

Synergistic effect of combined GDNF and Amodiaquine attenuates behavioral deficits and protect dopaminergic neurons of Parkinson's disease animal model through tyrosine kinase Ret receptor

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Research

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Abstract

Parkinson's disease (PD) is one among the most leading neurodegenerative disease after Alzheimer's disease, with a prevalence of approximately 0.5–1% among those 65–69 years of age. Efforts to vitiate this disease are ongoing, and several treatment modes such as Glial cell line-derived neurotrophic factor (GDNF) have been in place since 1993. Glial cell line derived neurotrophic factor (GDNF) protects, regenerates, and improves the metabolism of substantia nigra pars compacta neurons (SNpc), and it increases the high-affinity dopamine uptake. It has been recently reported that amodiaquine could attenuates the behavioral deficits of an animal model of Parkinson's disease, nevertheless its mechanism is obscure. We sought to demonstrate the mechanism of neuro-protection effect of amodiaquine and ascertain its corroborative effect when used together with GDNF. We show herein that combined therapy (GDNF and amodiaquine) ameliorated behavioral deficits of PD animal models as compared to single-factor treatment. TH positive neurons increased significantly upon combined therapy treatment, and besides, GDNF and amodiaquine interact functionally to protect dopaminergic neurons through the PIK-3/Akt pathway. We also found that combined therapy (GDNF and amodiaquine) mediates its action through a distinct trans-membrane tyrosine kinase Ret receptor by amplifying its effect. Slight elevated aspartate aminotransferase (AST) were noticed in amodiaquine treated groups, alarming the bio-utility. These findings collectively suggest an interplay between GDNF and amodiaquine and co-express to exert neuronal protection hence a promising approach in PD therapy. Despite its undisputed effect on neuro-protection, we report that amodiaquine may not be safe, particularly in translation to human beings' trial settings.

Background

Parkinson's disease, a midbrain dopaminergic neuron degeneration, is one of the foremost neurodegenerative diseases, second after Alzheimer's disease [7, 9, 48] with a prevalence of approximately 0.5–1% among those 65–69 years of age [36]. This prevalence would rise to 30% by 2030 in an aging population [9]. The current treatment of this disease, for example, L-3, 4-dihydroxyphenylalanine (L-DOPA), is symptomatic and causes severe side effects like dyskinesia [22, 39]. Efforts to vitiate this disease are ongoing, and several treatment modes such as Glial cell line-derived neurotrophic factor (GDNF) have been in place since 1993 [31]. GDNF protects, regenerates, and improves the metabolism of substantia nigra pars compacta neurons (SNpc), and it increases the high-affinity dopamine uptake [12, 31, 39]. Although GDNF elicited substantial outcome in rodents and non-human primates, it has failed to provide a conclusive clinical benefits in a randomized controlled trial in Parkinson's disease-human being cases thus biasing the beneficial outcome of GDNF in treatment of Parkinson's disease [5, 33, 47]. It has been recently reported that amodiaquine could attenuates the behavioral deficits of an animal model of Parkinson's disease, nevertheless its mechanism is obscure. We sought to demonstrate the mechanism of neuro-protection effect of amodiaquine and ascertain its corroborative effect when used together with GDNF.

Amodiaquine is an antimalarial drug (4-aminoquinoline class) that has recently discovered to possess Nurr1 (Nuclear receptor related one) agonistic activity, thus protects dopaminergic neurons in a mouse model of Parkinson's disease [22]. Nurr1 (also called NR4A2) is a member of the nuclear receptor superfamily of transcription factors, first identified from mouse brain cDNA library in 1992 [38] and localized to human chromosome 2q22-q23 [10]. Several lines of evidence have indicated that Nurr1 is essential for the development, migration, and survival of dopaminergic neurons [38, 41]. Defects in the Nurr1 gene or altered expression of the gene in SN associated with PD and certain psychiatric disorders, such as schizophrenia, manic behavior, and predisposition to cocaine addiction [11, 20, 27, 42].

Considering that both GDNF and amodiaquine elicit neuroprotection and behavioral effects, it is of paramount importance to explore the mechanism involved in synergistic protection of nigrostriatal neurons. To the best of our knowledge, so far, no studies have been performed exploring the effects of a combined GDNF and Amodiaquine administration in-vivo. Hence, in the present study, we aimed at investigating whether treatment with combined exposure to GDNF and amodiaquine improves dopaminergic neurons' survival and augments dopamine release as compared to a single-factor treatment and the mechanism involved. To confirm the neuroprotective effect of single factor treatment, we first divided the 6-OHDA animal model into three groups, i.e., Control, amodiaquine, and GDNF. We later combined the two therapies by injecting first the GDNF, followed by amodiaquine after 24hours. The results showed that combined therapy (GDNF and amodiaquine) ameliorated behavioral deficits of the PD animal model as compared to single-factor treatment. TH positive neurons increased significantly upon combined therapy treatment, and besides, GDNF and amodiaquine interact functionally to protect dopaminergic neurons through the PIK-3/Akt pathway. We also found that combined therapy (GDNF and amodiaquine) mediates its action through a distinct trans-membrane tyrosine kinase Ret receptor by amplifying its effect.

Results

Amelioration of behavioral deficits after Amodiaquine, GDNF and combined therapy treatment in 6-OHDA mice model

Apomorphine challenge

In the fourth week, animals found to have developed Parkinson's disease were divided into four groups (control, AQ, GDNF, and combined). In the 6th week (2nd week after treatment) their mean turns/30 min were 129 ± 7 , 13 ± 2.3 , 45 ± 4 , 31 ± 3.8 for control, AQ, GDNF, and combined therapy group respectively. Two weeks later (8th week) the mean turns/30 min were 202 ± 2 , 4.7 ± 0.8 , 28 ± 2 , 16 ± 1.6 for control, AQ, GDNF, and combined group respectively. These results show that the amodiaquine group displayed less rotation after the second and fourth weeks of treatment compared to GDNF and the combined group.

This indicates that amodiaquine had caused robust dopaminergic neurons protection compared to other groups (Fig. 1A).

Contralateral bias recovery

Percent of contralateral bias recovery was calculated and normalized as % of contralateral bias recovery = $[1 - (\text{lateral turns in 20 trials} - 10) / 10] \times 100\%$. On the 6th week (2 weeks after treatment) results were $60\% \pm 3$, $80\% \pm 1$, $160\% \pm 3$, $100\% \pm 2$ for control, AQ, GDNF and combined therapy group respectively. At 8th week, their recovery changed to $45\% \pm 4$, $95\% \pm 3$, $175\% \pm 4$, $114\% \pm 2$ for control, AQ, GDNF, and combined therapy group respectively. The percent of contralateral bias recovery for the control group declined over time, unlike the experimental groups, which elicited significant improvement of which GDNF group found to have a high percentage of recovery (Fig. 1B).

