Title of Manuscript:

***Liposomal-formulated curcumin[Lipocurc™] targeting HDAC (Histone Deacetylase) , prevents apoptosis and improves motor deficits in Park 7 (DJ-1)- Knockout rat model of Parkinson's disease : implications for Epigenetics-based Nanotechnology-driven Drug Platform**

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**Contributed equally towards the study

Abstract

Background: Converging evidence suggests dysregulation of epigenetics in terms of histone-mediated acetylation/deacetylation imbalance in Parkinson's disease. Targeting HDAC (histone deacetylase) in neuronal survival and neuroprotection may be beneficial in the treatment and prevention of neurodegenerative disorders. Few pharmacological studies use the transgenic model of Parkinson disease to characterize the neuroprotection actions of a lead compound known to target HDAC in the brain.

Objective: In our study, we investigated neuroprotective effects of liposomal-formulated curcumin: Lipocurc™ targeting HDAC inhibitor in the DJ-1(Park 7)-gene knockout rat model of Parkinson's disease. Group I (DJ-1 KO- Lipocurc™) received Lipocurc™ 20mg/kg iv 3 x weekly for 8 weeks; Group II: DJ-1 KO controls (DJ-1 KO-PBS) received iv Phosphate-Buffer-Saline (PBS). Group III: DJ-1-Wild Type (DJ-1WT-PBS) received PBS. We monitored various components of motor behavior: rotarod, dyskinesia, open-field behaviors, both at baseline and at regular intervals. Towards the end of the 8 weeks, we and measured neuronal apoptosis and dopamine (DA) neuron-specific tyrosine hydroxylase levels by immunohistochemistry methods at post-mortem.

Results : We found that DJ-KO group I and Group II , as compared with DJ-1 WT group exhibited moderate degree of motor impairment on the Rotarod test. Lipocurc™ treatment improved the motor behavior motor impairment to a greater extent than the PBS treatment. There was marked apoptosis in the DJ-1 WT group. Lipocurc™ significantly blocked neuronal apoptosis: the apoptotic index of DJ-1-KO-Lipocurc group was markedly reduced compared with the DJ-KO-PBS group (3.3 vs. 25.0, $p < 0.001$). We found preliminary evidence Lipocurc™ stimulated DA neurons in the substantia nigra. The ratio of immature to mature DA neurons in substantia nigra was statistically higher in the DJ-1-KO-Lipocurc™ group ($p < 0.025$).

Conclusion: We demonstrated for the first time Lipocurc™ 's anti-apoptotic and neurotrophic effects in the DJ-1- KO rat model of Parkinson's disease. Our promising findings warrant randomized controlled trial of Lipocurc™ in translating the novel nanotechnology-based epigenetics-driven drug discovery platform towards efficacious therapeutics in Parkinson's disease.

Key words: Curcumin , Liposome , Epigenetics , Parkinson's Disease, DJ-1 Knockout

1.Introduction

Parkinson's disease (PD), with a population base-rate of 1-2 % ranks as the second commonest neurodegenerative disorder in North America and Europe (1) In PD, the canonical motor symptoms of bradykinesia, resting tremor, muscular rigidity and gait imbalance, reflect progressive loss of dopamine (DA) neurons in zona compacta of substantia nigra (SNpc). There is converging evidence in support that epigenetics regulating gene expression of signal pathways in the brain plays important role in PD (**2**). Targeting epigenetics at various levels of regulation: DNA methylation, histone modification, histone deacetylase (HDAC), histone acetyltransferase (HAT)] and non-coding RNA (miRNA or miR) can open promising vistas in discovery of novel PD therapeutics. Signature epigenetic modifiers regulate neuronal development and apoptosis, fine tune synaptic plasticity, and coordinate multiple gene expressions of signal pathways (**3).** In PD post-mortem brains, miR-134b was deficient in the midbrain region (4**)**. Intriguing enough, the altered expression of DJ-1 and Parkin proteins in PD brains were related to down-regulation of miR-34b/c, even at the pre-motor stages of PD (**5)**. Altered expression of miR-1, miR-16-2, miR-22; miR-26a, miR29 and miR30 in lymphocytes has been reported in PD patients (**6)**.

Fusing epigenetics targets and nanotechnology to create epigenetics-nanotechnology drug platform is a relatively novel construct and has not been systematically explored in pharmaceutical development to modify the course of PD. Phospholipids-based liposomes have been shown to be non-toxic, confer additional advantage of enhanced localization of the drug at specific brain areas, and may synergize with the entrapped compound in exerting concerted neuroprotection, neuro-rescue and neurotrophic actions (**7**). These considerations raise the applicability and efficacy of nanoparticle-based drug delivery towards the development of CNS therapeutics.

