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Synergistic Neuroprotective Effect of Curcumin and Pioglitazone Against Intranigral LPS-Induced Sub-Acute Neurodegeneration in Rats

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Abstract

Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis and frontotemporal lobar dementia impose a pressing burden in most developed countries.

Parkinson's disease (PD) is clinically characterized as motor dysfunction disorder resulting from selective loss of substantia nigras dopaminergic neurons in the midbrain region. PD pathogenesis is multifactorial involving redox imbalance, glia-mediated inflammation and aggregated proteins accumulations that collectively lead to neuronal cell loss. Although traditional treatments relieve symptoms in early PD condition but they do not reverse the damage of dopaminergic neurons. Therefore, the present work was designed to investigate the neuroprotective efficacy of a novel combination of pioglitazone (PPAR-γ agonist) and curcumin in LPS-induced sub-acute dopaminergic neurodegeneration in rat model. We tested the effect of the combinations of pioglitazone (18 mg/kg.bw once daily) and curcumin (100 mg/kg.bw), in comparison to each one alone for five consecutive days before intranigral injection with lipopolysaccharide (LPS; 10 µg/2µl sterile saline/injection) against LPS-mediated neurotoxicity. The experimental results indicated that pretreatment with combined curcumin and pioglitazone, rather than each one alone, have powerful and synergistic anti-inflammatory effects by inhibiting the production of the cytokines TNF-α, IL-1β and IL-6 and inflammation-associated enzymes COX-2, iNOS through inhibition of NF-κB activity. Furthermore, this combination improved the brain antioxidant capacity by markedly elevating reduced glutathione content and catalase activity while reducing malondialdehyde level that were imbalanced by LPS. Moreover, pretreatment with pioglitazone and curcumin combination induced significant restoration in two of the inflammatory master regulator miRNAs (miR-155 and miR-124) levels toward normal profile expression. This study sheds light on the possible mechanism by which curcumin and pioglitazone combination might exert synergistic neuroprotective effect and provide benefit for those afflicted by neurodegenerative disorders. Further studies are still required to highlight in depth the efficacy of this combination for therapeutic applications.

Keywords: *Parkinson's Disease; Curcumin; Pioglitazone; LPS; miRNAs; Pro-Inflammatory Cytokines; NF-κB*

Abbreviations

BBB: Blood Brain Barrier; COX-2: Cyclooxygenase-2; GSH: Glutathione; IFN-γ: Interferon-Gamma; IL-1β: Interleukin-1beta; IL-6: Interleukin-6; iNOS: Inducible Nitric Oxide Synthase; LPS: lipopolysaccharide; MDA: Malondialdehyde; miRs: MicroRNAs; MMP-3: Matrix Metal-

loproteinase-3; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; MyD88: Myeloid Differentiation Factor 88; NF-κB: Nuclear Factor-Kappa B; NO: Nitric Oxide; PD: Parkinson's Disease; PPAR-γ agonist: Peroxisome Proliferator Activated Receptor-Gamma Agonist; PPRE: PPAR-γ Responsive Elements; ROS: Reactive Oxygen Species; SNpc: Substantia Nigra Pars Compacta; TLR4: Toll-Like Receptor 4; TNF-α: Tumor Necrosis Factor-Alpha; TZD: Thiazolidinediones; α-SYN: Alpha-Synuclein; γ-GCL: γ-Glutamyl Cysteine Ligase

Introduction

Parkinson's Disease (PD) is a common neurodegenerative disease that is characterized by motor dysfunction with symptoms including slowing of movement, rigidity and postural instability [1]. PD cases are 90% sporadic and 10% familial in reference to the disease etiology [2]. Numerous signaling pathways were correlated to PD development and progression. Oxidative stress and inflammation are key players in initiating and mediating dopaminergic neuronal cell loss in PD condition [3]. Deneyer., *et al*. [4] stated that "The disease is characterized by a progressive and selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leading to dopamine deficiency in the striatum". Because PD progression in is rapid and most of the up-to-date drugs do not prevent or slow down the dopaminergic neuronal loss, so applying neuroprotective strategies could provide novel insight in the management of Parkinson's patients. Many pathways are implicated in PD pathogenesis [4]. Recent studies in animal models have indicated that inflammation induced by administered lipopolysaccharide (LPS) can cause some characteristics of PD, such as extensive activation of microglial cells and selective loss of dopaminergic neurons in the nigrostriatal system [5]. LPS can elicit the release of cytokines and other inflammatory mediators via binding to specific receptors; the most important appear to be CD14 and LPS-binding protein (LBP) and the toll-like receptor (TLR) family which is a recently discovered group of transmembrane receptors [6,7]. In the central nervous system, it is found that systemic LPS injection upregulated its membrane CD14 receptor within specific cellular populations including microglia in the brain [7]. Thereafter, microglia was identified as the major LPS-responsive cell in the brain. LPS binds to TLR4 on microglia and induces microglial activation that results in neuronal damage.

The component of gram-negative bacteria endotoxin lipopolysaccharide (LPS) is widely used for selective induction of dopaminergic neurodegeneration of the substantia nigra (SN). It acts indirectly, via activation of microglial cells that release different inflammatory cytokines in rats and mice [5]. The binding of LPS to its receptor initiates the activation of various intracellular kinases and NF-κB signaling pathway [6]. NF-κB activation induces the expression of pro-inflammatory TNF-α and IL-1β as well as the production of NO and PGE_2 as soluble inflammatory mediators [7]. These soluble mediators damage nigral dopaminergic neuron by inducing oxidative stress and microgliosis [10]. Thus, inhibition of the microglial activation would be important for the prevention of the neurodegenerative process [8].

Pioglitazone (PPAR-γ agonist), is a member of thiazolidinediones (TZD) compounds, displayed a neuroprotective activity against inflammation-mediated model of neurodegeneration developed by intrastriatal injection of LPS in rats [9]. The neuroprotective mechanisms of pioglitazone-induced PPAR-γ activation were attributed to suppression of microglial activation following LPS injection [5,10]. Furthermore, pioglitazone specifically inhibits NF-κB, COX-2 and iNOS activation [11], as well as the transcription levels of TNF-α, IL-6 and IL-1β in stimulated microglia [12].

Curcumin the principal curcuminoid of Curcuma longa, is a safe supplement with low toxicity when administered at higher doses and can cross the blood-brain-barrier (BBB) [13]. Extensive studies including clinical trials have been conducted over the past quarter century to investigate the efficacy of curcumin against numerous diseases in humans. Curcumin was shown to exert potent anti-inflammatory activity through modulating several signaling pathways [14,15]. Previous reports indicated that curcumin blocks the induction of NF-κB, which consequently suppress IL-6 and TNF-α expressions [16]. It was also shown to activate PPAR-γ, thus potentiating the neuroprotective effects of pioglitazone (PPAR-γ agonist)in cancer model [17].

MicroRNAs (miRNAs) are widely expressed in brain tissue and play pivotal role in development, maturation and regulation of nervous system functions [18]. Recently, some miRNAs have been identified as crucial epigenetic regulators with specific altered expression

profile in neurodegenerative disorders including PD [19]. Many studies indicated a the role of specific miRNAs in modulating microglia and macrophages M1 and M2 polarization [20]. In particular, low expression level of inflammatory miR-155 and elevated expression of the anti-inflammatory miR-124 were linked to microglia resting state [21]. MiR-155 expression is elevated in response to inflammatory stimuli including, LPS, IFN-γ and TNF-α, an observation that is also implicated a microglia-activation in response neurotoxic agent [22]. On the other hand, over-expression of miR-124 has been reported to induce down-regulation of IL-6, TNF-α and iNOS as confirmed by the observation that highest levels of miR-124 were detected in resident microglia [22].

Accordingly, developing combined-therapy based on using novel combinations of well-documented compounds like curcumin and pioglitazone may offer new formula of potential neuroprotective activities by targeting multiple cellular pathways simultaneously to effectively suppress the dopaminergic neuron loss. Therefore, in the current study, we investigated the effect of pioglitazone and curcumin pre-treatment alone and in combination as novel strategy against intranigral LPS- induced subacute dopaminergic neurodegeneration in rats.