Rotarod test

Rotarod test disclosed motor activity of the amodiaquine and combined group to be higher compared to other groups, i.e., 30 ± 4 , 38 ± 5 , 36 ± 4 , 38 ± 3 on the 6th week for control, AQ, GDNF, and combined therapy group respectively. In the 8th week, animals in AQ, GDNF, and combined therapy group could finish the maximum velocity (40 rpm for 300 seconds) set on the rotarod apparatus, contrary to the control group, which dropped to 24 ± 3 (Fig. 1C).

The results above reveal that animals developed significant behavioral impairments; nevertheless, amodiaquine, GDNF, and combined therapy prompted attenuation of these damages. In contemplation of the artistic effect of a single factor or combined treatment, RT-PCR and western blotting were performed.

Combined therapy (GDNF and AQ) interact to promote dopaminergic neurons protection

We rendered to investigate whether combined therapy exhibits more effects as compared to the single factor treatment (GDNF or amodiaquine) in the 6-OHDA animal model. Importantly we implored RT-PCR to test if it can protect TH+ and DAT+ neurons, which are regarded to be dopaminergic neurons markers. DAT positive neurons were remarkably altered upon combined therapy treatment; the delta-delta ct revealed fold changes of 13, 45, and 72 and P values of $P = 0.000274$, $7.4663E-9$, $1.7106E-10$ (one way ANOVA followed by Bonferroni post hoc test) for Amodiaquine, GDNF and combined therapy respectively. Similarly, TH positive neurons were also increased to a significant level upon combined therapy treatment compared to single-factor therapy, i.e., increased by 10 folds compared to 2.8 and 6.5 of AQ and GDNF. We also tested the RET genes as it is considered to be a GDNF receptor. Results show a fold change of 4.9, 15 and 21 for AQ, GDNF, and combined therapy treatment, respectively. Collectively, these findings suggest that GDNF and AQ interact functionally to promote dopaminergic neurons protection (Fig. 2).

Combined therapy confers dopaminergic neurons protection in 6-OHDA animal model by restoring TH expression

We further sought to test the effect of combined therapy targeting tyrosine hydroxylase (TH), which is one of the dopaminergic neurons markers. We performed western blotting (Fig. 3A.) and immunohistochemistry (Fig. 4), incorporating a single factor and combined treatment. By analysis of variance (ANOVA) followed by Bonferroni post hoc results reveals that the number of TH positive neurons was significantly increased in combined therapy compared to single-factor treatment (p is less than 0.005) Fig. 3A. These results suggest that combined therapy is more suitable to halt Parkinson's disease as it rescues degenerative dopaminergic neurons.

GDNF and amodiaquine interact functionally to protect dopaminergic neurons through PIK-3/Akt pathway

It was described earlier that once GDNF bind to its receptors, it may induce the activation of mitogen-activated-kinase (MAPK) through phosphorylation of extracellular regulated kinase (ERK), P-38, JUNK and or PI3-K/Akt pathway. Importantly, Akt, which is downstream of PI3-K, has been implicated in neuronal regulation both in the central and peripheral nervous systems. Previous studies, Kim et al. [22] reported that amodiaquine could abolish behavioral deficits in the 6-OHDA animal model, which means amodiaquine may contain similar cellular effects like GDNF. We first tested the role of Akt and its impact on amodiaquine in 6-OHDA Parkinson's disease animal model and whether the combined therapy would dual its effect. Interestingly Akt in the amodiaquine treated group was slightly higher compared to GDNF treated group while the combined therapy group significantly augmented the Akt level in the ventral midbrain of the animal model. These findings collectively suggest an interplay between GDNF and amodiaquine and co-express to exert neuronal protection.

Combined therapy (GDNF and amodiaquine) mediates its action through a distinct transmembrane tyrosine kinase Ret receptor by amplifying its effect

Ret, an orphan receptor tyrosine kinase, which is expressed during vertebrae embryogenesis in motor, dopamine, and noradrenaline, has been demonstrated previously that GDNF binds to and induce phosphorylation. Similarly, ret proteins were also found to bind to GDNF and mediate survival and growth responses. There is clear evidence that GDNF is crucial for the development, maintenance, and protection of dopaminergic neurons through tyrosine kinase Ret receptor, whether amodiaquine does the same is perplexing. We first sought to test the expression of kinase receptor-ret, employing RT-PCR on correspondent groups. Interestingly ret was similarly expressed on AQ as the GDNF group and highly expressed upon combined therapy treatment (Fig. 2). Moreover, we further wanted to elucidate the effect

of combined therapy on western blotting, and we found that results are more significant compared to the other groups $**p < 0.005$ versus corresponding control (Fig. 3C).

Combined therapy could not counteract the weight loss

Previous studies reported that GDNF might not be safe as weight loss was observed in clinical trials. Several mechanisms were stipulated on how GDNF causes this. Lapchak [26] reports that GDNF may gain access to ventricular spaces and distribute to hypothalamic nuclei, where it alters neurotransmission to affect food intake and eventually may cause weight loss. Our results proved that there was a subsequent weight reduction today five in both GDNF and combined therapy treatment group ($*p < 0.05$ students t-test) Fig. 4b. In contrary to the amodiaquine group, the weight loss was not significant after five days of treatment. This indicates that amodiaquine is safe in terms of weight loss, nevertheless cannot attenuate the weight loss effect of GDNF.

Amodiaquine may not be safe for the liver

Despite its undisputed effect on neuro-protection, it is, however, necessary to consider its implications when translated to human beings' trials. Previous studies reported that AQ has a deleterious effect, especially to the liver. To elucidate this, ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were determined as described by Mien et al. [18]. Our results revealed elevated AST (121.3333 ± 22.17105 and 121.3333 ± 22.88134) for AQ and combined therapy, respectively, unlike ALT, which appeared to be within the average values range. This may indicate that more precautions are warranted before it translated to human beings' clinical trials.

Discussion

We have demonstrated that GDNF and amodiaquine enhanced behavioral deficits in 6-OHDA Parkinson's disease animal model; moreover, a combination of it might be superior in the treatment of Parkinson's disease. Previous studies had proved that GDNF alleviates behavioral deficits by exerting neuroprotection to the dopaminergic neurons of the substantia nigra and striatum [3, 4, 28, 31] similarly to amodiaquine [22] on 6-OHDA Parkinson's disease animal model it has proved to attenuate behavioral deficits and enhance its dual functions. Our study, which is the combination of two therapy avenues, is per Oh et al. [37], who combined two gene therapies in the treatment of midbrain dopaminergic neurons toxic insults and found the synergistic action of the two gene therapy.