We are not aware of any known drug discovery platforms to translate successfully molecular footprints within the epigenetics complex machinery for the treatment and prevention of PD. Curcuminoids, extracted from the curry plant Curcuma longa , exert multiple neurotropic actions in the brain. Curcumin, the bioactive component of turmeric, is commonly used as a spice in India and as a nutritional supplement, was isolated from the rhizomes of Curcuma Longa (*Zingiberaceae*) is identified chemically to be [(1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. There is a body of evidence showing that curcumin interacts with anti-inflammatory, anti-oxidant and epigenetic signal pathways in reducing alpha-synclein(SYN) toxicity (**8**). Orally administered curcumin exhibits poor systemic bioavailability and rapid first-pass metabolism, hence making it difficult to provide sustained therapeutic benefits (**9**). In the past decade, there has been phenomenal interest in developing targeted brain delivery of drug candidates for neurodegenerative disorders. Multifaceted nanotechnology-based delivery systems : lipid nanoparticles, micelles, nano-gels, nano-emulsions, polymeric nanoparticles, dendrimer/dimers, are being tested for safety and enhanced bioavailability for developing CNS therapeutics (**10**). To the best of our knowledge, none of the patented nanotechnology-based delivery systems for curcumin have been tested in genetic models of Parkinson's disease. We have previously shown that liposome-based curcumin formulation, LipocurcTM developed by SignPath Pharma. Inc. PA USA, when administered through the intravenous route, passed through the blood brain barrier and localized in the hippocampus, striatum and cerebral cortex in the rat (**11)**. In a very recent pharmacokinetic study of Lipocurc™ in the Beagle dogs, at post-2-hour and 8-hour infusion of 10 mg/kg iv, curcumin and its major metabolite, tetrahydrocurcumin (THC), distributed differentially among different peripheral organs : the lungs and the liver, and the brain region (**12**). The tissue partition coefficients for curcumin and THC were higher for the 8-hour infusion than the 2-hour infusion, with the highest uptake of curcumin in the lungs , followed by the liver. The data indicated that the distribution of curcumin into the various tissues followed the transport dependent mechanism and regulated by the enzyme activity reductase. The overall pattern of brain uptake at 2-hr and 8-hr infusion was similar to that found in bolus intravenous administration of Lipocurc™ in the rodent species.

In the present study we chose the transgenic DJ-1 gene knockout(DJ-1 KO) rat model of Parkinson's disease to evaluate the action of the patented liposomal curcumin formulation: Lipocurc™. The autosomal recessive genetic form of PD arising from point mutation of DJ-1 gene has drawn emerging interest: mutation of DJ-1 gene at Park7 gene locus at chromosome 1P36 leads to familial early-onset PD consisting of the classical motor symptom complex **(13)**. The full HGNC [HUGO (Human Genome Organization) Gene Nomenclature Committee] assigns ID number: 16369 to Park7 gene and approved Park7 Gene designation while acknowledging DJ1 and DJ-1 as synonyms of Park 7 gene loci. (**14**) The affected individuals exhibit atypical symptoms including sleep disturbances, morning-foot -dystopia, as well as psychiatric symptoms of psychotic episodes and anxiety. L-DOPA therapy in the cohort of PD is associated with dyskinesia. The detailed mechanisms underlying the link of impaired oxidative stress and the onset and progression of PD remain ill-defined.

The DJ-1 protein functions as the master sensor of oxidative stress regulating mitochondrial function and coordinates transcription and gene expression, and modulates diverse signal transduction pathways (**15**). DJ-1 protects and rescues DA neurons by upregulating intracellular glutathione, as well as inhibiting alphaSYN aggregates (**16)**. In an embryonic stem-cell derived DA neuronal model, DA neurons deficient in DJ-1 exhibit increased sensitivity to oxidative stress and proteasomal inhibition (**17)**. Reduced levels of endogenous DJ-1 in DA neurons are associated with increased sensitivity to neurotoxin insults (**18)**. There is a moderate degree of variability in the DJ-1 KO mice. Subtle motor deficits occurred with varying loss of dopamine neurons (DA) in the SN (**19)**. We note that there is a paucity of data whether transgenic models of PD respond differently towards drugs in terms of reducing the severity and frequency of Parkinsonian symptoms and dyskinesia**.**

The objective of the present study is to examine whether the patented liposomal curcumin: Lipocurc™ , exerts neuroprotection through improving motor impairment and reducing apoptosis: programmed cell death in the DJ-1 KO rat model of PD. Our results demonstrated for the first time that sub-chronic treatment with Lipocurc™ is efficacious in the transgenic PD model.

2. MATERIALS AND METHODS

2.1 Animals

DJ-1 KO rats were produced by the highly targeted genomic editing bioengineering technology: ZFN [(Zinc Finger Nuclease).Sigma Aldrich Corp. USA (SAGE Lab.) MO USA and purchased through special collaborative research arrangements between SignPath Pharma., USA, and Michael J Fox Foundation NY, USA. For DJ-1 KO control, we ordered Long Evans-hooded rats from Sigma Aldrich Corp. as DJ-1 Wild Type (DJ-1 WT0 control. The animals were kept under standard laboratory conditions at 22¡C, maintained on 12 hours light/dark cycle, and were allowed water and standard rat chow ad libitum. The rats were housed two per cage with enriched milieu and were acclimatized for 1 week prior to start of the study. All procedures were carried out and approved in accordance with the guidelines and regulations set by Animal Use Subcommittee (AUS) of UCAC University of Western Ontario London Ont. Canada.

2.2Drug Preparation and Treatment:

We obtained Lipocurc ™ directly from SignPath Pharma. Inc. PA USA. Curcumin was synthesized to 99.2% purity by Sami Labs, Sabinsa Corporation, Bangalore, India. Liposomal curcumin : the patented formulation (Lipocurc ™) was synthesized at Polymun Scientific GmbH, Vienna Austria in accordance with the encapsulation protocol developed by Li et al (20,21). Curcumin was conjugated with the lipid: 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine/1, 2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) : DGP. The method for optimizing encapsulation of curcumin by liposomes consisted of testing different ratios of lipid compositions to curcumin (w/w) ranging from 10:1 to 4:1 in vitro. The ratio of 10:1 was the optimal encapsulation ratio for inducing growth inhibition and apoptosis in two human colorectal cancer cell lines (**21**). In the vivo xenograft mouse model of human pancreatic cancer, lipocurc™ at the intravenous dosage schedule of 20 mg/kg three times weekly for 28 days, produced the maximal response in reducing tumor growth by 52 % when compared with non-treated control mice (20). The procedure for preparing Liposomal curcumin: Lipocurc ™ was as follows: curcumin and DGP were dissolved in *tert*-butanol and filtered through a 0.22-μm pore size filter for sterilization. The vials containing DGP and curcumin (10:1 ratio) solution were frozen in a dry ice-acetone bath and lyophilized for 24 h to remove the *tert*-butanol. The vials were stored at −20¡C and warmed up to room temperature immediately prior to use. Lipocurc ™ was stored in dark ampoules and prepared prior to each drug treatment session in 0.9% saline solution. DJ-1 KO and DJ-1 WT (Long Evans hooded rats) received equal volumes of of phosphate buffered saline (PBS). All the chemicals and laboratory reagents including the phosphate buffer in the study purchased were of the finest reagent grade.

