

Role of Secondary Mediators in Caffeine-Mediated Neuroprotection in Maneb- and Paraquat-Induced Parkinson's Disease Phenotype in the Mouse

Sharawan Yadav · Satya Prakash Gupta ·
Garima Srivastava · Pramod Kumar Srivastava ·
Mahendra Pratap Singh

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Abstract Maneb and paraquat are known to induce Parkinson's disease (PD) phenotype, however, caffeine offers neuroprotection. Nitric oxide (NO) acts an important mediator in PD phenotype and tyrosine kinase (TK), nuclear factor kappa B (NF-kB), p38 mitogen activated protein kinase (p38 MAPK) are known to regulate its production. The present study aimed to elucidate the role of caffeine in the regulation of NO production and microglial activation and their subsequent contribution in dopaminergic neuroprotection. The animals were treated with caffeine and/or mane and paraquat along with controls. In a few sets of experiments, the animals were also treated with aminoguanidine, an inhibitor of inducible NO synthase, pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-kB, genistein, an inhibitor of TK or SB202190, an inhibitor of p38 MAPK. Tyrosine hydroxylase (TH)-immunoreactivity and anti-integrin α M (OX-42) staining were performed to assess the number of dopaminergic neurons and activation of microglia, respectively. NO was measured in terms of nitrite, however, the expressions of p38 MAPK, interleukin (IL)-1 β , NF-kB and TK were checked by western blot analyses. Maneb and paraquat induced the number of degenerating dopaminergic neurons, microglial cells, nitrite content, expressions of IL-1 β , p38 MAPK, NF-kB and TK and caffeine co-treatment reduced

the level of such alterations. Reductions were more pronounced in the animals co-treated with aminoguanidine, PDTC, genistein or SB202190. The results obtained thus demonstrate that caffeine down-regulates NO production, neuroinflammation and microglial activation, which possibly contribute to neuroprotection.

Keywords Parkinson's disease · Maneb · Paraquat · Caffeine · Nitric oxide · Neuroprotection

Introduction

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder after Alzheimer disease and is clinically characterized by muscular rigidity, resting tremor, bradykinesia and postural instability [1, 2]. Clinical symptoms appear only after the degeneration of more than 50% of dopaminergic neurons in the substantia nigra pars compacta, which extends to the striatum through axons [2, 3]. Despite first scientific description of the disease in 1817 by James Parkinson, complete molecular pathogenesis, exact contributory factors and permanent cure are yet elusive [1–3]. However, ageing, genetic factors and environmental exposure to pesticides and heavy metals have emerged as the putative risk factors.

Various chemicals, such as 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), N, N'-dimethyl-4, 4'-bipyridinium dichloride (paraquat), manganese ethylene-bis-dithiocarbamate (mane), rotenone and cypermethrin have been used to develop animal models to understand PD pathogenesis and treatment outcomes [4–8]. Paraquat produces neurodegeneration due to its structural similarity with 1-methyl-4-phenylpyridinium (MPP⁺), a toxic metabolite of MPTP. Paraquat inhibits electron transport

S. Yadav · S. P. Gupta · G. Srivastava · M. P. Singh (✉)
Council of Scientific and Industrial Research–Indian Institute of
Toxicology Research (CSIR–IITR), Mahatma Gandhi Marg,
Post Box-80, Lucknow 226 001, UP, India
e-mail: singhmahendrapratap@rediffmail.com

P. K. Srivastava
Banaras Hindu University, Varanasi 221 005, India

chain complex-I while maneb inhibits complex III, leading to free radical generation, which ultimately leads to neurodegeneration [4–6, 9, 10]. Maneb and paraquat, either alone or in combination are found to induce PD phenotype in the mouse [6, 11]. Combined exposure exhibits more pronounced changes in the indices of the nigrostriatal dopaminergic neurodegeneration and overall oxidative stress than alone [5, 12]. Maneb- and paraquat-induced animal model is of environmental relevance, as these pesticides are commonly and concurrently used by the farmers worldwide [5, 11–13].

Oxidative stress, caused by enhanced reactive oxygen and nitrogen species and reduced antioxidants machinery, have been found to be one of the main culprits of PD [2, 10]. Nitric oxide (NO), is known to regulate the lipid peroxidation in maneb- and paraquat-induced PD phenotype [14]. Maneb- and paraquat-induced PD phenotype is contributed by NO, which in turn is produced by inducible NO synthase (iNOS) involving nuclear factor kappa B (NF- κ B), p38 mitogen activated protein kinase (p38 MAPK) and tyrosine kinase (TK) pathways [14]. An interaction among secondary signalling molecules, which are released after microglial activation and/or during lipid peroxidation, contributes to the nigrostriatal dopaminergic neurodegeneration [14, 15]. The role of NO in the nigrostriatal dopaminergic neurodegeneration is further supported by the fact that NOS inhibitors encounter the progression of PD [14]. Similarly, microglial activation is reported as one of the critical events in the pathogenesis of maneb and paraquat- induced PD phenotype in rodents [15].