Furthermore, we quantified the expression of TH and DAT genes in the mRNA level by performing reverse transcriptase RNA in single factor treatment and combined therapy. Tyrosine hydroxylase and dopamine transporter are one of the dopaminergic phenotype genes, and they get to be reduced in Parkinson's disease [32]. In the current study, the control group showed a significant reduction of TH and DAT, while the experimental groups revealed a substantial increase of TH and DAT. Moreover, by comparing the number of folds, the combined therapy group elicited a higher number of TH and DAT folds with respect to other groups. Concurrently, we performed the western blotting analysis and immunohistochemistry to elucidate whether the effect could be the same on the protein level, in this case employing TH antibody.

Combined therapy could elicit higher TH folds and significant positive neurons compared to single-factor treatment. Collectively, our results give proof that combined therapy confers dopaminergic neurons protection in the 6-OHDA animal model by restoring TH expression and, at a particular standpoint, gives an impression that GDNF and AQ interact functionally to promote dopaminergic neurons protection. It is already known that GDNF protects dopaminergic neurons by restoring TH fibers in the injured dopaminergic neurons, and this concurs our results after 6-OHDA animal model being treated with GDNF independently [12, 45].

Multiple lines of evidence indicate that PI3-K/Akt mediates the survival effect of GDNF [25]. Its roles had further been explored in the survival of the sympathetic neurons of the superior cervical ganglion [43], spinal motor neurons [24], cerebella granules cells [30], and midbrain dopamine cells [46]. Whether it does the same in 6-OHDA Parkinson's disease animal model was explored in our study. We found that both GDNF and amodiaquine protect dopaminergic neurons through the PIK-3/Akt pathway. Interestingly, when it was given as combined therapy, the Akt folds were increased significantly. Our findings collectively suggest an interplay between GDNF and amodiaquine and co-express to exert neuronal protection. Another emphasis was to investigate whether the tyrosine receptor –ret is involved in the co-expression effects prompted by the combined therapy of GDNF and amodiaquine.

Tyrosine kinase –ret receptor is the member of receptor tyrosine kinase superfamily that form a complex signaling component of Glial cells line-derived neurotrophic factor (GDNF) and its family ligands [15]. It is expressed in dopaminergic neurons, motor neurons, somatic sensory neurons, enteric neurons, sympathetic and parasympathetic neurons during their development and maintenance. Kramer & Liss, [25] reported that GDNF binds to tyrosine kinase receptor-Ret and induce phosphorylation. After it phosphorylates, activate other signaling cascades, including PI3-K/Akt, MAPK (JNK, P38, and ERK5), which will functionally stimulate cell survival, growth, differentiation, and neuritogenesis [25].

Surprisingly, our results show that Ret was also stimulated when we treated the animal with amodiaquine only and was highly stimulated when we combined the two therapy. This could be due to the fact that amodiaquine is an agonist of the nuclear receptor-related 1(Nurr1), an orphan member of the nuclear receptor superfamily which is highly expressed in the developing and adult ventral midbrain [22] and this might be the one added up the dual function.

In this current study, we noticed a dramatic weight loss after the animal was treated with GDNF. Our results comply the previous studies that reported a weight reduction after the subjects (Rodents-[26], monkeys-[39], and human beings-[5, 33, 47] being treated with GDNF. We thought amodiaquine could mimic the weight reduction effect of GDNF when combined therapy was applied, but that had never been so. This seems to be a physiological effect as postulated in different theories that GDNF may gain access to ventricular spaces and distribute to hypothalamic nuclei where it alters neurotransmission to affect food intake and eventually may cause weight loss [26]. The weight reduction was not statistically significant in the amodiaquine treated group when a comparison was made between day 0 (Before treatment) and day five after treatment.

Despite its undisputed effect on neuro-protection, we report that amodiaquine may not be safe, particularly in translation to human beings' trial settings. In the current study, we have found that the amodiaquine and combined therapy group had a slight elevation of AST. These elevated values are alarming the safety and utilization by human beings. It was reported earlier that amodiaquine might cause severe idiosyncratic drug reactions (IDR) that included hepatotoxicity and agranulocytosis [29, 34]. These findings collectively suggest an interplay between GDNF and amodiaquine and co-express to exert neuronal protection hence a promising approach in PD therapy. Amodiaquine safety should not be underated.

Methods And Materials

Subjects and 6-OHDA-Parkinsonism model

We obtained about 60 adult male mice (30–35 g) from the animal facility of Xuzhou Medical University, and the experimental protocols endorsed by the institutional animal care and use committee. Mice housed in four different cages with the controlled environment: 12/12-h light/dark cycle (lights on at 7 am) and room temperature of 23 ± 1 °C with ad libitum access to food and water.

We used the following materials: 6-OHDA (medchemexpress (china) CAS NO: 28094-15-7), Amodiaquine (medchemexpress (china) CAS NO: 6398-98-7), Primary antibodies: Antityrosine hydroxylase TH-ab112 (Rabbit,1:200 Abcam), Anti Ret [EPR2871] ab134100 (Rabbit,1:1000 Abcam), Beta-actin monoclonal (Mouse,1:5000 Proteintech), AKT mouse monoclonal (Mouse,1:2000 Proteintech), Protein -Glial cell-line derived neurotrophic factor (GDNF)-enquire BioReagents), For immunohistochemistry (IHC), the ZLI-9018 KIT purchased from ORIGENE was used. Nitrocellulose filter was from ExCell Bio (Excell bio-Shanghai China).

6-OHDA lesion

Animals were anesthetized with sodium pentobarbital [6] and placed in the stereotaxic apparatus with the bite bar set at 0 mm. The skull was exposed, and the burr hole was made using a high-speed dental drill. All animals were administered 6-hydroxydopamine (6-OHDA, Medchemexpress, 10ug in 2 ul of 0.9% saline containing 0.2% ascorbic acid) in the left substantia nigra (3.0 mm posterior to the bregma, 1.3 mm lateral, 4.7 mm ventral to the dural surface) [13]. The sham-operated animals received vehicle only (0.2% ascorbate in 0.9% saline) at the same coordinates. The injection carried out with a 10-ml Hamilton microsyringe, performed over 4 min, and an additional 4 min was allowed before the needle was removed, as described in the previous studies [2, 4].