2.3Study Protocol: DJ-1 KO rats were randomly assigned to following groups: 1)Group I DJ-1 KO Lipocurc™ (n =4) received 20mg/kg Lipocurc™ iv ; 2)Group II (n = 4) DJ-1 KO PBS iv . For control, the DJ-1 WT (Wild type) [Long Evans rat) group (n =4) received PBS IV. All animals were injected through the caudal tail vein three times daily for eight weeks. Injection volume was 1cc/kg for each rat, and this measure was readjusted on each day of injection for weight change. On the third day prior to injections, each rat was tested on the catalepsy test, followed by Rotarod test (**22**) to examine the motor integrity of the rats. Each rat completed two trials per session for the duration of the eight weeks. If the rat remained on the rod after 60 seconds, the speed was increased in a linear fashion (rpm1=14, rpm2=20, rpm3=25). During the last week of injections, all rats were assessed using a Sony camcorder on a number of behavioral tests [(open-field test (**23**), cylinder test (**22**) and the AIMS (Abnormal Involuntary Movement Scale) (22, **24)**] which allowed for frame-by-frame analysis. Throughout the study, we monitored closely the general health of the rats. No marked changes were found in the feeding behavior, grooming and reactivity towards handling. At the end of the study, the rats acquired weight gain of approximately 150 gm from the baseline weight of 250 gm. The rats weighed in at 250g and 400g at the end of the study. Seventy-two hours after the last injection, the rats were euthanized using sodium phenobarbital. Brains were extracted and post-fixed for a total of 24 to 48 hours. Following extraction brains were cytoprotected using 30% sucrose and 4% paraformaldehyde at 4¡C to allow for coronal sectioning. The tissue sections were prepared for immune staining with TUNEL assays and Tyrosine hydroxylase (TH) labeling assays of DA neurons **(25).** A Zeiss Axioskop microscope and Q Imaging Micropublisher 3.3 RTV camera were used, transferring the data to a computer for taking photographs of the relevant images under 60 x magnifications. In both TUNEL and TH assays, two independent investigators rated the slides and manually counted the number of apoptotic or TH (+) cells. The data were entered into the Statistical Package for the Social Sciences (SPSS) for statistical analysis.

2.3 Neuro-behavioral Analysis

2.3.a.Catalepsy (26) The catalepsy test (“passive immobility”) consisted of a horizontal metal rod suspended 1-3 inches off the ground by two vertical steel rods assuming an iron stand configuration. The animal was typically placed with its forepaws on the bar and the time was recorded as the duration the animal remained on the bar with both paws without falling off or

initiating movement. We considered the catalepsy test to be positive only if the animal rested on the horizontal bar with both paws extended for over 15 seconds. It was assumed that a normal animal would change its position within seconds, but a cataleptic animal would remain in the abnormal posture and position associated with varying degrees of muscle rigidity for a prolonged period of time.

2.3.b Rotarod test (22): The rotarod was purchased from Bluefine Inc., India, and conformed to North American electrical requirements. It is a motorized rotating cylindrical apparatus measuring 7cm diameter that was accelerated in a stepwise 60-second fashion to challenge the rats to remain mobile until the animal fell off the drum, or swirled around without making an attempt to walk. The total time for the animal to remain on the rod was electronically recorded and the stop response was monitored by pressure activation underneath the cylinder. Severity of motoric impairments was derived from magnitude of rotarod scores.

2.3.c Cylinder Test (22): The cylinder measured 20 cm in diameter, and 30 cm in height was constructed to prevent escape. The cylinder test was a behavioral estimate of akinesia. The cylinder test was conducted to assess forelimb use in 25 touches-sessions. Spontaneous use of both forepaws, as well as the use of only one forepaw was calculated on the basis of 25 touches during one minute observations of recorded exploratory behavior measures.

2.3. d Open Field test (23) the open field test assessed the locomotor activity over 5 minutes to examine changes in exploratory behavior and initiation of movements. The open field test is typically in the form of an enclosed platform (101cm in diameter by 101cm width by 35cm height) divided into 16 equal squares. The animals were placed in the center of the field and allowed to roam freely for five minutes. Locomotor activity was measured with two methods: the number of squares covered in the allotted time, as well as the number of outside versus inside the designated square. The greater the motoric impairments, the less time the rat would spend exploring the area.

2.3.e Abnormal Involuntary Movement Scales (AIMS) (22,24) The AIMS (Abnormal Involuntary Movement Scale) test was used to rule out any treatment induced dyskinesia that may have arisen and may have resulted in abnormal movements. Each rat was rated on a scale of 0 (absent) to 4 (most severe) on each of the four subscales of abnormal movements (limb dyskinesia, axial dystonia, oral dyskinesia, and contraversive rotation). Limb Dyskinesia was assessed on the basis of 1 being 1-2 bouts of abnormal movement, 2 indicated 3 or more bouts of abnormal movement and a score of 3-4 indicated persistent abnormal movements. Axial dystonia was assessed on the basis of 1 being contralateral bias in head orientation, 2 indicated contralateral biases in uppermost parts of the body; 3-4 indicated severe contralateral bias in upper extremities. Oral dyskinesia was assessed on the basis of 1 being 2-3 bouts of chewing movements, 2 indicated more than 3 bouts of chewing, and 3-4 indicated continuous chewing or the presence of prolonged tongue protrusions. Contraversive rotation is specific to L-DOPA induced dyskinesia in which 1 means 2-3 contraversive turns, 2 indicated more than 3 contraversive turns and 3-4 indicated continuous contraversive turns.