Caffeine is one of the most widely consumed psycho-stimulants and is consumed by around 70% of the population globally [16]. An inverse relationship between PD and caffeine consumption is reported in epidemiological and animal based studies [8, 17–20]. The neuroprotective effects of caffeine are reported to be mediated by its antagonistic action on adenosine A_{2A} receptor, leading to enhanced dopamine release into the striatum [13]. Additionally, its phosphodiesterase inhibitory activity leads to pronounced availability of cyclic adenosine monophosphate, which augments the protein kinase activity, thereby enhancing the expression of growth factors, modulation of toxicant responsive genes and anti-apoptotic property [8, 19–25].

The present study was undertaken to investigate the effect of caffeine in the regulation of p38 MAPK, TK and NF- κ B and inflammatory molecule interleukin-1 β (IL-1 β) expressions, microglial activation and nitrite content and the possible contribution of these secondary signalling molecules to the nigrostriatal dopaminergic neuroprotection in maneb- and paraquat-induced PD phenotype in the mouse.

Materials and Methods

Chemicals

Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore, Billerica, MA, USA. Streptavidin peroxidase complex and normal goat serum were procured from Bangalore Genei Private Limited, Bangalore, India. Anti-integrin α M (OX-42), anti-IL-1 β , anti-tyrosine hydroxylase (anti-TH), anti-TK, anti-NF- κ B and anti-p38 MAPK antibodies and compatible secondary antibodies were procured from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Ammonium chloride (NH₄Cl), hydrogen peroxide, ethanol, potassium dihydrogen orthophosphate, potassium permanganate and dimethyl sulfoxide (DMSO) were procured locally either from Qualigens or from SISCO Research Laboratory (SRL), Mumbai, India. All other chemicals were purchased from Sigma–Aldrich, St. Louis, MO, USA.

Animal Treatment

Male Swiss albino mice (20–25 g) were obtained from the animal house of the Council of Scientific and Industrial Research-Indian Institute of Toxicology Research (CSIR–IITR), Lucknow. Animals were housed in a room under the standard conditions of temperature, humidity and 12/12 h light/dark cycle. The CSIR-IITR animal ethics committee approved the study. Animals were fed commercially available pellet diet and drinking water ad libitum. Animals were treated intraperitoneally with paraquat (10 mg/kg) and maneb (30 mg/kg) in combination for 9 weeks along with saline treated controls [14]. In a few sets of experiments, the animals were also treated with caffeine, intraperitoneally (20 mg/kg). In another few sets, the animals were treated with aminoguanidine (30 mg/kg), an inhibitor of iNOS, pyrrolidine dithiocarbamate (PDTCC; 50 mg/kg), an inhibitor of NF- κ B, genistein (10 mg/kg), an inhibitor of TK and SB202190, an inhibitor of p38 MAPK (5 mg/kg) along with respective controls. The animals were sacrificed via cervical dislocation; the brain was dissected out immediately and kept in liquid nitrogen. The striatum was dissected out for the measurement of nitrite content and preparation of protein samples for western blotting as reported previously [14]. TH-immunoreactivity and immunohistochemistry for microglial activation were performed on the coronal brain sections across the substantia nigra as described elsewhere [7, 19].

Tyrosine Hydroxylase (TH)-Immunoreactivity

TH-immunoreactivity was performed followed by the unbiased counting of the TH- positive neurons in the

substantia nigra. In brief, the sections were cut from the perfused brain ($n = 3$ in each group) dissected coronally through median eminence and non-specific labelling in the sections was blocked. The sections were incubated in monoclonal anti-TH antibody, washed and re-incubated with secondary antibody, followed by streptavidin peroxidase complex for 30 min. The colour was developed with 3, 3'-diaminobenzidine (DAB) and the sections were dehydrated in graded ethanol, mounted with DPX and visualized under the microscope (Leica Microscope DM6000B, Germany). The images were captured at lower and intermediate magnification ranges and the TH-positive cells were counted as described previously [7, 26].

Microglial Activation

The microglial activation was performed in the substantia nigra region of the brain ($n = 3$ in each group) as described elsewhere [27]. In brief, the coronal sections were taken and incubated with 3% v/v hydrogen peroxide for 30 min at room temperature. After this, the sections were washed with phosphate buffered saline and incubated with 0.1% triton X-100 and 5% normal goat serum in phosphate buffered saline for 30 min. The sections were again incubated for 48 h at 4°C with anti-OX42 primary antibody (1:500 dilution in 0.1% triton X-100 and 5% normal goat serum) followed by incubation in biotinylated goat anti-rabbit (1:1000) secondary antibody for 2 h at room temperature. The sections were washed with phosphate buffered saline and transferred in a solution containing streptavidin-horseradish peroxidase (HRP) complex for 30 s and the colour was developed with DAB. The sections were dehydrated in graded ethanol, mounted with DPX and visualized under the microscope as above.