Elevated body swing test

Body asymmetry conducted, as previously described by Sanberg [40]. Briefly, the animal was first placed into a standard cage on the table for habituation and to attain a neutral position (all four paws on the ground). The animal was held approximately 3 cm from its tail base and elevated above the surface

(3 cm) in the vertical axis. A swing recorded whenever the animal moved its head out of the vertical axis to either side and before attempting another rhythm, the animal must return to the vertical position for the next swing to be counted. Oscillations recorded by using a hand counter. The frequency of initial turning of the head or upper body contralateral to the lesioned side was calculated in 20 consecutive trials and normalized, as follows: % contralateral recovery = $[1 - (\text{lateral turns in 20 trials} - 10) / 10] \times 100\%$ [8, 17, 40]. Body asymmetry was assessed at three different times: (i) 14 days after 6-OHDA lesion, (ii) 28 days after 6-OHDA lesion, and (iii) 2 and 4 weeks after treatment. Animals with left side nigra 6-OHDA lesion will exhibit right side biased swings [40].

Apomorphine challenge.

Mice were challenged with apomorphine (0.6 mg/kg, s.c.) at 14 and 28 days after 6-OHDA injection, 2 and 4 weeks after treatment. Animals will exhibit swings to the side with more dopaminergic neurons upon apomorphine injection (Sanberg, 1995), [1, 40, 44]. The number of net rotations was evaluated in plastic tubes (19 cm diameter, 22 cm high) in 30 minutes using the video tracking system ANY-maze (Stoelting, Wood Dale, IL, USA) [3, 4]. Four weeks after surgery, animals with an insufficient number of net rotations (< 1 clockwise rotation/min) [35] were discarded (about five mice). Apomorphine challenge was assessed at three different times: (i) 14 days after 6-OHDA lesion, (ii) 28 days after 6-OHDA lesion, and (iii) 2 and 4 weeks after treatment.

Rotarod

The rotarod was performed as described by Jiang et al. [21, 44]. Briefly, the accelerating rotarod apparatus (Insight Scientific Equipments, Ribeirão Preto, SP, Brazil) consists of a grooved metal roller (6 cm in diameter) and separated 9-cm-wide compartments elevated at 16 cm. The spindle speed was increased to 40 rpm over a maximal period of 300 s, and the time spent on the accelerating rotarod and the corresponding rpm were determined. Rotarod performance was assessed at three different times: (i) 14 days after 6-OHDA lesion, (ii) 28 days after 6-OHDA lesion, and (iii) 2 and 4 weeks after treatment.

GDNF, Amodiaquine and combined treatment

In order to reveal the robust effect of the combined therapy, the PD mice model was divided into four groups. The first group was the control, which received vehicle administration; in short, the PD model was injected normal saline (2 ul) through the same coordinates and continuously received an intraperitoneal injection of normal saline (10 ul/g) for 10 days similar to amodiaquine group. The amodiaquine group received a dose as previously described by Kim et al., [22, 23] briefly; Amodiaquine (medchemexpress-china) CAS NO: 6398-98-7) was dissolved in 0.9% physiological saline at 4 mg/ml and administered to mice at 40 mg/kg intraperitoneally, for ten days in total at the interval of 24hrs daily. GDNF group received 2 ul of GDNF at the concentration of 4ug/ul [6] through the same coordinates used to administer 6OHDA (3.0 mm posterior to bregma, 1.3 mm lateral and 4.7 mm ventral to the cranial surface). The combined therapy group received both GDNF and amodiaquine (the same protocol).

Weight

Previous studies have reported that one of the factors noticed in the failure of clinical trials was weight loss [5]. The same observation reported in 2019 [47]. The leading cause of weight loss for patients treated with GDNF is elusive. Some studies suggest that intranigraly -administered GDNF may gain access to ventricular spaces and distribute to hypothalamic nuclei, where it alters neurotransmission to affect food intake and eventually may cause weight loss [19]. A similar effect documented by Lapchak, a study that showed that GDNF distributes from lateral ventricle to fourth through the third ventricle and labels the hypothalamus, and this would indicate that GDNF alters hypothalamic neurotransmission which is necessary for feeding behavior [26]. In the current study, we wonder if amodiaquine administration could mimic or counteract the effect of GDNF on weight loss.

Immunohistochemistry

Under sodium pentobarbital anesthesia, mice were transcardially perfused with 100 ml PBS followed by 150 ml of cold 4% paraformaldehyde in 0.1M phosphate buffer (0.1M PB). The brains were removed, fixed for 24 h at 4 °C in the same fixative, processed, and embedded in paraffin. Coronal brain sections (5 µm-thick) were cut on a microtome (Leica RM2155, Nussloch, Germany). Parts were deparaffinized in xylene and rehydrated in a gradient of ethanol and distilled water. After being washed three times with 0.01M PBS (for 5 min each), the sections were incubated in H₂O₂ solution for 5–15 min to block the endogenous peroxidase activity then washed three times with PBS. In order to reduce non-specific staining, the sections were incubated with goat-serum at room temperature for 10-15mins then serum removed without washing. Incubated with the primary antibody in 37°C for 60 min and washed three times with 0.01M PBS, then sections were incubated with a biotinylated goat anti-rabbit IgG. After three piles of washing with 0.01 M PBS, horseradish enzyme-labeled streptomycin was added to react for 10-15mins at room temp. After three times wash with 0.01M PBS, the sections were stained for peroxidase reaction by incubation with a mixture of diaminobenzidine (DAB) for 5–10 min at room temperature. This was followed by recoloration by incubating with hematoxylin for 20seconds, then dehydrated, cleared, and mounted with galvanol and were examined under a light microscope.

Western blot

The proteins of the ventral midbrain were prepared according to the previous studies [14, 28]. In brief, animals (n = 4 per group) were sacrificed, brains rapidly removed, and the ipsi- and contra-lateral ventral midbrain were dissected and immediately put on dry ice for immediate use and in a -80°C fridge for further use. Tissues were homogenized in RIPA buffer (Beyotime, China) with phosphatase inhibitor and protease inhibitor (Phenylmethylsulfonyl fluoride) at a cocktail of 100: 1 (RIPA: PMSF). Proteins homogenates centrifuged at 4°C, 15,000 × g for 30 min. The concentrations of proteins determined by the BCA Protein assay kit (Beyotime, China). 20 µg proteins separated by 10% SDS-PAGE and then were electrotransferred (100V, 1 hour) to nitrocellulose membranes. We used 5% skim milk in Tris-buffered saline to block the membrane in 2hour then incubated overnight at 4°C with one of the following primary antibodies: Antityrosine hydroxylase TH-ab112 (Rabbit,1:200 Abcam), Anti Ret [EPR2871] ab134100

(Rabbit,1:1000 Abcam), Beta-actin monoclonal (Mouse,1:5000 Proteintech), AKT mouse monoclonal (Mouse,1:2000 Proteintech). The next day, membranes incubated with their respective secondary antibodies. After blotting, the bands on the filter were scanned and analyzed with an image analyzer (Lab Works Software UVP upland, CA, USA).