2.4. Immunohistochemistry

2.4. a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) Staining for apoptosis.

Immunohistochemistry assay kit was bought from ImmnoStar Inc., WI, and USA. TUNEL staining is a method for detecting fragmentations of DNA resulting from apoptosis within the cells. Slices were incubated with 0.2% Triton X-100 in PBS at room temperature. Sections were then pretreated by proteinase K digestion. Sections were then incubated using DAB for 1-2 minutes in order to intensify the staining process. Counterstain was done using Gill's hematoxylin. Sections were then dehydrated. Cells within the striatum and hippocampus that had undergone apoptosis were identified using a Zeiss Axioskop microscope and Q Imaging Micropublisher 3.3 RTV camera which transfers the data to a computer which will photograph the images under 60 x magnifications. Apoptotic neurons were counted manually by two independent investigators to account for potential bias, and subsequently the data were entered into the Statistical Package for the Social Sciences (SPSS) for data analysis. The criteria for apoptotic cell identification were as follows: nuclear fragmentation, nuclear condensation, membrane blebbing and coloration change (**23**).

2.4.b Tyrosine Hydroxylase (TH) Staining for Dopamine neurons The TH antibody kit was also purchased from Immunostar Inc., WI, USA. This kit had wide species cross-reactivity because it recognized an epitope in the catalytic core of the TH molecule where extensive species homology existed. TH antiserum was quality control using standard immunohistochemical methods. The antiserum demonstrates significant labeling of rat catecholamine neuron systems using indirect immunofluorescent and biotin/avidin-HRP techniques. This antibody does not cross react with phenylalanine hydroxylase or tryptophan hydroxylase. Catecholaminergic neurons within the substantia nigra were identified using a Zeiss Axioskop microscope and Q Imaging Micropublisher 3.3 RTV camera which transferred the data to a computer for image processing and photography under 60 x magnifications. Neurons were counted manually by two independent investigators to

reduce potential rater bias. The data were entered into the Statistical Package for the Social Sciences (SPSS) for statistical analysis.

2.5. Statistical analysis: To compare the effects of Lipocurc treatment with saline-treatment in the DJ-1 KO and DJ-1 WT rats on the battery of neurobehavioral tests, we use the 2-way mixed ANOVA followed by Tukey test For the Cylinder test, open-field behavior score expressed as continuous variable, we compare the score at the baseline and at the end of the 8-week period. Both parametric and non-parametric tests were used. With saline-treated another 2-way mixed ANOVA. We set level of significance at $p < 0.05$ for two-tailed t- test. For the immunohistochemistry data : the ratio of immature to mature tyrosine hydroxylase-labeled DA neurons, and apoptosis index , we used chi square test for comparing Lipocurc™-treated group with PBS-treated DJ-1 KO and PBS-treated DJ- 1 WT groups. In view of the small sample size, we estimated the treatment effect using Cohen's d effect size method **(27**) which took into account the standard deviation of either of the two groups but is independent of the sample size. In drug efficacy studies, Cohen's d effect size is often used to complement the conventional ANOVA or MANOVA (time x treatment effect).

3. RESULTS

3. 1: DJ-1 KO rats showed motor impairment on the rotarod test. Lipocurc™ treatment improved rotarod performance

The results of the present study of Lipocurc™ treatment in DJ-1 KO rats , as shown in Figure 1, showed significant Session ($F(4, 36) = 9.25$, $p < .001$), Treatment ($F(2, 9) = 9.054$, $p < .01$), and interactions ($F(4, 36) = 2.52$, $p < 0.01$. ANOVA was conducted with Session-week (7 levels) as the within-subjects factor and Treatment (3 levels) as the between-subjects factor using Greenhouse Geisser correction. We observed a significant main effect of Session, $F (2, 12) = 7.80$, $p < .01$, however, there was no main effect of Treatment, $F (1, 6) = .252$, p = ns, or Treatment by Session interaction, $F (2, 12) = 0.53$, p = non-significant. Finally, there were no treatment body weight differences on the last rotarod session ($F (2, 12) = .0224$, p = not significant) compared to the baseline, indicating that treatment did not influence body weight, suggesting that Lipocurc™ did not affect metabolic processes.

For comparing the treatment effect of Lipocurc™ compared with placebo, we used the formula of Cohen d's effect size as follows: [(Change of Rotarod score of Lipocurc™ -treated DJ-1 KO group – Change of rotarod score of PBS-treated DJ-1 KO group)/ SD of the either of the two group]. We found that Cohen's d effect size (27) analysis favors drug effect since the effect size is greater than 0 and is determined to be 0.48 rated as moderate according to the formula of Cohen's effect size.