Measurement of Nitrite Content

Nitrite content was estimated ($n = 3$ in each group) as reported previously [14]. Briefly, 10% w/v tissue homogenate was incubated with NH_4Cl (0.7 mM) and Griess reagent [0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride and 1% sulfanilamide in 2.5% orthophosphoric acid] at 37°C for 30 min. The reaction mixture was centrifuged at $10,000\times g$ at 4°C for 10 min and the absorbance of the supernatant was measured at 550 nm. The nitrite content was calculated as $\mu\text{moles/ml}$ using a standard curve for sodium nitrite (10–100 μM).

Western Blotting of NF- κB , TK, IL-1 β , and p38 MAPK

Western blot analysis was performed ($n = 3$ in each group) as described elsewhere previously [14, 19, 28] with some modifications. In brief, 10% tissue homogenate was

prepared in lysis buffer containing 20 mM tris-HCl, pH 7.4, 2 mM ethylene-diamine-tetra-acetic acid (EDTA), 2 mM ethylene glycol-bis (β -aminoethylether)-N, N, N', N'-tetra-acetic acid (EGTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF), 30 mM sodium fluoride, 30 mM sodium pyrophosphate, 0.1% sodium dodecyl sulphate (SDS), 1% glycerol, 1% triton X-100 and protease inhibitor cocktail. The resultant homogenate was centrifuged at $10,000\times g$ for 10 min and the supernatant was taken. The protein content was measured [29] and $\sim 100 \mu\text{g}$ protein was denatured with SDS buffer. The denatured protein was separated on (8–12%) polyacrylamide gel and transferred onto PVDF membrane. Non-specific labelling was blocked with tris buffered saline containing 5% non-fat dry milk for 2 h. The blots were incubated with any of the primary antibodies (anti-NF- κB , TK, IL-1 β or p38 MAPK; dilution: 1:1000) overnight. Anti-mouse or anti-rabbit alkaline phosphatase-conjugated secondary antibody was used to detect the target proteins, visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) chromogen solution as substrate. The relative band density was calculated with respect to the β -actin blot developed under similar conditions using the computerized densitometry system (Alpha Imager System, Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test was used for comparisons between various groups. The data are expressed as means \pm standard error of means (SEM). The differences between groups were considered statistically significant when the P value was less than 0.05.

The various levels of significance in this study are symbolically depicted as $*(P < 0.05)$; $^\#(P < 0.01)$ and δ ($P < 0.001$) and numerically designated as 1, 2, 3 and 4 in comparison with control, maneb- and paraquat-treated, maneb- and paraquat- and caffeine-treated and maneb- and paraquat- and PDTC-treated animals, respectively.

Results

TH-Immunoreactivity

The number of TH-positive cells is increased in the substantia nigra of caffeine and maneb and paraquat co-treated animals as compared with maneb and paraquat alone co-treated animals. However, treatment of caffeine alone did not change the TH-immunoreactivity and the number of TH positive cells in the substantia nigra. Aminoguanidine, PDTC, genistein and SB202190, partially but significantly,

Fig. 1 TH immunoreactivity of dopaminergic neurons in maneb- and paraquat-induced PD phenotype in mouse substantia nigra in the presence or absence of caffeine co-treatment along with aminoguanidine, PDTC, genistein and SB202190 (n = 3 in each group) **(a)**. The number of TH-positive cells expressed as percent of control **(b)**. The control values were considered 100% in each replicate and experimental values were calculated accordingly for each experiment, therefore, there is no error bar in the control in **b**. Significant changes are expressed as 1 δ ($P < 0.001$) in comparison with control; 2 δ ($P < 0.001$) in comparison with maneb and paraquat and 3 δ ($P < 0.001$) in comparison with maneb and paraquat and caffeine