RT-PCR

Total RNA was reverse transcribed into cDNA using PrimeScript™, RT Master Mix [37], and used for quantification of mRNAs encoding TH, DAT, and RET. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA used as an internal control. The PCR program run at 25 °C for 10 min, then 42 °C for 30 min and 85 °C for 5 min [16]. Results were analyzed with a delta-delta Ct method.

Statistical analysis

All data were shown as mean ± SD. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test and the statistical significance level was set at $p < 0.05$ for all tests.

Liver hepatotoxicity test

Although amodiaquine was reported to halt Parkinson's disease motor symptoms, we rendered to determine whether the dosage we used is safe to the liver or not. Previous studies reported that AQ is toxic to the liver as it may cause severe idiosyncratic drug reactions (IDR) that included hepatotoxicity and agranulocytosis, necrosis, and presence of inflammatory cells on histopathological analysis [29, 34]. In order to attain this, the liver test was done as described in the protocol ("<http://www.bio-protocol.org/e931> Vol 3, Iss 19, Oct 05, 2013). Briefly, blood was collected from the orbital sinus with a microhematocrit blood tube (heparinized). The dropper was used to push out the blood in the heparinized blood tube, and about 300 µl of blood was collected in the 1.5 ml polypropylene test tube. Centrifuged at 1500 x g, 4 °C for 15 min. Cell-free supernatant plasma was carefully taken (about the half volume of blood) and place it in a properly labeled polypropylene test tube and transferred, about 150 µl plasma into the sample cups. The ALT and AST were further determined as in this protocol ("<http://www.bio-protocol.org/e931> Vol 3, Iss 19, Oct 05, 2013," 2013).

List Of Abbreviations

GDNF-Glial cells line derived neurotrophic factor, Nurr1-Nuclear receptor related one, Alanine transaminase (ALT), Aspartate transaminase (AST).

Declarations

Ethical approval

Experimental protocols endorsed by the institutional animal care and use committee of Xuzhou medical university.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Code availability

Not applicable

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Authors' contributions

PK is the investigative lead responsible for the research ideas, design, experimental works, analysis and manuscript write up under the supervision and guidance of DG. All authors read and approved the final manuscript

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Not applicable

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Footnote

The primers we used:

GAPDH; forward 5'- AGG TCG GTG TGA ACG GAT TTG -3',

reverse 5'- TGT AGA CCA TGT AGT TGA GGT CA -3',

RET forward 5'- ACAAGCACACTACTCTCAGG -3',

reverse 5'- CATTGACCAGGACTACTAGC-3'

TH; forward 5'-AGC CCC CAC CTG GAGTAT TTT G-3',

reverse 5'-AGC AAT CTC TTC CGC TGT GTA TTC-3',

DAT; forward 5'-GCT GGC ACA TCT ATC CTC TTT GG-3',

reverse 5'-CAA TGC TGA CCA CGA CCA CAT AC-3',

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Table

	Control	AQ	GDNF	Combined
ALT(U/L)	50.66667 ±18.20867	40.66667 ±3.399346	38.66667 ±8.219219	42 ±21.22891
AST(U/L)	95.33333 ±3.399346	121.3333 ±22.17105	92.66667 ±6.599663	121.3333 ±22.88134

Table 1: AST and ALT values measured from the amodiaquine group, GDNF, and combined therapy group. Normal range: ALT 25-60 U/L; AST: 50-100 U/L; The bolded (AQ and combined therapy) means elevated hence abnormal values. n=4/group, data are expressed as mean ± SD. AST (aspartate aminotransferase), ALT (alanine aminotransferase).

Figures

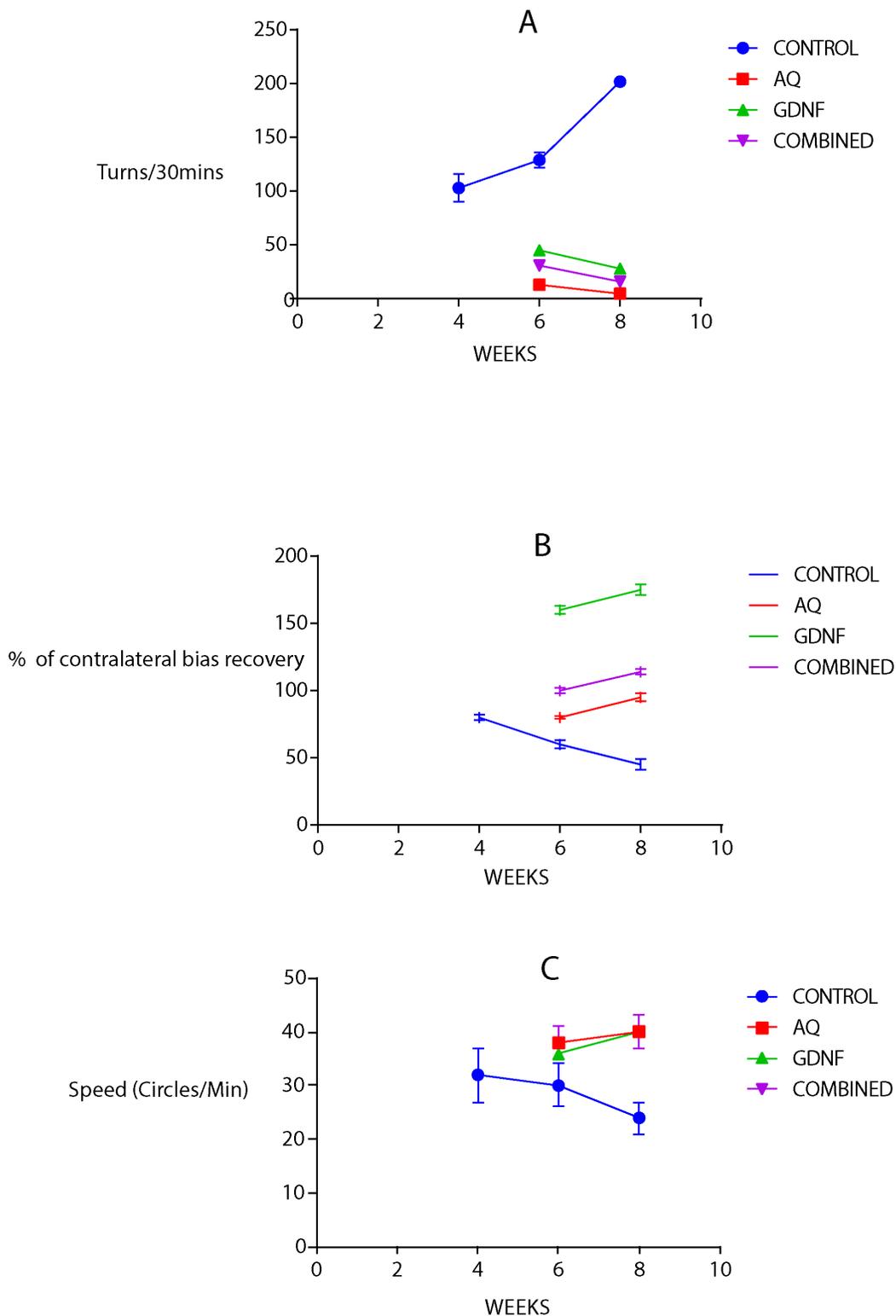


Figure 1

Treatment response of amodiaquine, GDNF or combined therapy at 6th and 8th week. Amelioration of behavioral deficits after Amodiaquine, GDNF, and combined therapy treatment in 6-OHDA mice model, n=5 in each group. (A) Apomorphine challenge: Animals developed PD on the 4th week and divided into four groups and then treated with either amodiaquine or GDNF or combined therapy, the results are the mean number of turns/30mins expressed as mean±S.D. (B) % of contralateral bias recovery measured on