3.2. Cylinder and Open Field Tests Lipocurc™-treated DJ-1 KO rats showed forepaw preference similar to PBS-treatedDJ-1 WT group

A series of one-way ANOVA were conducted comparing treatment on various dependent measures. Data for one animal in the Lipocurc™ -treated DJ-1 group was excluded due to malfunction of recording equipment. The results of the cylinder test, as shown in Figure 2, found that there were no significant effects for either one paw or two paws, $F(2, 8) = 1.437$, p = not significant . Although there was no significant difference between these groups, it seems that with respect to means, the Lipocurc™ –treated DJ-1 KO group and DJ-1 WT controls preferred to use 1 paw (front paw) more often than PBS-treated DJ-1 KO group. Similar results were obtained using the non-parametric test of Wilcoxon rank sum test. Figure 3 represents the means and standard error of the mean for the open field test. The data indicated that there were no significant effects between the treatment groups whether observing the number of inside squares, $F (2, 9) = .602$, p = ns, or outside squares, $F (2, 9) = .087$, p = ns. These results suggest there were no differences in exploratory behaviors between the 3 groups

3.3. No dyskinesia or catalepsy was observed in Lipocurc™ -treated DJ1 KO; PBS- treated DJ1 KO and DJ-1 WT groups

There were no differences between groups. In fact there were no observations of dyskinesia measured with AIMS test in any of the groups: AIMS scores were zero among all the groups. Since no variation was found, statistical analysis was considered to be not necessary.

3.4 Lipocurc™ protected against apoptosis and activated dopamine neurons in DJ-1 KO rat

A one-way ANOVA was conducted comparing treatment groups to the number of apoptotic cells determined using two methods: 1) morphological analysis according to established criteria : membrane blebbing, chromatin condensation and nuclear fragmentation ; 2) the apoptotic index defined as the number of TUNEL (+) apoptotic cells to the total number of cells counted within a predetermined microscopic field **(25).** The immunohistochemistry data as shown in Figure 4 (A, B, C), and Table 1 showed that the PBS-treated DJ1 KO group had significantly more TUNEL(+) apoptotic cells in the striatum (Mean =62.00, SEM =2.58) compared to both the Lipocurc™ treated DJ-1 KO group (Mean = 8.25, SEM

=2.39), and the PBS-treated DJ-1 WT group (Mean = 7.75, SEM= 1.75), $F(2, 7) = 49.278$, $p < .001$, $\eta = .264$, power= 1.00 . We subsequently conducted Post-hoc analyses to determine which group means significantly differed from the other groups. Tukey's HSD test revealed that the PBS-treated DJ-1 KO group significantly differed from both the Lipocurc™ -treated DJ-1 KO and PBS-treated DJ-1 KO, $q(3, 62) = 16.25$, $p < .001$, and DJ-1 Lipocurc™- treated group, $q(3, 62) = 17.78$, $p < .001$. The data suggested that unprotected PBS-treated DJ-1 KO group exhibited extensive apoptosis as compared with the DJ-1 WT group: the apoptosis was to be attributed to down-regulation of the DJ-1 gene. On the other hand, Lipocurc™ treatment significantly protected the DJ-1 KO group from apoptosis in the striatum, hence preserving the integrity of the striatum. The frequency of apoptotic cells in the Lipocurc treated DJ-1 KO group was almost indistinguishable from the DJ-1 WT group treated with PBS. By contrast, we did not detect any change in the frequency of apoptosis in the hippocampus among the three groups: PBS-treated DJ-1 WT group, LipocurcTM- treated DJ-1 KO group and PBS-treated DJ-1 KO group (Figure 5).
[2]

As shown in Table 1, the apoptotic index in the substantia- nigra- pars-compacta (SNpc) of PBS-treated DJ-1KO group: 24.8, was statistically higher than the Lipocurc™ - treated DJ-1 KO group 3.3 ($p < 0.001$). In addition, the DJ-1 KO rats can be differentiated from DJ-1WT in terms of the presence of extensive apoptosis in the striatum as assessed by morphological observations in the KO group but not in the WT group (Figure 4). The data provided indirect evidence that Lipocurc™ rescued the substantia nigra pathway from apoptosis in the DJ-1 KO rats as compared in the PBS-treated DJ-1 KO group, since Lipocurc™ substantially antagonized the apoptosis in the nigra-striatal pathway in the DJ-1 KO rat . The apoptotic index from the Lipocurc™ treated DJ-1 KO rats did not differ significantly from the PBS-treated DJ-1 WT group $q(3,62) = .85$, $p > .05$. Levene's test of homogeneity of variance was non-significant among the three groups ($p = 0.74$).

In the DJ-1 KO rat model, we do not find substantial loss of Tyrosine Hydroxylase [TH (+) DA neurons] as the DJ-1 KO group rats as compared with DJ-1 wild type group (Figure 4 D, E, F). We carried out a log transformation of the total number of TH (+) DA neurons for the three groups to partially normalize the skewed distribution of the cell count (Table 2). A one-way ANOVA showed that no significant differences occurred with respect to log-transformed total number of TH(+) DA neurons among three groups: DJ-1KO PBS treated; DJ-1WT PBS-treated and DJ-1 KO Lipocurc™ treated groups: F :3.26, df : 2, $p = 0.11$. The marginal DA neuron loss: 14.8% in the DJ-1 KO rats was relatively unaffected by Lipocurc™.

While Lipocurc™ did not appear to change the total number of TH (+) DA neurons in DJ-1KO rats, we have preliminary morphological evidence that Lipocurc™ is capable of shifting the balance of mature and immature DA neurons. We carried out a more detailed morphological quantification of the newly identified "immature DA neurons", based upon the mitotic figures and size and shape of the nuclei. We derived a ratio to describe the shift in the pool of mature cells to immature cells. The Lipocurc™- treated DJ-1 KO rats, showed higher ratio of immature (TH+) cells to mature (TH+) cells (Table 2). One-way ANOVA was carried out to identify any difference among the three groups. Our results indicated that the three groups behaved differed from each other with respect to the DA neuron maturity index defined as the ratio of immature DA neurons to mature DA neurons. [Standard weighted means analysis (F ratio: 10.28; df: 0.64; $p < 0.05$)] and the difference was statistically significant. In the Lipocurc™-treated group the ratio was: 0.578, whereas in the PBS-treated DJ-1 KO group the ratio was 0.095 and in the DJ1 WT group the ratio was 0.086. Tukey HSD test confirmed that Lipocurc™ increased the ratio of immature to mature DA neurons in the striatum at a statistically significant level ($P < 0.025$).