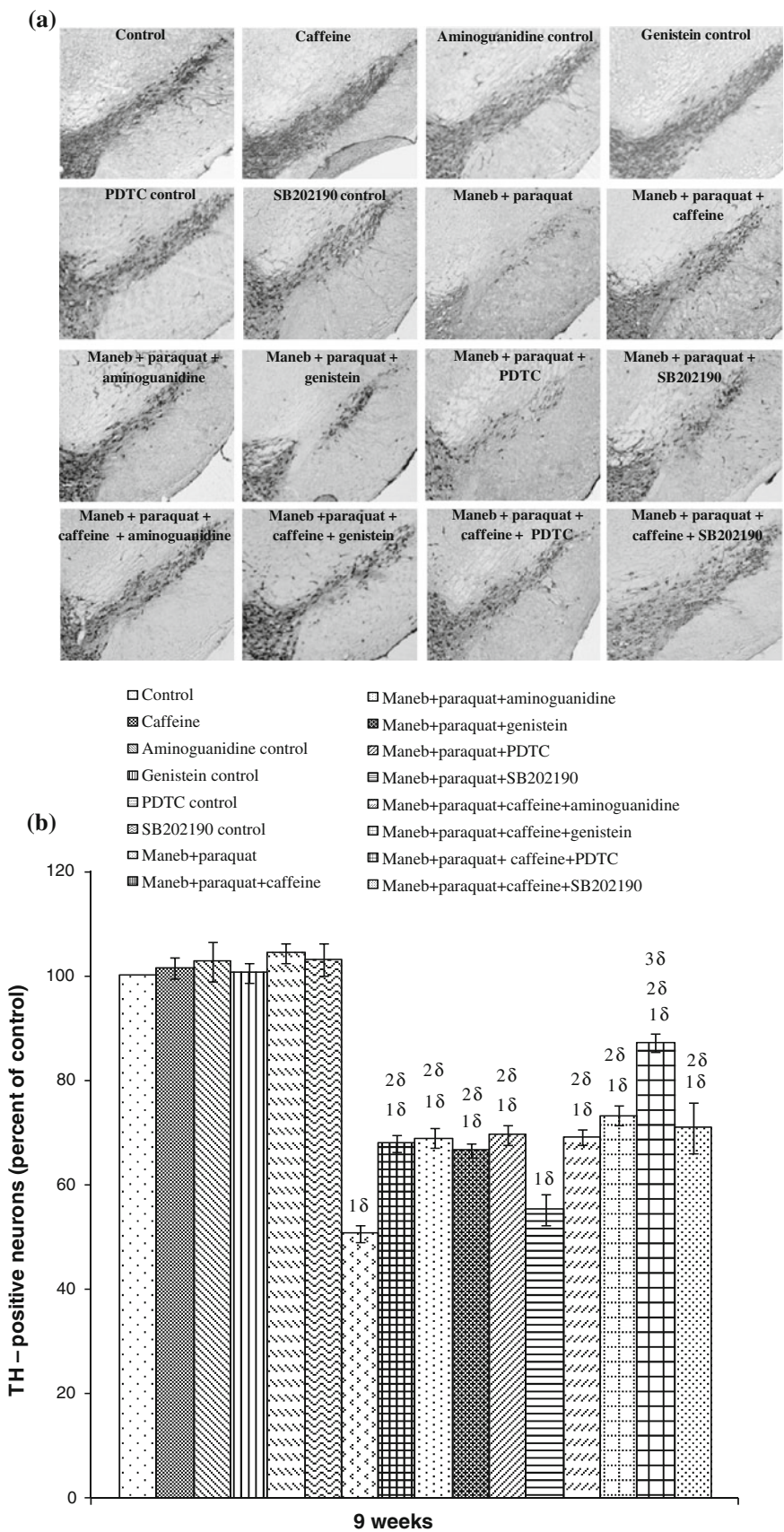
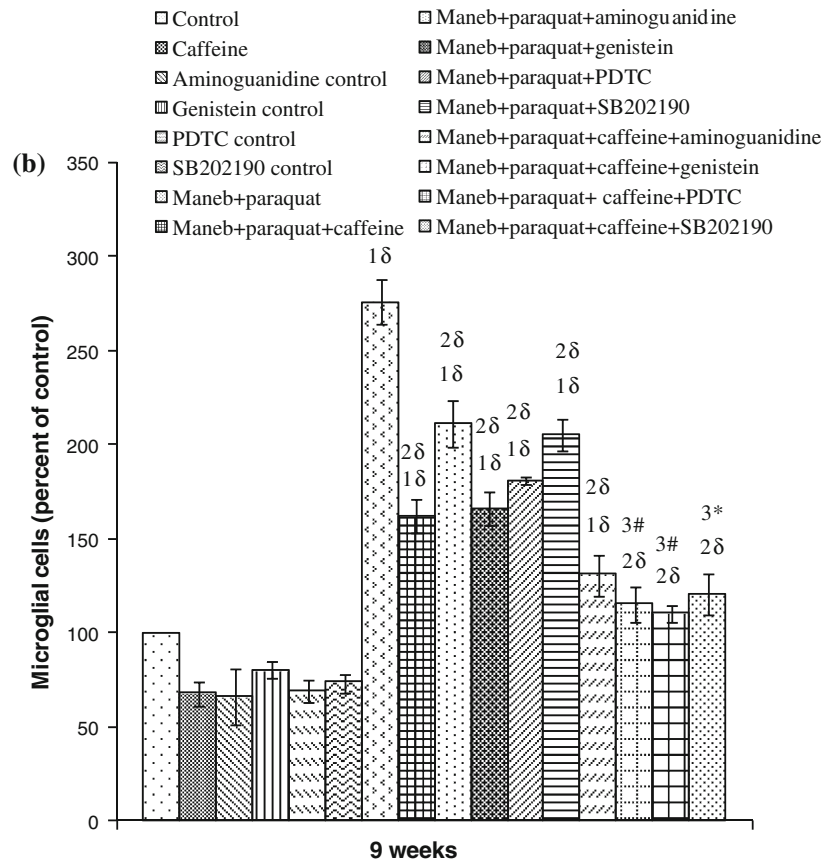
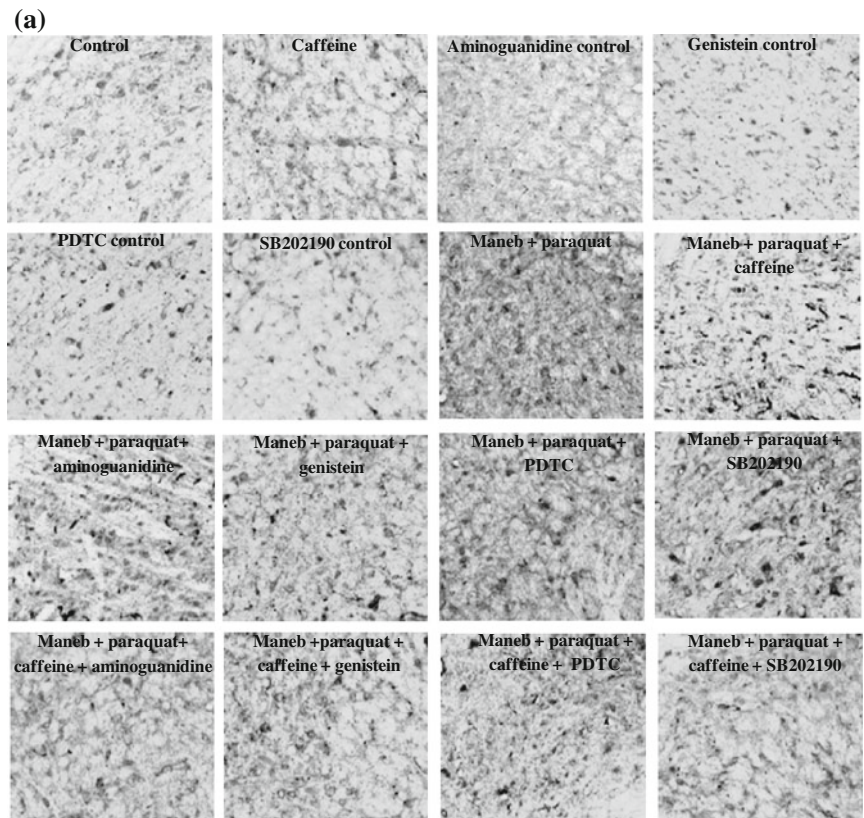