the 4th week (Before forming groups), 6th and 8th week. The results are mean percentage expressed as mean±S.D. (C) Rotarod test: The maximum circles were 40c/5 mins, results are represented as mean circles ± S.D.

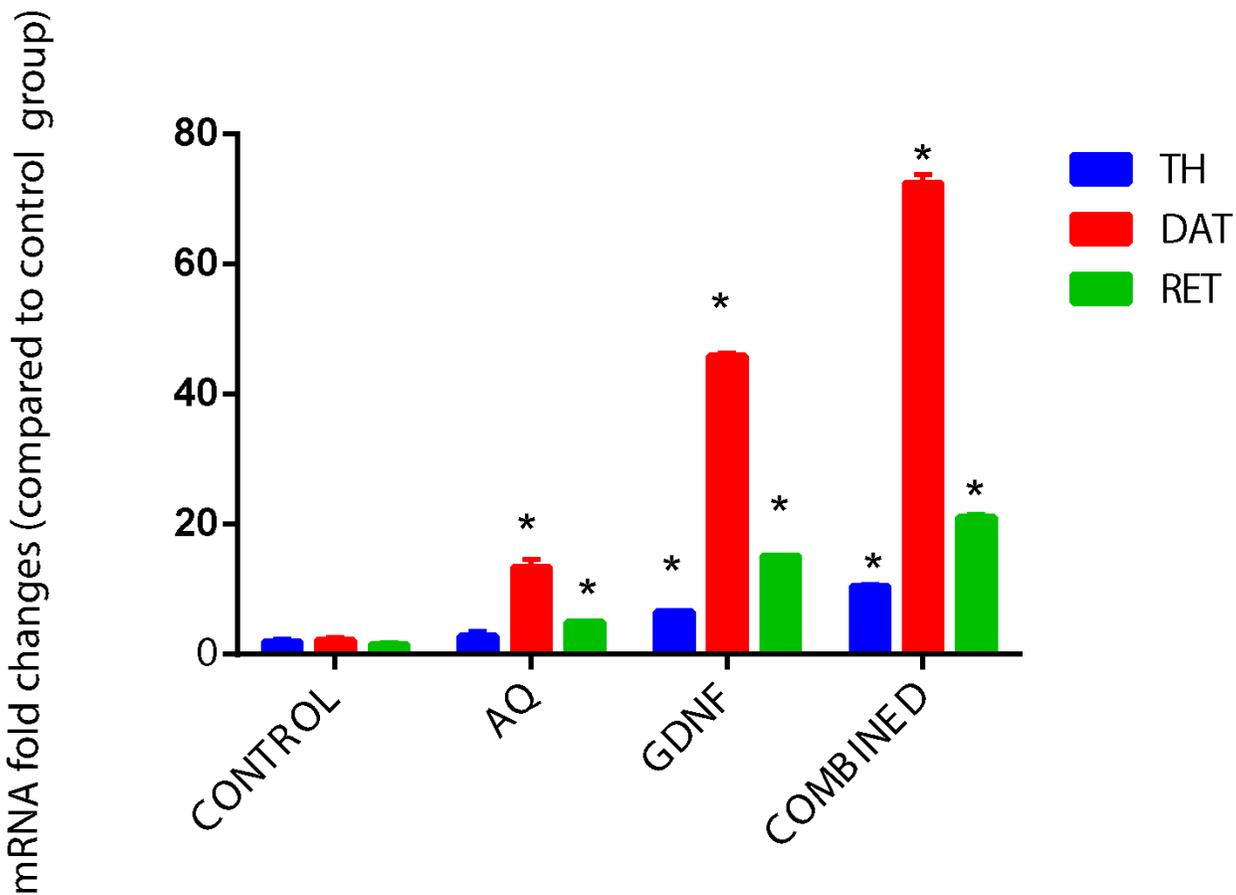


Figure 2

Effect of combined therapy on TH, DAT, and RET mRNA. In the 8th week, substantia nigra of left ventral midbrain was dissected out, and cDNA prepared from total RNA followed by real-time PCR. DAT (red

color) seems to have expressed more especially in the combined therapy group. The fold change in all experimental groups was significant ($*p < 0.001$ vs. control group, one way ANOVA followed by Bonferroni post hoc test). Data are expressed as mean \pm SD, $n=4$ /group. TH-Tyrosine hydroxylase, DAT-Dopamine transporter, RET- Receptor kinases-ret.