Materials and Methods

Animals and experimental design

All experiments were performed on forty, three-month-old male Sprague-Dawley (250 - 300g) rats. The animals were supplied and maintained at a medical research institute in which the principles approved by the ethics committee of animal research. Rats were housed in groups of 4 - 5 per cage under artificial light of 12hr-light: dark cycle (light on 8 a.m.), at a controlled temperature (20 - 25°C) and at humidity level maintained between 35 - 70% with free access to water and standard commercial food containing 55% sugars, 25% proteins, 4% fiber, 4.5% lipids and 6% ash.

Subacute dopaminergic neurodegeneration in rat model was established by intranigral LPS injection using stereotaxic surgery. The experimental design of different rat groups (with eight rats per group) is illustrated in the figure 1, in which rats were randomly selected and divided into three major groups: (1) Mock-treated rats (control group), were subjected to the infusion of 2 µL of 0.9% sterile saline into the SNpc using stereotaxic apparatus. (2) LPS-treated group (LPS-Trx group), were given 10 µg LPS/2 µl sterile saline/injection, into the SNpc using stereotaxic apparatus. (3) LPS pre-treated pioglitazone and/or curcumin groups (LPS + Piog and/or Cur group), were pre-treated with neuroprotective agents (Pioglitazone and/or Curcumin) for 5 days via oral gavage in vehicle suspension. On the sixth day, the rats were intranigrally injected with 10 µg LPS/2 µl sterile saline/injection. The treated rats were subdivided into: (3a) LPS + Piog group; were pre-treated with Pioglitazone (Actos; Takeda Chemical Industries, U.S.A., 18 mg/kg bw once daily as clinically dosed) [11]; (3b) LPS + Cur group, were pre-treated with the Curcumin (100 mg/kg bw dissolved in absolute ethanol once daily) [23]; (3c) LPS + Piog and Cur group, were pre-treated with both Pioglitazone (18 mg/kg bw once daily) and Curcumin (100 mg/kg bw dissolved in absolute ethanol once daily).

Induction of subacute dopaminergic neurodegeneration by stereotaxic surgery

Unilateral LPS (10 µg in 2 µl sterile saline in groups 2 and 3) or vehicle (2 µL of 0.9% sterile saline in group 1) injection was performed through a 5-µl Hamilton syringe under isoflurane anesthesia (5% in O_2 for induction and 2% in O_2 for maintenance) in a stereotaxic frame with the nose bar set at 2.3 mm. The substantia nigra, on left side, was infused at the following stereotaxic coordinates that taken from [24] and confirmed by a Rat Brain Atlas [25]; Antero-posterior 5.0 mm from bregma, medio-lateral -2.0 mm from midline and dorso-ventral -8.0 mm from the top of the skull. All injections were completed in a total volume of 2 µl at a rate a 1 µl/min with a further 5 minutes allowed for the complete diffusion of the drug and then slowly withdrawn from the brain. Following surgery, the rats assessed for body changes until scarifying four days later for postmortem analyses. Then on the 10th day of the experiment, the animals were submitted to behavioral tests. After that, they were euthanized (decapitation) and blood was collected and brain tissues were removed from postmortem analyses.

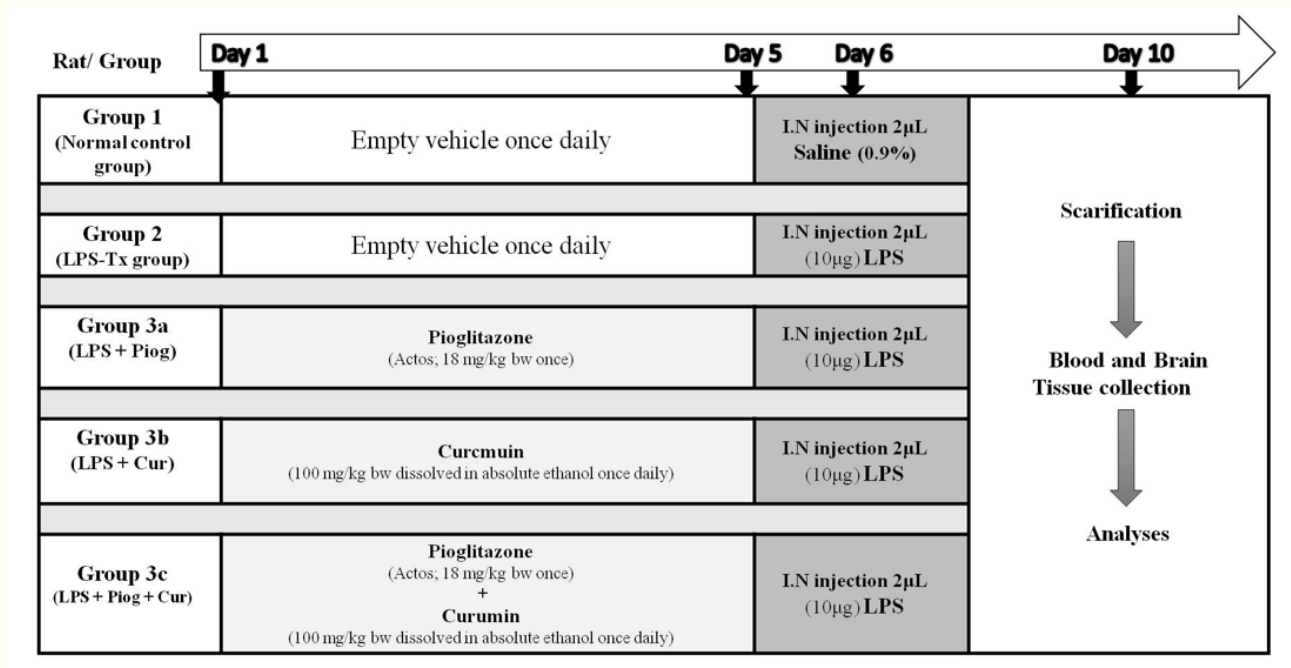

Figure 1: *Experimental design of LPS-induced subacute dopaminergic neurodegenerative rat model and groups classifications.*

Samples collection

Blood was collected from different groups at day 10 of the experiment to test the neurotoxicity of LPS toxin and neuroprotective efficacy of Curcumin and/or Pioglitazone. From each group, 4 - 6 rats were killed by cervical decapitation and blood was collected by cutting inferior vena cava during scarifying into empty tubes. After coagulation, sera were collected and stored at -80°C for later analyses. Brain tissue was rapidly removed, washed with cold saline solution. Using a rat brain matrix, the mid brain, containing the substantia nigra was collected and part of striatum was dissected out, snap frozen, weighed and stored in an Eppendorf tube with 1.5 ml RNA later (RNA stabilization) solution for extraction of total RNA. The remaining section was rapidly frozen with liquid N_2 and stored at -80°C until use for preparation of the total and nuclear extracts, as well as for enzyme assays.

Behavioral evaluations

Motor behavioral pole test

The motor-behavioral pole testing was used, with some modifications, to assay the movement disorders in rats, according to a previous of inducing selective degeneration in nigral and striatal study [26]. Briefly, rats were placed at the top of a 70 cm vertical wooden pole with a rough-padded surface with a 2 cm diameter. On the day before testing, the rats were placed at the top of the pole and exposed to 5 trials. The total time spent to reach the base of the pole and hold all paws on the floor was recorded. For each trial of five descents, the best time was recorded. If the rat was unable to turn completely downward, fell, or slipped down the pole, a default value of 120s was measured as a complete impairment. The pole test was performed at 0 day, then at days 6,7,8,9 and 10 during the experiment.

Quantitation of apomorphine-induced rotational behavior

On the 10th day of the experiment, rats were subcutaneously injected with 0.5 mg/kg apomorphine-HCL (dopamine receptor agonist) (Sigma-Aldrich, USA) and each rat was placed in the test cylinder and the number of contralateral full-body rotational turns for each rat was recorded, immediately after apomorphine injection, over 30 minutes' period. The cause of excess abnormal rotational behavior was due to the unilateral nigrostriatal degeneration.