4 Discussion:

Our study demonstrated for the first times that the neuroprotective efficacy of the liposomal formulated epigenetic modulator, curcumin, in DJ-1 KO model of PD. Our finding of Lipocurc™ in DJ-1 KO rats agrees with previous studies of curcumin in the preclinical models of Parkinson disease: 6-OHDA (6-hydroxydopamine) model (28, 29) and the homocysteine model (30). In the recent 6-OHDA study in hemiparkinsonian mice, 7-day administration of curcumin at the dosage of 200 mg/kg i.p., inhibited the glial cell response to inflammation and restored the loss of DA neurons (29). Curcumin's anti-inflammatory effect in the 6-OHDA lesioned mice was accompanied by maintenance of the antioxidant enzyme, Cu/Zn superoxide dismutase expression in the striatum (29). Lipocurc™ exerted anti-Parkinsonian effect at a dosage which did not induce any dyskinesia or aberrant behavioral changes, as shown by the lack of changes in open-field behavior and AIMS score throughout the treatment period. We further showed that the DJ-1 KO rats displayed the core phenotype of PD: early neurobehavioral deficits and extensive programmed cell death (apoptosis) in the nigro-striatal region. Surprisingly enough, the hippocampus mediating the non-motor symptoms of PD: cognition impairment, affective changes and behavioral control, was spared of apoptosis. The 4-8 month old DJ-1 KO most likely represents early stage of PD, since no spontaneous catalepsy was noted, along with lack of substantial loss of DA neurons. We further showed that Lipocurc™ exerted its neuroprotective effects through inhibiting apoptosis and attenuating motor impairment.

Our finding of the anti-apoptotic effect of Lipocurc™ in DJ-1 KO rat model extends an earlier neuroprotection study of encapsulating curcumin by the nanoparticle complex : the patented Nanocurc™ formulation (**31**). In the athymic mice, Nanocurc™ at the dosage of 25 mg/kg. ,inhibited neuronal apoptosis via caspase 3 and 7. The anti-apoptotic property of Nanocurc™ appears to be correlated with its antioxidant activities of reducing hydrogen peroxide and the ratio of free to oxidized glutathione. Curcumin prevents auto-oxidation of DJ-1 protein most likely through synchronizing the intracellular redox network comprising superoxide dismutase, glutathione, superoxide oxygen (SOA), inducible nitric oxide (iNO) (**32,33).** The auto-oxidation of DJ-1 protein occurs more frequently in aging recognized as an independent risk factor for PD (1**)**.

These considerations raise the question whether epigenetics mediate the complex links of oxidative stress and DJ-1 in neurodegeneration. Cur cumin has been identified as a potent modifier of epigenetics signal pathways at multiple sites: HDAC (Histone Deacetylase) [class 1, 3, 8], HAT (histone acetyltransferase), and non-coding RNA (abbreviated miRNA or miR) miRNA-22, miRNA-186a and miRNA-199a (8). In high throughput epigenetic screening assay using HeLa nuclear extract, curcumin was found to be more potent in inhibiting HDAC than valproic acid and sodium butyrate (34**)**. The inhibition constant Ki of cur cumin (539 NM) was comparable to Ki of Trichostatin A (504 nM) but more potent than valproic acid (Ki 564 μ M) and sodium butyrate (Ki 365 μ M). In our study, we did not conduct a parallel series of experiments to determine whether liposomal curcumin is more potent than the free curcumin in competing for HDAC binding in in vitro assay. Liposomal curcumin most likely releases the free curcumin at the various brain regions, once the compound passes through the blood-brain barrier. With advances in small animal positron emission tomography (PET) and the availability of radio-labeled curcumin, it may be possible to conjugate the curcumin with lipid and follow the pharmacokinetic fate of liposomal curcumin from the injection site via blood brain barrier to be localized in selective brain regions with real time PET micro-imaging technology.

In DJ-1 KO mice, DJ-1 expression is upregulated by the prototypal HDAC inhibitor, sodium phenylbutyrate (**35)**. Our finding of extensive apoptosis in DJ-1 KO rat model may be explained in terms of the hypoacetylation hypothesis of neurodegeneration (36**)**. The model predicts that as a result of imbalance between HAT and HDAC activities, there is overall reduction in the neuronal level of histone acetylation. Impaired loss of deacetylation of histone is likely the pivotal early event triggering apoptosis. Our in vivo study result is consistent with an earlier in vitro study showing that when cortical neurons were challenged with oxidative stress load, acetylation homeostasis is shifted towards marked loss of transcription coactivator complex : cAMP -response-element-binding-protein-(CREB)-coupled-p300/histone acetylation transferase (HAT) activities (36). The epigenetic cascade has deleterious consequences at the downstream in terms of impaired synaptic plasticity . More significantly, putative HDAC inhibitors like suberoylanilide hyroxamic acid (SAHA) block oxidative stress-induced apoptosis in the mouse cell line fusing motor neuron and neuroblastoma cell line, and protect against polyglutamine toxicity (37, 38). Polyglutamine neurotoxicity parallels Huntington's chorea , whereas DJ-1 KO rat model mimics PD. Taken together, the findings suggest that imbalance in acetylation homeostasis coupled with the inflammation downstream , may be the missing clue linking oxidative stress and apoptosis in PD.