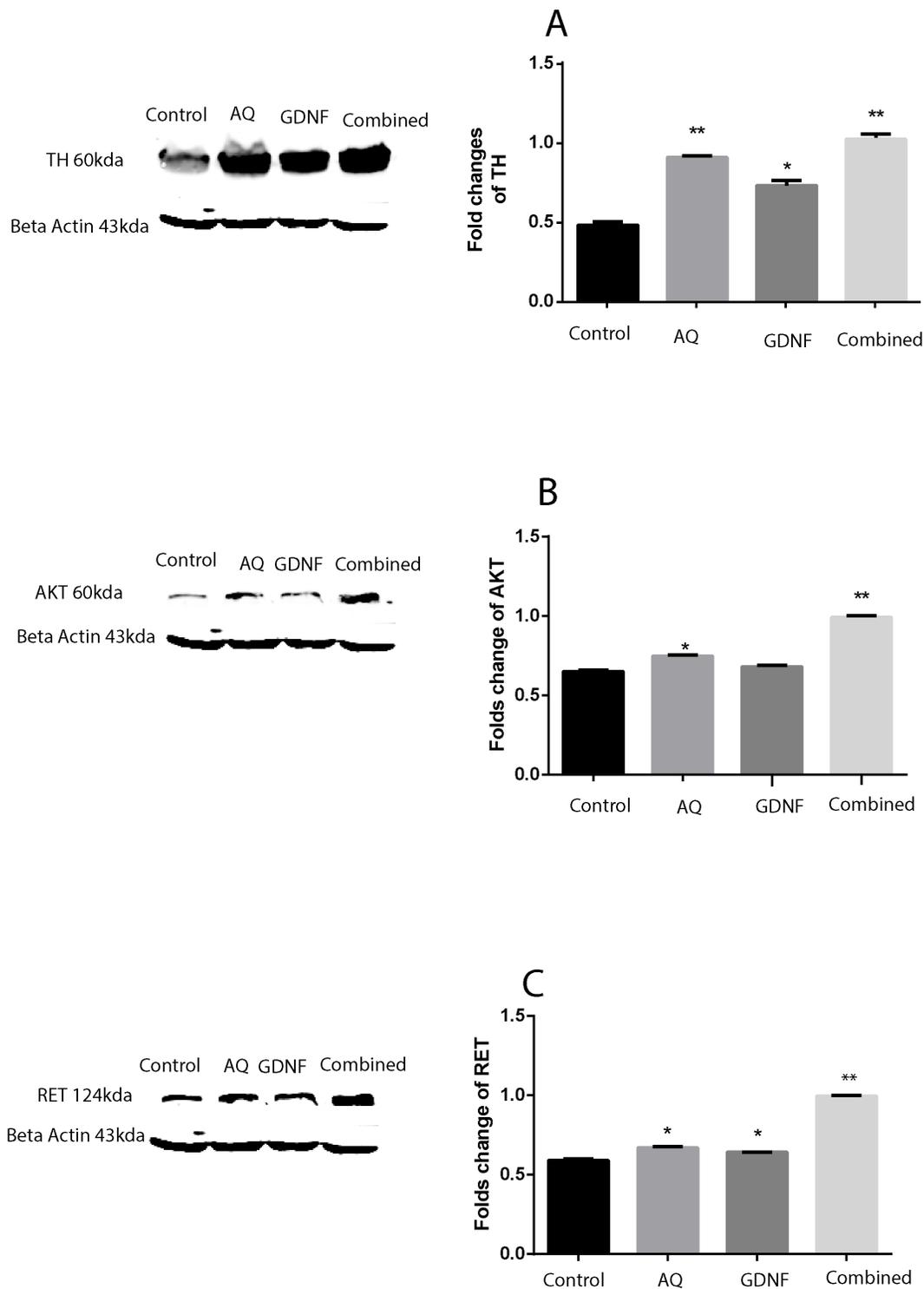


Figure 3

Quantification of TH, AKT, and RET immune-content in the lesioned substantia nigra of 6-OHDA in comparison to control, AQ, GDNF, and combined therapy treated group. (A) TH expression upon treatment. (B) AKT levels in the control group, AQ, GDNF, and combined therapy. (C) RET expression in each group: * $p < 0.05$, ** $p < 0.005$ versus corresponding control, Data are expressed as mean \pm SD, $n = 4$ /group.

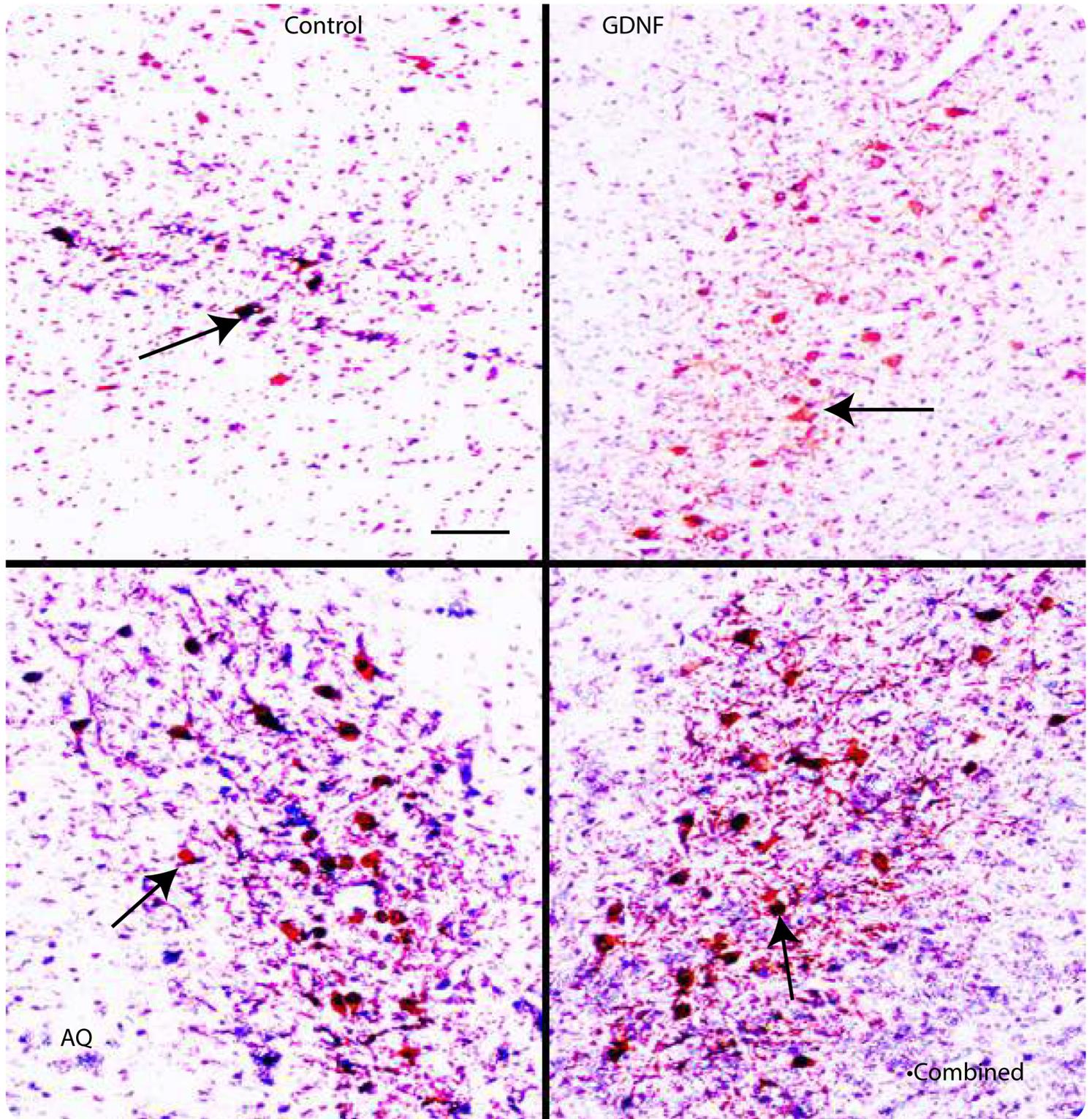


Figure 4

Immunohistochemical analysis of 6-OHDA-lesioned mouse treated with AQ, GDNF, and combined therapy. The marked depletion of TH positive neurons was observed in the control group; in contrast, plentiful positive neurons noted in both AQ and combined treatment. Scale bar 200 μ m and arrow indicate nucleus of the dopamine neuron.