Sera biochemical analyses

Measurement of sera aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

Both sera AST and ALT activities (U/L) were assayed, according to the previously described colorimetric protocol of Reitman and Frankel method [27]. Briefly, sera samples were incubated with substrate/buffer reagent mixture (100 mM phosphate buffer pH 7.2 containing 4.0 mM α-ketoglutarate and 80 mM L-aspartate (for AST activity) or 80 mM DL-alanine (for ALT activity), at 37°C for 30 min. Then, 4 mM 2, 4-Dintrophenylhydrazine color reagent was added and the mixtures were further incubated at R.T for 20 minutes before addition of 0.4 M NaOH. Samples were further incubated at R.T for 5 min to develop the color and the O.D of were measured against reagent blank at 546 nm within 1 hr.

Determination of serum albumin level

Serum albumin concentration (g/dl) was analyzed according to Doumas., *et al*. method [28]. Briefly, an albumin (in the standard or samples) incubated with an acidic buffered bromocresol green (BCG) color solution (0.18 g/d bromocresol green in 74 mM succinate buffer, pH 4.2), for 5 minutes at R.T, forming a green color that was measured at 630 nm within 30 minutes. The color intensity was directly proportional to the amount of the albumin in a serum sample.

Determination of serum total bilirubin level

Serum bilirubin level (mg/dl) was assayed according to the reference method of Jendrassik and Grof [29]. Briefly, bilirubin in the sample was coupled with diazotized sulfanilic acid, in the presence of caffeine, giving azo dye with green color, which was read at 578 nm.

Determination of blood urea concentration

Blood urea concentration (mg/dl) was measured according to the method described by Fawcett and Scott [30]. The principle of the method is based on enzymatic hydrolysis of urea in the sample or standard into ammonium ions. The ammonium ions formed reacts with Salicylate and hypochlorite, in the presence of the catalyst nitroprusside, to form a green indophenol color, measured at 542 nm.

Determination of serum creatinine concentration

Serum creatinine level (mg/dl) was measured according to the Bartels., *et al.* reference assay [31]. The assay is based on the reaction of the creatinine with alkaline sodium picrate, forming red color, measured at 495 nm.

Analyses of oxidative stress-related parameters

Measurement of total lipid peroxidation (MDA estimation)

MDA level (nmol/g tissue) determination was carried out according to Kei Satoh procedure [32]. Striatum part of brain tissue was homogenized (10% w/v) in 5 - 10 ml ice-cold buffer (50 mM potassium phosphate, pH 7.4). Then, the homogenate was centrifuged at 4000 rpm at 4°C for 15 min and the resultant supernatant was collected for assay of the lipid peroxidation (LPO). Briefly, in acidic medium, MDA in the supernatant reacts with thiobarbiturate for 30 min at 95°C to form pink color complex that was measured at 534 nm.

Determination of reduced glutathione (GSH) level

Reduced GSH level (mmol/g tissue) was determined according to the reference method of Brehe and Burch [33]. Striatum part of brain tissue was homogenized (10% W/V) in ice-cold 1.15% potassium chloride- 0.01 M sodium phosphate buffer, pH 7.4. The homog-

enate was centrifuged at 10,000 rpm for 20 minutes at 4°C and the collected supernatant was used for determination of reduced glutathione content. Briefly, reduced GSH was reacted with 5,5'-dithiobis-2-nitrobenzoate giving a yellow colored 2-Nitro-5-thiobenzoate that was measured at 412 nm.

Determination of catalase activity

Catalase activity (U/g tissue) was assayed according to Abei reference method [34]. Striatum part of brain tissue was homogenized (10% w/v) in 5 - 10 ml ice-cold lysis buffer (50 mM potassium phosphate buffer with pH 7.4 (containing 0.1% (v/v) Triton X-100 and 1 mM EDTA). The supernatant was collected, after centrifugation of the ice-cold homogenate at 4,000 rpm for 15 minutes at 4°C and used for determination of serum catalase activity. In brief, catalase enzyme reacts with a known amount of H_2O_2. The reaction is, then, stopped with catalase inhibitor after exactly one min. The remaining H_2O_2 reacts with quinone and 4-aminoantipyrine, in the presence of peroxidase, to form a pink-colored complex, that was measured at 510 nm and the color intensity was inversely proportional to activity of catalase in the tissue homogenates.

Preparation of total cell lysates and nuclear extracts followed by ELISA and colorimetric assay

Preparation of total cell lysates and nuclear cell extracts

Part of frozen left-side injected brain striatal tissues was washed with ice-cold PBS (pH, 7.4) and then lysed in homogenization buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4, containing 4 mM EDTA, 1% (vol/vol) Triton X-100, 1 mM NaF, supplemented with 1 mM tetrasodium pyrophosphate (TSPP), 0.1mM Sodium vanadium oxide, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10μg/ml each of aprotinin and leupeptin) (all from Sigma Chemical Co.). The mixture was vortexed and freezed in dry ice bath for 15 min with frequent agitation. Then, cycles of thawing/ freezing were repeated twice. The homogenate of the total cell extract was divided into aliquots of 300μl each and stored at -80°C for further analysis of TNF-α, IL-6, IL-1β and Dopamine by ELISA. Nuclear extracts were prepared as previously described by Schreiber., *et al.* method [35], with some modifications. Briefly, 500 mg of frozen left-side injected brain striatal tissue was homogenized in 5ml of ice-cold extraction buffer A (0.6% NP-40, 150 mM NaCl, 10 mM HEPES pH 6.9, 0.5 mM PMSF, 1mM EDTA) (all from Sigma Chemical Co.). The homogenates were centrifuged at 2,000 rpm for 30 s at 4°C, to eliminate any unbroken tissue. The supernatants were incubated for 10 minutes on ice, then centrifuged at 5,000 rpm at 4°C for 5 minutes. The crude nuclear pellets were resuspended in 200 μl ice-cold extraction buffer B (20 mM HEPES pH 7.9, 25% (vol/vol) glycerol, 0.5 mM PMSF, 20 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 5 mg/ml each of aprotinin, leupeptin and pepstatin) (all from Sigma Chemical Co.) and incubated in ice for 20 min with frequent agitation. The homogenates were centrifuged at 12,000 rpm at 4°C for 20 min. the supernatants were collected as nuclear extracts and stored at -80°C for analysis of NF-κB by Colorimetric Assay.

ELISA for quantifying TNF-α, IL-6, IL-1β and dopamine in total cell lysates

The aliquot of the total cell lysates was used to determine the concentrations of TNF-α, IL-6, IL-1β and dopamine from commercially available rat TNF-α (Prprotech, cat# 900-K73), rat IL-6 (BioLegend's MAX™, cat# 430502), rat IL-1β (Enzo, cat# ADI-900-131), rat Dopamine (MyBiosource, cat# MBS262606) Kits. These kits with high sensitivity were used according to the manufacturers' instructions (0.25 μg/ml for rTNF-α, 7.8 pg/ml for rIL-6, 25.6 pg/ rIL-1β and 15.6 pg/ml for rDopamine). Five to ten animals per group were analyzed and each sample was in duplicate.

Colorimetric assay of NF-κB in nuclear extracts

NF-κB estimation in nuclear extracts of brain striatal tissue samples was performed according to Renard., *et al.* protocol [36], with some modifications. This assay is very sensitive to determine the amount of activated NF-κB in nuclear extracts of striatal tissue samples. The assay is based on an ELISA principle, except that the NF-κB was captured by a ds-oligonucleotide probe, adsorbed on microwell plates, containing the consensus binding sequence for NF-κB instead of by an antibody. The bound NF-κB was then detected using 1ry anti-NF-κB Ab, followed by a 2ry horseradish peroxidase-labeled Ab. Finally, the results are quantified by a colorimetric reaction in "O.D / 25 μg nuclear extract".

