النسيجية المرضية ، والتي تتفق مع النتائج البيوكيميائية والجزيئية لمسار إشارات PI3K / AKT. **الخلاصة:** تشير هذه البيانات إلى أنه يمكن اعتبار exosomes مجتمعة ومتبرع H2S خطًا محتملاً وفعالًا لعلاج PD.