In view of the established neuroprotective role of DJ-1 in regulation of neuronal apoptosis, it is not surprising in gene knockout model, marked neuronal apoptosis occurs. Preliminary evidence in postmortem PD brains in humans showing that dysregulation of expression DJ-1 and Parkin proteins is associated with decrease of miRNA 3 b/c (14). MicroRNA -34b/c previously shown to be the key player in maintaining DA neuronal homeostasis regarding survival and cell death signals, was found to be downregulated in PD brains (14). Hence the altered acetylation-miRNA link regulating the DJ-1 gene and oxidative stress most likely mediates the cell fate towards apoptosis and neurodegeneration. In the DJ-1 KO model, Lipocurc™ can replace the loss of the mitochondrial protein DJ-1 in buffering against oxidative stress and redox potential instability and becomes the de facto DJ-1 protein. DJ-1 has been found to up-regulate intracellular glutathione, as well as preventing the oligomer formation of αSYN which is soluble under normal physiological conditions (4). Curcumin, when administered through intraperitoneal route, has recently found to be efficacious in reversing the severity of abnormal oral-facial-movement (AOFM) rodent model of tardive dyskinesia as induced by the first generation antipsychotic : haloperidol (**39**). In the AOFM model , curcumin upregulated the anti-apoptotic protein, Bcl-xL in the striatum (39). It is highly likely that the lipid of Lipocurc™: DGP, synergizes with free curcumin in achieving the optimal anti-oxidant and anti-apoptotic state for neural repair. No data exists whether Lipocurc™ exerts similar action in L-DOPA dyskinesia model.

Our results unmask an intriguing paradox regarding the double-edged pharmacological effects of curcumin in modulating apoptosis. In tumor cells, curcumin favors pro-apoptosis, but inhibits apoptosis in in vitro and in vivo models of neurodegenerative disorders (40**)**. Conceivably, in tumor cells apoptosis may be functioning at the suboptimal level. On the other hand, apoptosis may be accelerating at a catastrophic level leading to ultimate cell death in neurodegenerative

disorders :PD and Alzheimer dementia (40, 41). Intriguing enough, the hippocampus in DJ-1 KO rats in our study was spared of apoptosis. In DJ-1 KO model the SNpc is highly vulnerable towards cell death most likely through enhanced expression of apoptosis-related proteins like Dopamine Transporter (DAT) (42). On the other hand, the reduced expression of BDNF (Brain-Derived -Neurotrophic -Factor) and DVTP (Dopamine Vesicle Transport Protein) may select out the SN preferentially for apoptosis (42). On the other hand, in our DJ-1 KO rat model, hippocampus was privileged in sparing the demise of apoptosis. The enriched neurogenic and vascular niche for neural repair and regeneration in the hippocampus may also protect against apoptosis in DJ-1 KO rat model (43).

Our study indicates that the ratio of immature to mature TH (+) DA neurons was higher in the Lipocurc™ DJ-1 KO group compared with PBS-treated DJ-1 KO or PBS-treated DJ-1 WT group. The findings are consistent with curcumin's stimulatory effect on neurogenesis mediated through transcriptional regulation of synaptic plasticity at the level of HDAC-HAT (**44)**. Caution must be exercised, however, to interpret our morphological finding since we do not use standard immnohistochemical markers like doublecortin for characterizing immature DA neuron. It is likely that HDAC inhibition can reprogram the cellular aging in PD through activating the adult neural stem cell, since DJ-1 is co-expressed with nestin , the neural stem cell marker and neuronal-specific glial fibrillary acidic protein (GFAP) and closely correlated with differentiation of neural stem cell (45).Lipocurc™ may mobilize the so-called neuroregenerative reserve in the DJ-KO rat model of PD. Taken together, our findings with, Lipocurc™ in inhibiting HDAC provide preliminary indirect evidence for the role of epigenetics in coordinating and synchronizing the neurogenesis program in the specific brain regions: the hippocampus and the substantia nigra **(** 45,46)

Studies in DJ-1 gene knockout in mice have yielded discrepant findings regarding the severity of DA neuron loss (47, 48, 49, 50, 51, and 52): the discrepancy probably reflects the different clinical stages of PD. Deleting DJ-1 at the 1st coding exon resulted in age-dependent behavioral deficits: gait abnormality and reduced grip strength; however, the nigrostriatal DA system remained relatively intact (48, 49). Adenovirus vector–driven over-expression of α SYN, on the other hand, damaged both neurons and glia and created a phenotype resembling advanced stage of PD: severe behavioral dysfunction and marked DA neuronal loss in SNpc proceeded by dystrophic axons and dendrites. (50,51). In our study, widespread marked apoptosis in DJ-1 KO rats occurred despite marginal loss of TH (+) cells in the SNpc and striatum. This is not surprising for gene knockout studies. In the 12020T-Leucine-rich repeat kinase 2 (LRRK-2) transgenic mice, DA neuronal loss was minimal but marked increase in the frequency of apoptosis with fewer neurite outgrowths (52).

Our study has methodological limitations in terms of small sample size, the single dosage schedule and the relatively short time of treatment. In addition, we did not examine the changes in the inter-related network of epigenetics targets: HDAC, HAT , methylation of DNA and microRNA and to correlate the epigenetics changes with histological data. From the technological perspective, it is difficult to run parallel epigenetic assays with immunohistochemistry on the same tissue samples from the various groups of animals. Unbiased immune-histochemistry and apoptosis cannot be conducted in predetermined brain sections, while sparing the residual brain tissues for assaying the multiple epigenetic targets: HDAC, HAT and miRNA. The experimental protocol requires conducting an identical series of studies to evaluate the postmortem changes in epigenetic networks from DJ-1 KO rats treated with Lipocurc™ compared with control in relation to the immunohistochemical findings reported in our present study.