Western blotting analysis of iNOS and COX-2:

Immunoblots were performed as described by Burnette (Burnette 1981), COX-2, iNOS and β-actin immunoblots were performed on prepared total cell extracts of striatal tissue. Primary antibody to COX-2 (sc-7951) and iNOS (sc-650) were used. Antibody binding was detected following appropriate secondary antibody using chemiluminescence detection and equal loading was confirmed by probing with β-Actin monoclonal antibody (sc-81178).

Isolation of total RNA and quantitative real-time PCR

Total RNA was extracted from brain striatal tissues stored in RNA later solution according to Chomczynski and Sacchi procedure [37] using BIOZOL RNA Isolation Kit (Life Technologies, Inc.). The concentration of RNA in the homogenate was determined by measuring the O.D at 260 nm and the RNA purity was estimated by 260/280 ratio and confirmed by running RNA samples on a standard 1% agarose gel. Then, cDNA was synthesized from total RNA samples using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc. USA), while miRNA was reverse transcribed into cDNA via *miScript II RT Kit* (*Qiagen*, Hilden, Germany), according to the manufacturer's guidelines. cDNA was further amplified by PCR using specific forward and reverse primers, illustrated below, in SYBR Green PCR Master Mix (FastStart Universal SYBR Master; Roche Diagnostics, Basel, Switzerland) and the amplified PCR product was analyzed with a 7500 ABI PRISM Sequence Detector System according to the manufacturer's instructions (Applied Biosystems, Cheshire, UK). Quantitative real time RT-PCR was used to measure the relative mRNA expression levels of miR-124, miR-155, TNF-α, IL-1β, IL-6, iNOS, COX-2 and APE1. The primers for miR-124: AGGCACGCGGTGAATGCC; miR-155: GTTAATGCTAATTGTGATAGGGGT; U6 F: GGAACGATACAGAGAAGATTAGC, R: AAATATGGAACGCTTCACGA; TNF-α F: GCACAGAAAGCATGATCCGAG, R: CCTGGTATGAAGTGGCAAATCG; IL-1β F: AAGCCAACAAGTGGTATTCTC, R: GGACAGGTATAGATTCTTCCC; IL-6 F: GGTACATCCTCGACGGCATCT, R: GTGCCTCTTTGCTGCTTTCAC; iNOS F: GCAGAATGTGACCATCATGG, R: ACAACCTTGGTGTTGAAGGC; COX-2 F: AGGCCTCCATTGACCAGA, R: TCATGGTAGAGGGCTTTCAAC; APE1 F: GCTTGGATTGGGTAAAGGA, R: TTCTTTGTCTGATGGAGCTG; β-actin F: CCGTGAAAAGATGACCCA, R: AGAGGCATACAGGGACAACA. The relative expressions of indicated genes and miRNAs were normalized to β-actin and U6 internal controls, respectively. The fold change of miRNA and mRNA expression was calculated based on the threshold cycle (Ct) value using the following formula: $2^{-\Delta\Delta CT}$ [38].

Statistical analysis

All experiments were performed in duplicates and the results were expressed as mean ± S.D in the different experimental groups. Raw data were firstly analyzed by one-way analysis of variance (ANOVA) tool in the Statistical Package for Social Sciences, version 17.0 (SPSS Inc, Chicago, IL, USA), for multiple comparison of the statistical differences among all experimental groups, followed by Fisher's protected least significant difference (LSD) as *a post hoc test* with statistical significance adjusted at $P < 0.05$, to statistically compare each two experimental groups together. Statistical significance when compared to control group $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$when compared to induced group.

Results

Curcumin/pioglitazone combination improves biochemical indices and oxidative stress parameters

Intranigral LPS injection showed non-significant changes in both liver and kidney profile compared with control group (Table 1). These results indicate no significant hepatotoxicity or nephrotoxicity in the LPS-induced and Pioglitazone/Curcumin-pretreated versus control rats in all liver and kidney markers, indicating absence of LPS-induced hepatotoxicity or renal toxicity at the applied dose injected intranigrally.

Treatment with either Pioglitazone or Curcumin exerted neuroprotective effect, in part, by improving the antioxidant capacity as represented by markedly elevating GSH and reducing MDA levels when compared with LPS-induced group, but still significantly different from the control group as shown in figure 2. Moreover, up-to-normal level restoration of GSH and MDA to the normal level of the control group was mediated by pretreatment with Pioglitazone and Curcumin combinations, indicating their powerful effects when present to-

Group	ALT (IU/L)	AST (Iu/L)	Albumin (g/dl)	T.Bilirubin (mg/d1)	Urea (mg/dl)	Creatinine (mg/d1)
Control	24.4 ± 1.8[b]	38.0 ± 2.9[b]	3.96 ± 0.28[b]	0.28 ± 0.021[b]	24.2 ± 3.5[b]	0.66 ± 0.05[b]
LPS-Tx	32.8 ± 2.5[a]	48.0 ± 3.3[a]	3.50 ± 024[a]	0.31 ± 0.03[a]	30.6 ± 7.4[a]	0.75 ± 0.04[a]
Piog	26.9 ± 2.4[b]	41.4 ± 3.2[b]	3.59 ± 0.28[a]	0.26 ± 0.03[b]	22.9 ± 3.7[b]	0.64 ± 0.03[b]
Cm.	26.0 ± 3.2[b]	44.8 ± 3.4[a]	3.91+ 0.13[b]	0.29 ± 0.03	21.5 ± 2.8[b]	0.70 ± 0.03
Piog + Cur	27.1 ± 2.3[b]	43.6 ± 4.3[ab]	3.91 ± 0.17[b]	0.27 ± 0.02[b]	23.1 ± 3.2[b]	0.66 ± 0.04[b]

Table 1: *Liver and kidney functions indices in the sera of different experimental rat groups.*

Notes: Values are expressed as mean ± standard error of the mean (n=8). [a]P < 0.05, significant change with respect to control group; [b]P < 0.05, significant change with respect to LPS group for Duncan's post hoc test.

Abbreviations: ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; LPS: Tnc, Lipopolysaccharide-Neated; Piog: Pioglitazone; Cur: Curcumin.

gether. Furthermore, Pioglitazone more than curcumin showed a marked enhancement in the activity of catalase enzyme in the striatum compared to rats treated with LPS (Figure 2).