Fig. 2 OX-42 staining (microglia) in control and maneb- and paraquat-induced PD phenotype in mouse substantia nigra in the presence or absence of caffeine pre-treatment along with aminoguanidine, genistein, PDTC and SB202190 (n = 3 in each group) **(a)** and the number of microglial cells in various treatment groups expressed as percent of control **(b)**. The control values were considered 100% in each replicate and experimental values were calculated accordingly for each experiment, therefore, there is no error bar in controls in **b**. Significant changes are expressed as 1 δ ($P < 0.001$) in comparison with control; 2 δ ($P < 0.001$) in comparison with maneb and paraquat; 3* ($P < 0.05$) and 3# ($P < 0.01$) in comparison with maneb and paraquat and caffeine



restored the level of TH-positive neurons in maneb and paraquat treated mice. The restoration was more pronounced in the animals that were co-treated with caffeine (Fig. 1a, b).

Microglial Activation

Maneb and paraquat significantly increased the number of activated microglia in the substantia nigra, as evidenced by OX-42 immunostaining; however, caffeine alone did not produce any significant change in the number of activated microglia in the studied tissue. Caffeine reduced the number of activated microglia in maneb and paraquat co-treated animals significantly. Although aminoguanidine, PDTC, genistein and SB202190 produced a significant decrease in the number of activated microglia in maneb and paraquat co-treated animals, the responses were more evident in the animals that were also treated with caffeine (Fig. 2a, b).

Nitrite Content

Caffeine co-treatment exhibited significant reduction in the nitrite content as compared with maneb and paraquat treated animals. However, caffeine vehicle did not alter the nitrite level significantly. Aminoguanidine, genistein, PDTC and SB202190 significantly attenuated the nitrite level as compared with maneb and paraquat treated animals and the responses were more pronounced in the animals that were also treated with caffeine (Fig. 3).

IL-1 β and p38 MAPK Protein Expressions

Caffeine alone did not produce significant change in the IL-1 β expression; however, maneb and paraquat significantly increased its expression level. Caffeine in combination with maneb and paraquat reduced the IL-1 β expression as compared with maneb and paraquat treated animals. Aminoguanidine, genistein, PDTC and SB202190 attenuated the IL-1 β expression as compared with maneb and paraquat treated animals and the expression level was more pronounced when the animals were also treated with caffeine. Similar trend was seen in the case of p38 MAPK expression but the changes were statistically significant only when the animals were treated with PDTC or SB202190 in combination with caffeine (Fig. 4a, b).

NF- κ B and TK Protein Expressions

The caffeine treatment did not alter NF- κ B and TK expressions as compared with the respective controls. Caffeine, even without NF- κ B or TK inhibitor, reduced NF- κ B and TK protein expressions in maneb and paraquat

treated mice. The reductions were more pronounced in the presence of the inhibitors of various pathways used in this study. However, the pronounced change was statistically significant only in the case of NF- κ B protein expression (Fig. 5a, b).