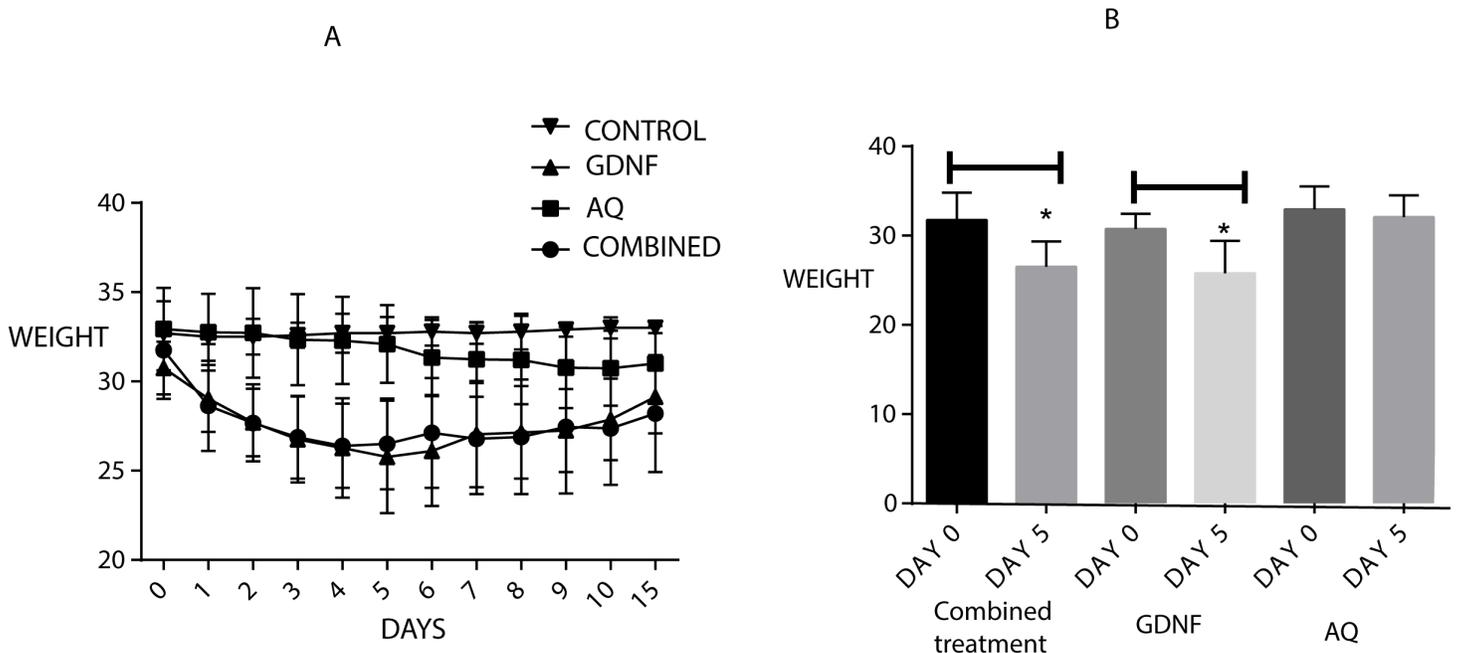


Figure 5

The primers we used: GAPDH; forward 5'- AGG TCG GTG TGA ACG GAT TTG -3', reverse 5'- TGT AGA CCA TGT AGT TGA GGT CA -3', RET forward 5'- ACAAGCACACTACTCTCAGG -3', reverse 5'- CATTGACCAGGACTACTAGC-3' TH; forward 5'-AGC CCC CAC CTG GAGTAT TTT G-3', reverse 5'-AGC AAT CTC TTC CGC TGT GTA TTC-3', DAT; forward 5'-GCT GGC ACA TCT ATC CTC TTT GG-3', reverse 5'-CAA TGC TGA CCA CGA CCA CAT AC-3', Fig 1: Treatment response of amodiaquine, GDNF or combined therapy at 6th and 8th week. Amelioration of behavioral deficits after Amodiaquine, GDNF, and combined therapy treatment in 6-OHDA mice model, n=5 in each group. (A) Apomorphine challenge: Animals developed PD on the 4th week and divided into four groups and then treated with either amodiaquine or GDNF or combined therapy, the results are the mean number of turns/30mins expressed as mean \pm S.D. (B) % of contralateral bias recovery measured on the 4th week (Before forming groups), 6th and 8th week. The results are mean percentage expressed as mean \pm S.D. (C) Rotarod test: The maximum circles were 40c/5 mins, results are represented as mean circles \pm S.D. Fig 2: Effect of combined therapy on TH, DAT, and RET mRNA. In the 8th week, substantia nigra of left ventral midbrain was dissected out, and cDNA prepared from total RNA followed by real-time PCR. DAT (red color) seems to have expressed more especially in the combined therapy group. The fold change in all experimental groups was significant (*p<0.001 vs. control group, one way ANOVA followed by Bonferroni post hoc test). Data are expressed as mean \pm SD, n=4/group. TH-Tyrosine hydroxylase, DAT-Dopamine transporter, RET- Receptor kinases-ret.

Fig 3: Quantification of TH, AKT, and RET immune-content in the lesioned substantia nigra of 6-OHDA in comparison to control, AQ, GDNF, and combined therapy treated group. (A) TH expression upon treatment. (B) AKT levels in the control group, AQ, GDNF, and combined therapy. (C) RET expression in each group: * $p < 0.05$, ** $p < 0.005$ versus corresponding control, Data are expressed as mean \pm SD, $n = 4$ /group. Fig 4: Immunohistochemical analysis of 6-OHDA-lesioned mouse treated with AQ, GDNF, and combined therapy. The marked depletion of TH positive neurons was observed in the control group; in contrast, plentiful positive neurons noted in both AQ and combined treatment. Scale bar 200 μ m and arrow indicate nucleus of the dopamine neuron. Fig 5: Weight change trend from day 0 (A day before treatment), day one after treatment to day 15. (A) For GDNF and combined therapy, weight decreased dramatically, unlike a slight change of weight change noticed in AQ and control group. (B) Statistical differences were quantified, day 0 against day 5 in combined, GDNF, and AQ treated group. * $p < 0.05$ Unpaired t-test, Data are expressed as mean \pm SD, $n = 6$ /group.