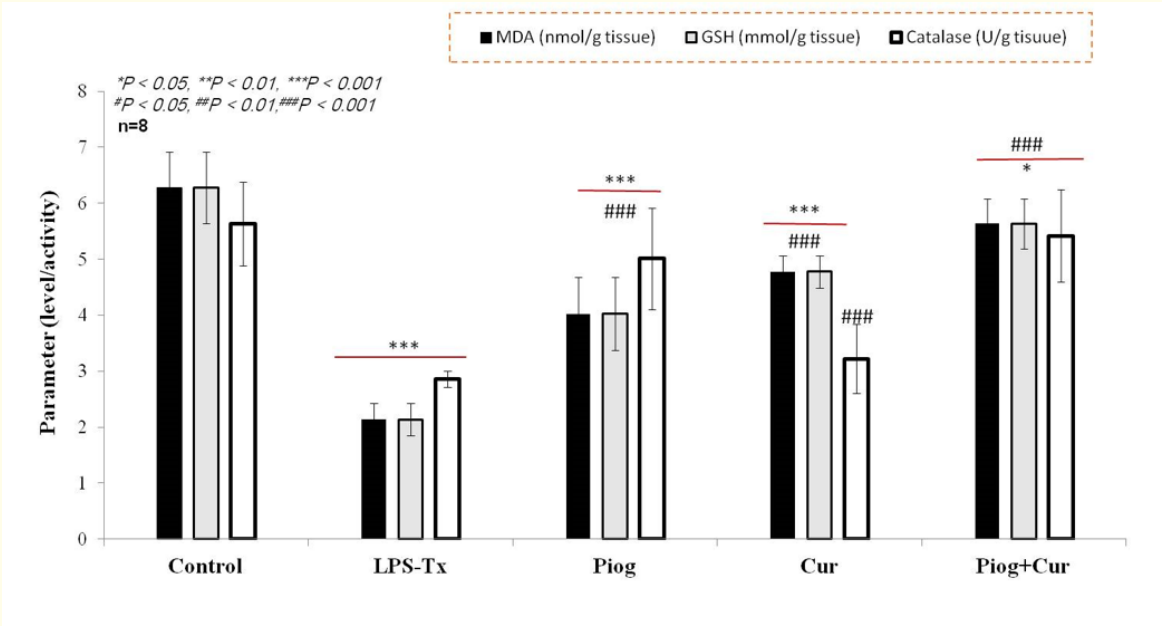

Figure 2: *Alterations in the levels of GSH in (mmol/g tissue), MDA in (nmol/g tissue) and catalase activities in the different experimental rat groups. A significant reduction (P < 0.001) in GSH level and Catalase activity as well as significant elevation (P < 0.001) in the MDA level were observed in LPS-induced group versus control group. Treatment with either Pioglitazone or Curcumin exerted antioxidant protective effect as represented by markedly elevated GSH and reduced MDA levels as compared with LPS-induced group but still significantly different from the control group. However, with regarding to catalase activity, Pioglitazone exerted more protective effect than Curcumin, by significantly increasing catalase activity (up to normalization) as compared with LPS-induced group. Moreover, combined pretreatment of Pioglitazone and Curcumin completely restored GSH, catalase and MDA close to the normal levels as in the control group. Data are expressed as means ± SD, * or # (P < 0.05), ** or ## (P < 0.01), *** or ### (P < 0.001), where *, **, or *** represents Control vs. LPS, Curcumin and/or Pioglitazone and #, ##, or ### represents LPS vs. Curcumin and/or Pioglitazone.*

Curcumin/Pioglitazone combination restored dopamine level and improved cognition in the experimental rats

A novel combination of curcumin and pioglitazone, rather than each one alone induced significant restoration of dopamine level in the striatum toward normalization, which was significantly reduced in LPS-induced rat group (Figure 3A). This indicates the efficacy of this combination in protecting the dopaminergic neurons against LPS-induced neurotoxicity.

Rats subjected to unilateral intranigral injection of LPS and received Curcumin and/or Pioglitazone had significant ($P < 0.05$) effect in different behavioral motor tests. At cognition assessment level, a significant ($P < 0.01$) protective effect of combined curcumin and pioglitazone pre-treated animals rather than of each one alone was assessed using apomorphine-induced rotational behavior and showed significant decrease in the number of rotations as compare to LPS-treated group toward normalization (Figure 3B). In LPS-induced group, subacute dopaminergic neurodegeneration induction was confirmed by significant ($P < 0.001$) increase in the number of rotations observed as compared to the normal control group. Furthermore, our result indicates that in motor behavior pole testing, the total time it took a rat to reach the base of the pole was significantly increased in LPS- induced rat group compared with the normal control group. However, the descend time was significantly reduced in pretreatment with novel combinations with curcumin and pioglitazone in comparison to either pretreatment with curcumin or pioglitazone alone, as shown in figure 3.

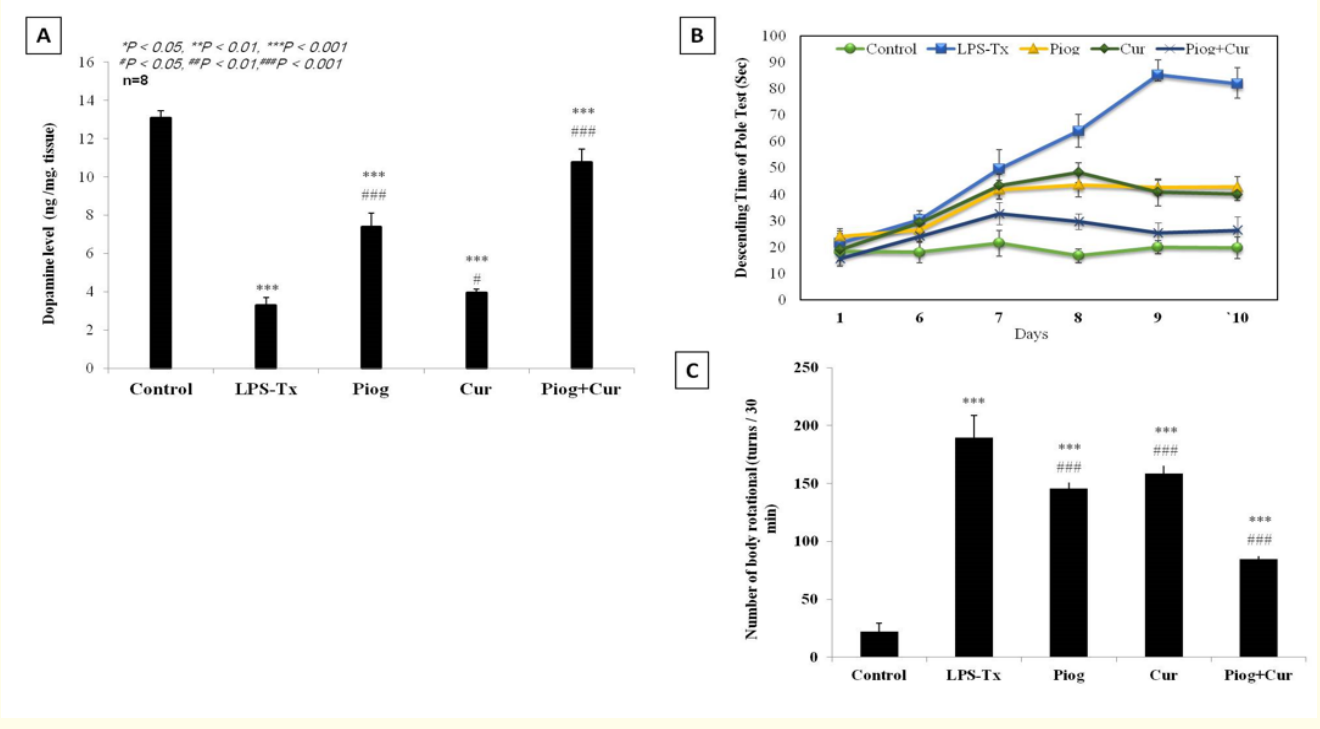

Figure 3: *Alterations of Dopamine (DA) level (ng/mg tissue) and behavior tests in different experimental rat groups. (A) Dopamine levels in striatal tissues are largely and significantly reduced after LPS induction compared with the normal control group ($P < 0.001$). However, in comparison to LPS-treated group, a significant elevation ($P < 0.001$) toward normalization was observed in case of pretreatment with pioglitazone and curcumin combinations rather than each one alone. (B) Apomorphine-induced rotation tests revealed significant rotational movements over 30 min periods in the LPS-induced subacute dopaminergic neurodegeneration group in comparison with normal control group. However, in pretreatment with novel combinations with curcumin and pioglitazone, rather than pretreatment with each one alone, shows significant decrease in the total number of rotations. (C) In motor behavior pole testing, the total time to descend a pole and to place the four paws on the floor was significantly increased in LPS-treated rats versus controls ($p < 001$). Compared to LPS group, the pretreatment with novel combinations of curcumin and pioglitazone rather than each one alone in LPS-treated rats significantly decreased the total descent time toward normalization. Data are expressed as means ± SD, * or # ($P < 0.05$), ** or ## ($P < 0.01$), *** or ### ($P < 0.001$), where *, **, or *** represents Control vs. LPS, Curcumin and/or Pioglitazone and #, ##, or ### represents LPS vs. Curcumin and/or Pioglitazone.*

Curcumin/Pioglitazone combination significantly attenuates the expression of Neuroinflammatory Cytokines, Inflammation-Associated Enzymes and NF-κB

The present study shows that LPS treatment significantly increases IL-1β and TNF-α mRNA levels (Figure 4A) as well as IL-1β, TNF-α and IL-6 protein levels (Figure 4B) compared with control group administered the empty vehicle, sterile saline alone. Pretreatment with either Pioglitazone or Curcumin followed by LPS injection shows very high significant downregulation in both mRNA and protein levels of inflammatory cytokines (IL-1β, TNF-α and IL-6) compared with LPS-induced rat group but still significantly than control groups. However, in case of pre-treatment with Pioglitazone and Curcumin combinations show marked reduction in mRNA and protein levels of these inflammatory cytokines, which became non-significantly different from control group as shown in figure 4. These data indicate that pre-treatment with pioglitazone and curcumin combinations show the synergistic anti-inflammatory effects on LPS-induced subacute dopaminergic neurodegenerative model. However, it seems that there are no preclinical studies on animal models examined the strong anti-inflammatory synergism between both Pioglitazone and Curcumin combinations.