Discussion

Maneb and paraquat in combination were found to induce degeneration of dopaminergic neurons, which is in accordance with the observations that maneb and paraquat in combination induce PD phenotype in the mouse [5, 6, 11, 12, 14, 26, 30, 31]. Caffeine was found to protect the degeneration of dopaminergic neurons in this study as reported in several chemically induced mice models of PD [8, 13, 19, 20]. In this study, increased nitrite content, an indicator of NO, was seen after maneb and paraquat exposures, as observed previously [14]. The nitrite content

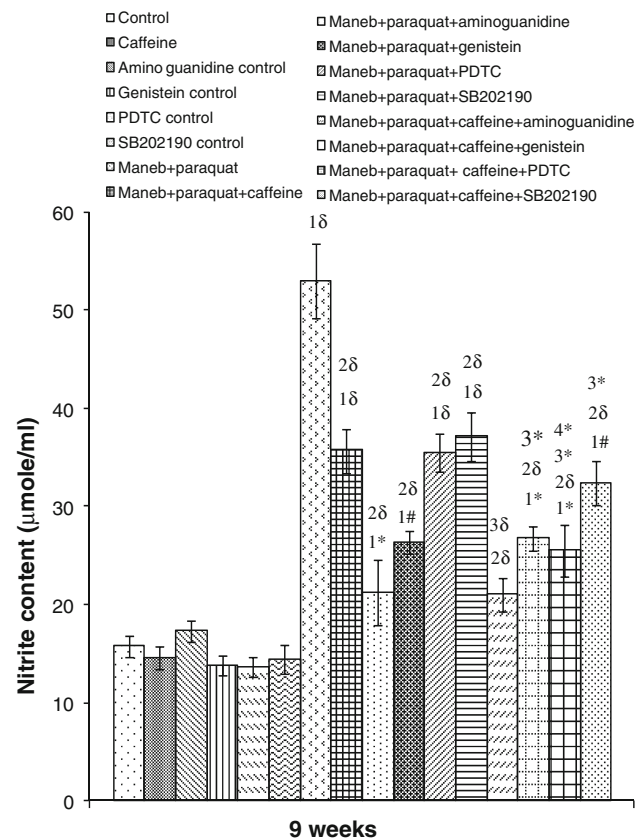


Fig. 3 Nitrite level in control and maneb- and paraquat-induced PD phenotype in mouse in the presence or absence of caffeine pretreatment along with aminoguanidine, genistein, PDTC and SB202190 ($n = 3$ in each group). Significant changes are expressed as 1* ($P < 0.05$), 1# ($P < 0.01$), 1 δ ($P < 0.001$) in comparison with control; 2 δ ($P < 0.001$) in comparison with maneb and paraquat; 3* ($P < 0.05$) in comparison with maneb and paraquat + caffeine and 4* ($P < 0.05$) in comparison with maneb and paraquat and PDTC