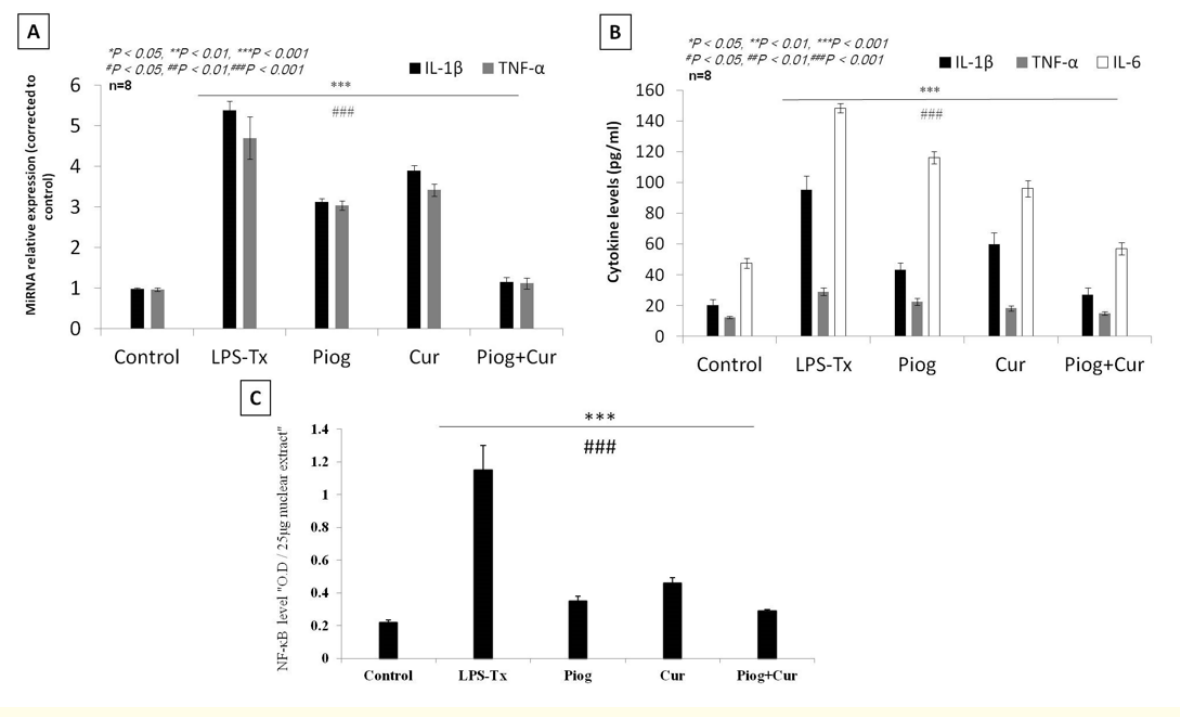

Figure 4: *Expression profiles of neuroinflammatory cytokines by qRT-PCR (A) and ELISA (B) in the different experimental rats' groups (c) as well as nuclear protein level of NF-κB (O.D / 25µg nuclear extract of striatum tissue). It has been observed that LPS treatment significantly increased both IL-1β and TNF-α mRNA levels (A) as well as IL-1β, TNF-α, and IL-6 protein levels (B) compared to control group. In addition to, pretreatment with either Pioglitazone or Curcumin followed by LPS injection, inhibited significantly the mRNA and Protein levels of inflammatory cytokines compared with LPS-induced rat group (P<0.001) but still very higher significant than control groups (P < 0.001). In case of pretreatment with both Pioglitazone and Curcumin followed by LPS injection, there is a significant decrease in mRNA and Protein levels of inflammatory cytokines compared with LPS-induced rat group (P < 0.001) which become not significantly different from control group (almost reach the normal level). However, in case of pretreatment with Pioglitazone + Curcumin combination followed by LPS injection, we observed significant decrease in the inflammatory-associated enzymes and NF-κB expression levels toward the control group. Data are expressed as means ± SD, * or # (P < 0.05), ** or ## (P < 0.01), *** or ### (P < 0.001), where *, **, or *** represents Control vs. LPS, Curcumin and/or Pioglitazone and #, ##, or ### represents LPS vs. Curcumin and/or Pioglitazone.*

Our data established that Intranigral injection of LPS resulted in a significant up-regulation in the mRNA expression levels of the inflammation associated enzymes COX-2 and iNOS (Figure 3). In the present study, we demonstrate that pretreatment with either Pioglitazone or Curcumin followed by intranigral LPS injection partially reduced the overexpressed mRNA levels of iNOS and COX-2 as compared with control groups. Both Pioglitazone and Curcumin combination, however, have been led to a marked reduction in iNOS and COX-2 mRNA expressions that became near to those of the control group (Figure 5A). We also extended our study by measuring the nuclear level of NF- κB as a molecular target in the anti-inflammatory mechanism by Pioglitazone and Curcumin combination, in which the over-expressed inflammatory-associated enzymes iNOS and COX-2 as well as the inflammatory cytokines TNF-α, IL1β and IL-6 are inhibited by reducing the nuclear level of NF-κB (Figure 4C). Compared with the LPS-induced group, there was a significant reduction of nuclear level of NF-κB in case of pretreatment with either curcumin or pioglitazone, which almost become non-significantly different from the control group in case of pretreatment with both pioglitazone and curcumin combinations (Figure 4C). This study demonstrates that NF-κB attenuation is considered as a direct causal link between the anti-inflammatory effects of Pioglitazone/Curcumin and the down-regulation of iNOS and COX-2 enzymes as well as TNF-α, IL1β and IL-6 cytokines. This study confirms our results which indicate that curcumin potentiating the anti-inflammatory effects of pioglitazone.

Expression profiles of mRNA levels of inflammation-associated enzymes APE-1, iNOS and COX-2 by qRT-PCR and protein levels of iNOS and COX-2 by western blotting indicated that pre-retreatment with either Pioglitazone or Curcumin followed by intranigral LPS injection significantly reduced mRNA levels of APE1, iNOS and COX-2 expression compared with LPS-induced rat group ($P < 0.001$) in addition to protein levels of iNOS and COX-2 (Figure 5); although still highly significant from control groups ($P < 0.001$). Pretreatment with both Pioglitazone and Curcumin followed by LPS injection, ameliorated the toxicity and significantly reduced the inflammatory enzymes expression compared with LPS-induced ($P < 0.001$).

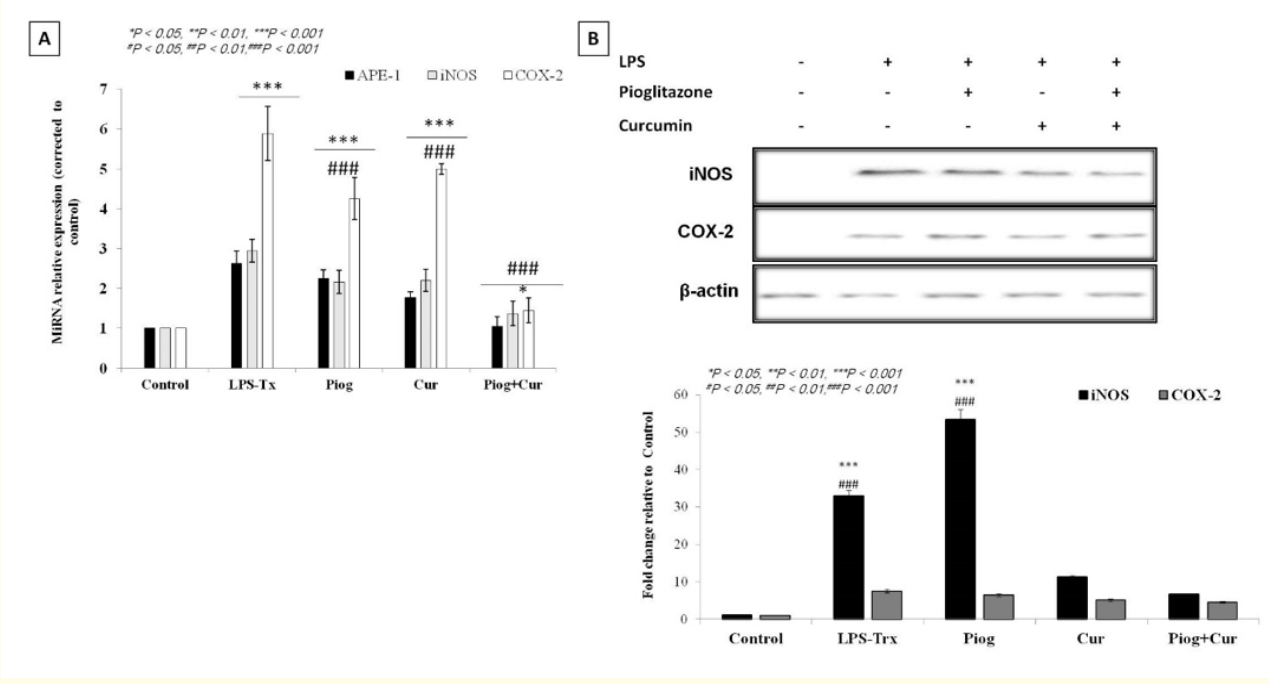

Figure 5: *Determination of mRNA Expression profiles of the inflammatory-associated enzymes APE-1, iNOS, and COX-2 in the different experimental rat groups. (A) Pretreatment with either Pioglitazone or Curcumin followed by intranigral LPS injection significantly reduced mRNA levels of APE-1, iNOS and COX-2 expression compared with LPS-induced rat group (P < 0.001); although still highly significant from control groups (P <0.001). (B) in parallel western blotting analysis confirmed the expression profile of both iNOS and COX-2 in pre-treated versus induced rats. Data are expressed as means ± SD, * or # (P < 0.05), ** or ## (P < 0.01), *** or ### (P < 0.001), where *, **, or *** represents Control vs. LPS, Curcumin and/or Pioglitazone and #, ##, or ### represents LPS vs. Curcumin and/or Pioglitazone.*

Curcumin/Pioglitazone combination regulates the epigenetic modulators; miR-155 and miR-124

In the present study, after intranigral LPS injection, miR-155, mRNA expression levels are up-regulated significantly by almost 4 folds versus control group. However, miRNA-124, which contributes to the M2 phenotype of the anti-inflammatory microglia, was found to be down-regulated by 0.79-fold (Figure 6). Pretreatment of Pioglitazone or Curcumin restored partially and preferentially the expression levels of both miRNAs towards the normal profile. These results indicate the anti-inflammatory mechanism could be partly modulated by miRNAs rebalance. Moreover, pretreatment of with Pioglitazone and Curcumin combination induced significant restoration of miRNA-155 and miRNA-124 levels compared to the LPS-induced group and almost reach the normal profile of the control group (Figure 6).

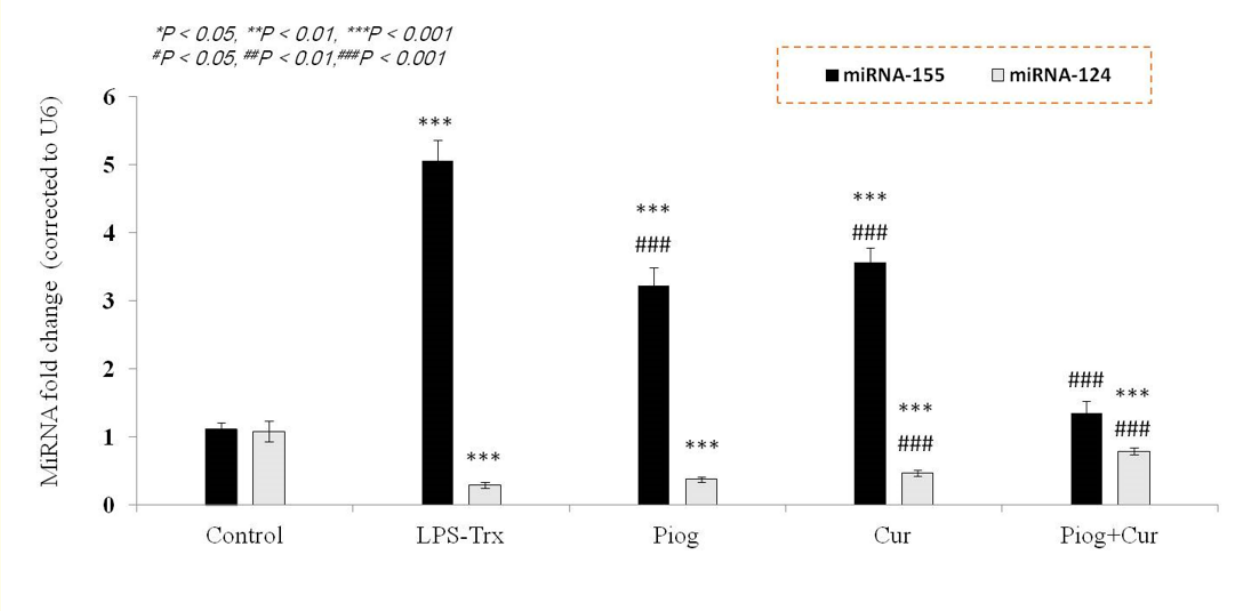

Figure 6: *Expression profiles of miR-155 and miR-124 in the striatal tissue by qRT-PCR in the different experimental rat groups. Experimental results show that miR-155 is up-regulated significantly (4 folds; P < 0.001) with concomitant reduction in miRNA-124 (0.79 fold) (P< 0.001) in LPS-induced versus control group. A marked reduction in miR-155 level was observed in both Pioglitazone-pretreated (1.8 fold) and Curcumin-pretreated (1.5 fold) compared with LPS-induced group (P < 0.001) and still significantly higher than control group (P < 0.001). Moreover, both Pioglitazone and Curcumin-pretreated groups showed significant reduction compared with LPS-induced group (3.7 fold) (P < 0.001) that became almost up to normal level when compared to control. However, the pretreated groups show marked elevation in miR-124 level compared with LPS-induced groups, but didn't reach normal levels of the control group. Data are expressed as means ± SD, * or # (P < 0.05), ** or ## (P < 0.01), *** or ### (P < 0.001), where *, **, or *** represents Control vs. LPS, Curcumin and/or Pioglitazone and #, ##, or ### represents LPS vs. Curcumin and/or Pioglitazone.*

Discussion

The endotoxin Lipopolysaccharide (LPS) is widely applied in research model for selective induction of the degeneration of SN dopaminergic neurons via activation of inflammatory mediators' release [39,40]. Hence intranigral injection of LPS is ideal for selective induction of subacute dopaminergic neurodegeneration in rodent and primate models through microglial activation [41,42].