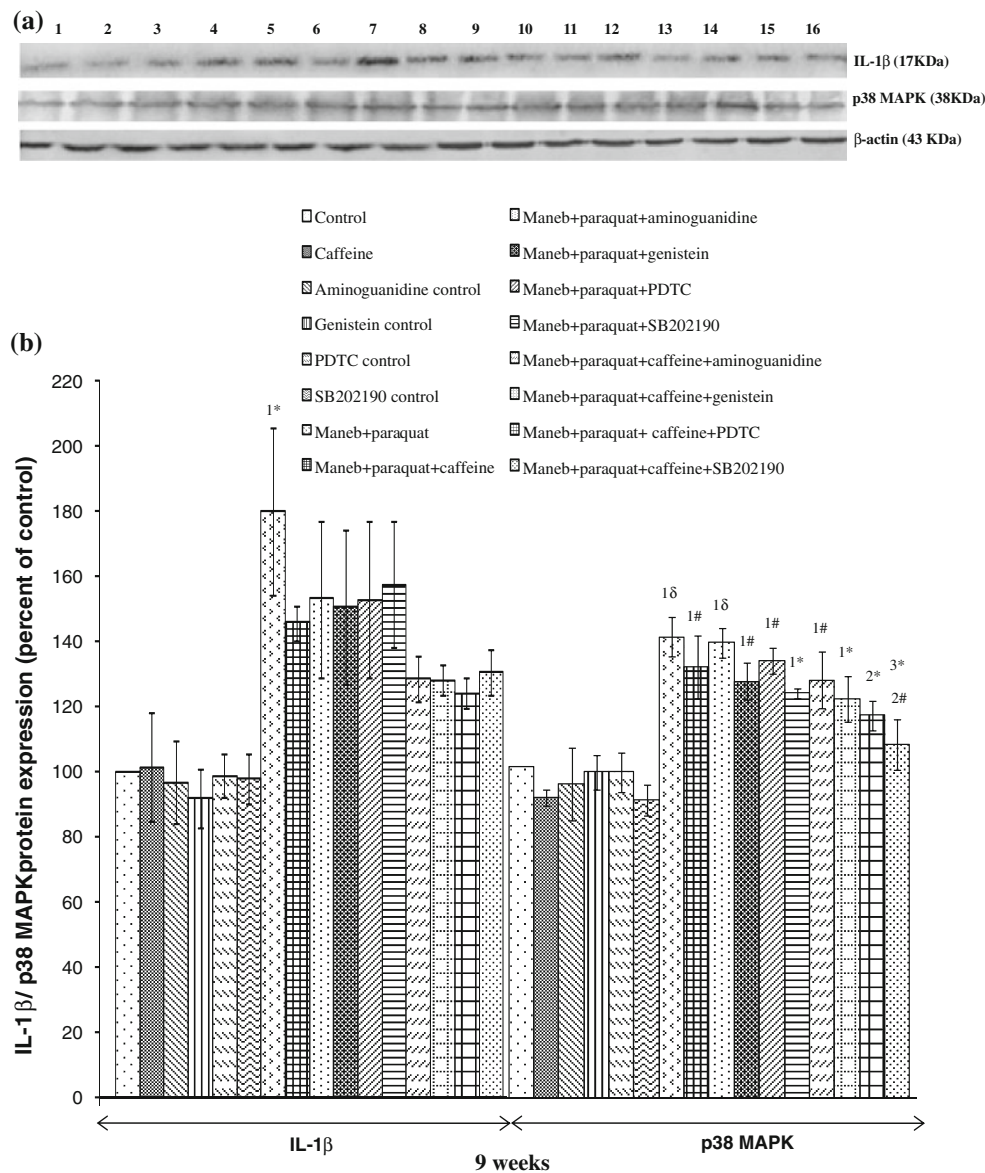


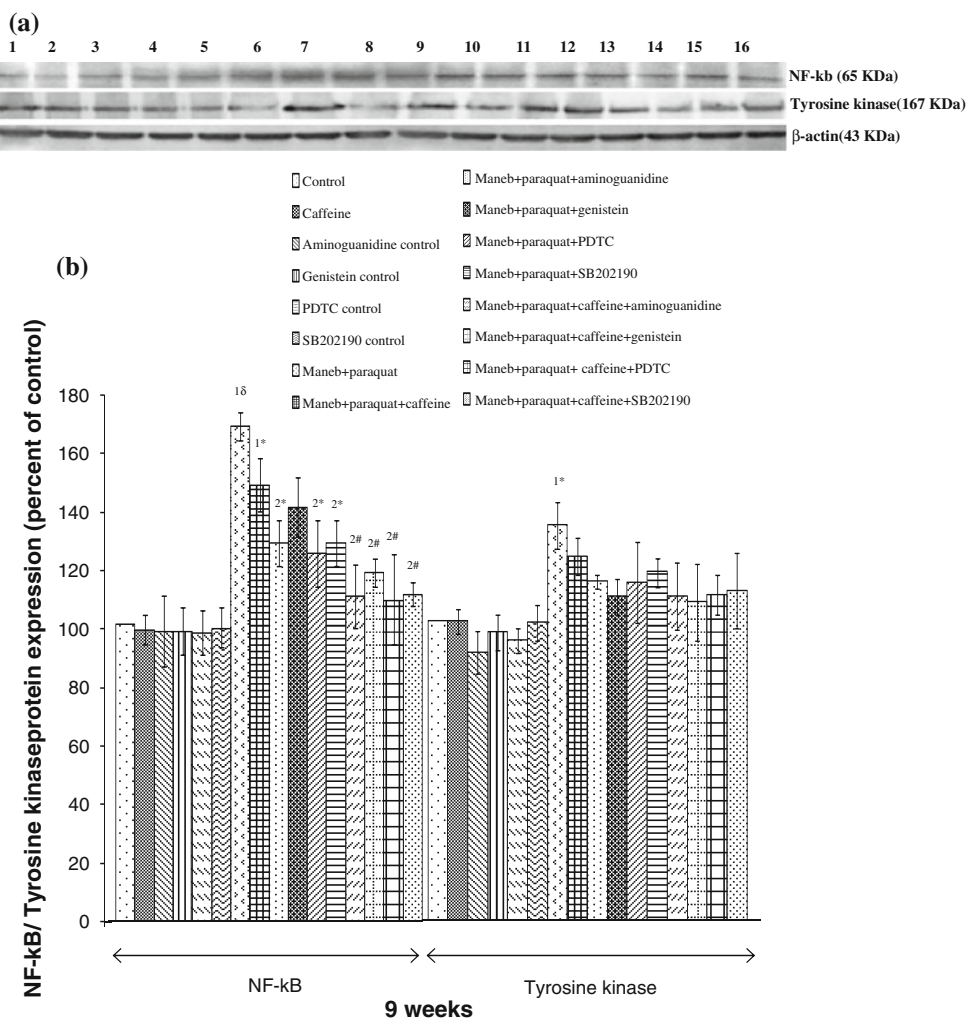
Fig. 4 Effect of maneb and paraquat and caffeine on IL-1β and p38 MAPK protein expressions in the presence or absence of aminoguanidine, genistein, PDTC and SB202190. IL-1β and p38 MAPK expressions are shown in **a**, bar diagram representing the band density ratio with respect to the housekeeping gene β-actin (expressed as percent of control) in **b**. The data are expressed as means ± SEM

(n = 3 in each group). Significant changes are expressed as 1* (P < 0.05), 1# (P < 0.01), 1δ (P < 0.001) in comparison with control; 2* (P < 0.05) and 2# (P < 0.01) in comparison with maneb and paraquat and 3* (P < 0.05) in comparison with maneb and paraquat and caffeine

and neurodegeneration were significantly reduced in the presence of aminoguanidine, an inhibitor of iNOS, PDTC, an inhibitor of NF-κB, genistein, an inhibitor of TK and SB202190, an inhibitor of p38 MAPK showing the involvement of multiple pathways in the regulation of NO production and neurodegeneration. The study showed that NO is critical in the nigrostriatal dopaminergic neurodegeneration mediated by maneb and paraquat [14]. Caffeine reduced the nitrite content, which is further altered by the inhibitors of various pathways, showing that NO is involved in caffeine-mediated neuroprotection.

Activation of microglial cells, the resident innate immune brain cells, was observed in the substantia nigra region after maneb and paraquat co-exposure. Previous studies have also shown that microglial activation is an important event in chemically-induced [32–36] and sporadic PD [37]. Activation of microglial cells is associated with excessive production of NO as activated microglial cells contain high level of iNOS. NO is known to increase glutamate release, which leads to excitotoxicity via the N-methyl-D-aspartate receptor. Aminoguanidine partially attenuated the effect of maneb and paraquat possibly by

Fig. 5 Effect of maneb and paraquat and caffeine on NF- κ B and tyrosine kinase protein expressions in the presence or absence of aminoguanidine, genistein, PDTC and SB202190. NF- κ B and tyrosine kinase expressions with respect to the housekeeping gene β -actin are shown in panel (a) and the corresponding bar diagram representative of the relative band density ratio (expressed as percent of control) in (b). The data are expressed as means \pm SEM (n = 3 in each group). Significant changes are expressed as 1* ($P < 0.05$) and 1 δ ($P < 0.001$) in comparison with control; 2* ($P < 0.05$) and 2# ($P < 0.01$) in comparison with maneb and paraquat



reducing the number of activated microglia. Caffeine treatment reduced the number of activated microglial cells as compared with maneb and paraquat treated animals, which could be responsible for the reduction of nitrite content in caffeine treated animals. Increased level of IL-1 β in maneb- and paraquat-induced PD and reduction in its extent in caffeine treated animals is in accordance with previous reports as activated microglial cells secrete the pro-inflammatory cytokine IL-1 β , which could exacerbate toxicity in dopaminergic neurons [38]. Genistein reduced the nitrite content, thereby reduced the nigrostriatal dopaminergic neurodegeneration as reported in a previous studies [14]. This is further supported by the role of NF- κ B in the regulation of IL-1 β expression and its subsequent association with neurodegeneration [39–43]. The decreased level of its expression in aminoguanidine treated group as compared with maneb and paraquat treated mice further suggests the involvement of microglial activation in maneb- and paraquat-induced PD phenotype and caffeine-mediated neuroprotection.

Nitrite content, which increased in maneb and paraquat treated animals, was reduced by the caffeine or inhibitors of various pathways, significantly by PDTC, an inhibitor of NF- κ B. Reduced nitrite content could be responsible for the reduced loss of TH-positive cells in the nigrostriatal tissues. This observation suggests that NF- κ B could be critical in the regulation of NO level in caffeine-mediated effects. Caffeine-mediated neuroprotection in maneb and paraquat treated animals could be contributed by the reduced microglial activation or nitrite content. The results obtained in this study demonstrate that caffeine offers neuroprotection involving NO, which in turn is regulated by multiple pathways, and reduced microglial activation.

Overall, the maneb- and paraquat-induced PD phenotype is contributed by NO (measured in terms of nitrite), which in turn regulates nigrostriatal dopaminergic neurodegeneration. In this study, caffeine was found to reduce the levels of NO, microglial activation and thereby protect dopaminergic neurodegeneration and the changes were more pronounced in aminoguanidine (inhibitor of NO

synthase enzyme) and caffeine both treated animals as compared with caffeine alone-treated animals. The NO level is reported to be regulated by NF- κ B, p38 MAPK and TK pathways in maneb- and paraquat-induced PD [14], therefore, the roles of these pathways in caffeine-mediated reductions of NO and microglial activation and subsequent nigrostriatal dopaminergic neuroprotection were assessed. The alterations in these parameters were checked in the presence/absence of NF- κ B inhibitor-PDTC, TK inhibitor-genistein and p38 MAPK inhibitor-SB202190. Although caffeine-mediated reductions in NO and microglial activation leading to dopaminergic neuroprotection were modulated by all these three inhibitors, more pronounced modulations were seen in PDTC-treated groups. The results obtained thus suggest that caffeine-mediated neuroprotection is regulated by NF- κ B, TK and p38 MAPK pathways; however, NF- κ B pathway seems to be predominant.

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Conflict of interest The authors report no conflicts of interest.

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