In the present work, we examine the key evidence contributing to our understanding of the potential role of inflammation-mediated degeneration of the dopaminergic (DA) nigrostriatal pathway in PD. The ultimate therapeutic challenge remains. A systematic assessment of potential disease-modifying compounds to further improve symptomatic therapy of motor Parkinson's disease (PD) symptoms concluded that pioglitazone could hold promise for the treatment of patients with this disease. Many researchers have expected pioglitazone to serve as an effective neuroprotective agent against Parkinson's disease (PD). Therefore, we conducted this study to investigate the association between pioglitazone use and PD by using a rat model. The neuro-protective effects of combined pioglitazone and curcumin were evaluated in the experimental model of subacute dopaminergic neurodegeneration, consisting of a unilateral intranigral injection of the LPS neurotoxin in male Sprague-Dawley rats. An attempt was made to correlate the neuro-protective action of this novel combination, rather than each one alone, to its anti-inflammatory and anti-oxidant effects. It has been reported that intranigral LPS injection induces an inflammatory response as well as oxidative stress in the striatum and SN, followed by a dopaminergic neuronal loss[15]. Evidences have indicated the neuro-protective effects of either pioglitazone or curcumin on several studied *in vivo* experimental models. However, there is no studies used the combination of pioglitazone and curcumin in experimental models of neurodegenerative disorders, such as Parkinson's disease. Our data indicate that intranigral LPS injection at the applied dose did not affect either liver or kidney functions profile when compared to control group.

Oxidative stress which is a hallmark of neurodegeneration pathogenesis, in particular to the dopamine-rich areas of the brain that are vulnerable to elevated ROS that is generated by mitochondrial dysfunction in addition to dopamine metabolism itself [43]. Hence, altered oxidative stress evidence after LPS injection was marked by an increase in striatal MDA and reduction in GSH levels, our results in parallel showed an increased production of MDA levels which is in agreement with the *in vivo* study by Ferger., *et al*. [44]. In addition, we found reduction of the GSH content that results from chronic oxidative which is consistent with Mostafa., *et al*. [45] finding.

Administration of either Pioglitazone or Curcumin alone exerted partial neuroprotective effect by inducing the antioxidant capacity as represented by the significant elevation in GSH and reduction of MDA levels versus the LPS-induced group. In agreement with the present work, a recent study by Nade., *et al*. [46] illustrated the dose-dependent effect of pioglitazone on lowering the MDA level in subacute dopaminergic neurodegenerative rat model. Interestingly and in agreement with our results which indicated that curcumin mediate an increase in the levels of endogenous GSH this was postulated to be mediated by enhancing astrocytic efflux of GSH [47] or through other mechanisms in both the neurons and astrocytes [48,49].

On the other hand, Pioglitazone was reported to enhance the activity of catalase antioxidant enzyme in the striatum compared to rat treated with LPS via regulation of catalase gene promoter through functional PPAR-γ responsive elements (PPRE) [50]. Furthermore, the results in this report indicate that dopamine levels in striatal tissues are largely and significantly reduced after LPS induction compared with control group. However, in comparison to LPS-treated group, a significant elevation toward normalization was observed in case of pretreatment with pioglitazone and curcumin combinations rather than each one alone.

Moreover, we assessed the altered behavioral function by employing two specific testes on the rats of the different experimental groups. The behavior pole testing showed that the total time the rat took to descend a pole was significantly increased in LPS-induced rat versus control group. However, the descend time was significantly decreased upon pretreatment with the combinations in comparison to either pre-treatment with curcumin or pioglitazone alone. We also observed that an increase by more than 20-fold in the apomorphine-induced rotational behavior, compared to a normal control group which might correlate with loss of dopaminergic neurons which was further confirmed by, as confirmed by measuring the dopamine level which, in part, confirm the efficiency of the administered combination.

Since microglia activation is implicated in neurodegeneration pathogenesis, we herein present the novel combination of curcumin and pioglitazone as an effective anti-inflammatory formula, suppressing NF-κB which is considered as a direct causal link between the

anti-inflammatory synergism of Pioglitazone/Curcumin combination and the down-regulation of iNOS and COX-2 enzymes as well as TNF-α, IL-1β and IL-6 cytokines. Several lines of previous investigation confirmed these results and indicated that the increased level of pro-inflammatory IL-1β, TNF-α and IL-6 in the substantia nigra after LPS injection as a causal factor for LPS-induced neuronal damage [51-53]. Moreover, previous studies shown that intrastriatal injections of LPS resulted in a significant upregulation of the striatal proteins expressions of COX-2 [10] and iNOS [52,54], that confirm our results. Herein we show that intranigral LPS injection significantly upregulates mRNA levels of neuroinflammatory cytokines including IL-1β and TNF-α and inflammation-associated enzymes including COX-2 and iNOS as well as protein levels of IL-1β, TNF-α and IL-6 COX-2 and iNOS compared with normal control group.

MicroRNAs are small endogenous molecular docking devices that act as regulators for diverse cellular processes, such as cell differentiation, proliferation and disease. These docking RNA molecules interfere with gene expression and regulates transcription pathways by different mechanisms including accelerating mRNA degradation or inhibiting its translation via binding to the 3'UTR of target genes [55]. Their defective biogenesis and/or functions have been identified in various pathological conditions, like inflammation, neurodegeneration, or autoimmunity. Resting microglia showed to express low level of miR-155 and a relatively high level of miR-124 [21]. However, activated microglia during inflammation leads to imbalance in the levels of these miRNAs [21]. In this report, we found that intranigral LPS injection induces miR-155 expression by around 4 folds, while miR-124 was down-regulated by 0.79-fold. These results are in concordance with previous studies that indicated that miR-155 is up-regulated in microglia in response to exposure to LPS [56-58] and low miR-124 level was detected [21]. Pre-treatment of Pioglitazone or Curcumin maintained partially and preferentially the expression levels of both miRNAs towards the normal profile. These results indicate the anti-inflammatory mechanism could, in part, modulated by miRNAs rebalance as proposed in figure 7. Moreover, pretreatment of with Pioglitazone and Curcumin combination induced significant restoration of miRNA-155 and miR-124 levels compared to the LPS-induced group and almost reach the normal profile of the control group. Curcumin was reported to regulate miR-155 both in *in vitro* and *in vivo* models via blockade of PI3K/AKT signaling pathways [59] which is also reported to play a neuroprotective role in subacute neurodegeneration pathogenesis[28]. Herein curcumin may play the same role, although the exact mechanism by which miR-155 and miR-124 are regulated by this novel combination is not well illustrated yet and will be investigated in further study.

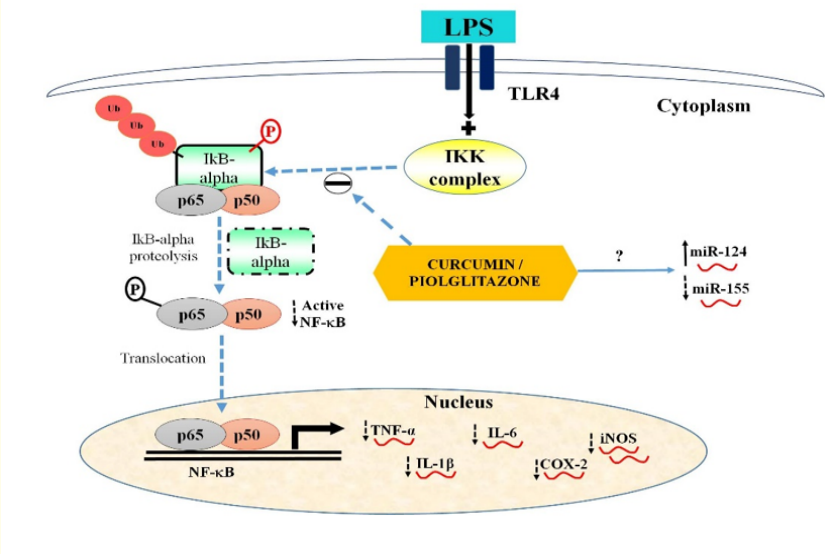

Figure 7: *Illustration of the proposed mechanism of the neuroprotective effect of the Curcumin and Pioglitazone combination at molecular levels.*

Conclusion

Taken together, all results of the current investigation, we indicate the synergistic neuro-protective efficacy of both pioglitazone and curcumin against toxicity induced by LPS in *"in vivo"* subacute dopaminergic neurodegenerative model. Moreover, this work is an attempt to answer questions about the different pleiotropic neuro-protective mechanisms and synergistic effects of this novel combination as well as the involvement of differential miRNAs regulation in neuroinflammation. Further studies are still required to highlight the exact mechanism by which the pioglitazone and curcumin combination affect neuronal integrity.

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Volume 11 Issue 12 December 2019