In summary, Lipocurc™ a unique promising drug candidate for PD treatment since the formulation exerts anti-apoptotic effect while at the same time plays a role in driving neurogenesis program. The paradigm of Lipocurc™ as potential PD therapeutics exemplifies the synergistic benefits of fusing advances in nanotechnology (53) and epigenetics (54). In the DJ-1 KO model, we have indirectly provided insight to the mechanism of action of curcumin as to the anti-apoptotic action through resetting the imbalance of acetylation-deacetylation in histone regulation in the SN-striatum. The antioxidant effects of curcumin synergize the HDAC inhibitor action. We hypothesize that once the neuroprotective phenotype of DJ-1 is lost; neurons in SN are highly vulnerable towards oxidative stress and apoptosis. In this respect aberration in acetylation homeostasis plays an important role. Sensitizing the apoptosis cascade is an essential component in triggering the onset of and accelerating the course of PD. Pharmacological antagonism of neuronal apoptosis can modify the PD course. Lipocurc™ is a unique pipeline drug transformed from the yellow curry extract incorporating both liposome-based targeted drug delivery and brain HAT-HDAC system in epigenetic reprogramming of neural regenerative and neural rescue networks in the brain. The HAT-HDAC balance regulates the on-and-off switch of gene expression in the interrelated cellular processes implicated as heuristic CNS drug targets : oxidative stress, inflammation, neuronal survival, apoptosis and neurogenesis. The odd couple : nanotechnology and epigenetics , create the unprecedented drug discovery platform not only for neurodegenerative disorders, but also for neuro-psychiatric disorders as well as cancer. Our promising results of Lipocurc™ in DJ-1 KO rat model of PD will catalyse randomized controlled trials of Lipocurc™ for the treatment of PD .

List of Abbreviations

ANOVA- analysis of variance
AIMS- abnormal involuntary movement scale
αSYN- alpha-synuclein
DA- dopamine
HAT: Histone acetytransferase
HDAC: histone deacetylase
KO- gene knockout
Lipocurc: abbreviated name of Lipocurc™ :patented compound owned by SignPath Pharma. Inc. USA
miRNA: non-coding microRNA
PBS- phosphate buffer saline
PD- Parkinson's disease
GMP- Good manufacturing practice

Competing Interests

Lawrence Helson MD is the CEO of SignPath Pharma Inc. The proprietary formulation of curcumin (Lipocurc™) is solely and exclusively owned by SignPath Pharma. Inc. USA.

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Authors Contributions

Dr. Simon Chiu and Dr. Lawrence Helson contributed equally towards the design and development of the project. Dr. Simon Chiu, in collaboration with the curcumin research team, assumes full responsibility for analysing and interpreting the results. Dr. Vladmir Badmaev highlighted the relevance of curcuminoids towards the development of CNS therapeutics. Dr. Simon Chiu wrote the first draft of the paper except for the Methods section contributed by Kristen Terpstra B.A Honors. Simon Chiu directed the dissection of all the brain regions. Dr. Yves Bureau, Kristen Terpstra, and Dr. Jirui Hou have contributed equally towards the study in refining the handling and intravenous injection of the animals, standardizing and conducting the behavioral testing, and conducting immunohistochemistry experiments for measuring apoptosis and tyrosine hydroxylase. Hana Raheb assisted in all phases of the study. Dr. Dr. Yves Bureau and Dr. Zack Cernovsky have shared with the team their statistical expertise in analysing the results. Dr. Mariwan Husni and Dr John Copen collaborated with the curcumin research team in organizing translational research in expanding the therapeutic horizons of curcuminoids to the spectrum of neuropsychiatric disorders : schizophrenia , Alzheimer dementia and depression. All members of the research team have reviewed all sections of the paper, agreed with the interpretation of the data and critically commented on the significance and findings reported in the paper**.**

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Group	Apoptotic cells/250 cells at 40x microscopic field	Apoptotic index (mean +/- SEM)
I: DJ-1(Park7)-WT: PBS-treated	7. 75 +/- 1. 75	3.40 +/- 0.70
II.DJ-1 (Park-7)-KO: Lipocurc™ - treated	8.25 +/- 2.39	**3.30 +/- 0.96
III. DJ-1 (DJ-1)-KO: PBS-treated	62.00 +/- 2.58	* 24.80 +/- 1.03

Table I: Effect of Lipocurc™ on Apoptotic Index of the striatum in DJ-1 (Park7) KO rats. Lipocurc almost completely prevented apoptosis in the striatum of DJ-1(Park7) KO rats : the apoptotic index of Lipocurc treated group was almost the same as the DJ-1(Park7)WT group ($p = 0.987$). Extensive apoptosis occurred in the unprotected PBS-treated DJ-1(Park7) KO group. Group III statistically different from Group II ($P < 0.001$)

Groups	Log. number of TH(+)DA neurons 10 x microscopic field Results expressed as mean +/- SEM	DA neuron maturity index : Ratio of immature DA neurons to Mature DA neurons Results expressed as mean +/- SEM
I: Park7(DJ-1)-WT: PBS- treated	2 .37 + 0.02*	0.086 + 0.020
II.Park 7 (DJ-1)-KO: Lipocurc™ - treated	2. 02 + 0. 21	0.578 + 0.156**
III. Park 7 (DJ-1)-KO: PBS- treated	1.93 + 0.07	0.094 + 0.017

Table II: Tyrosine hydroxylase-labelled [TH (+)] DA neurons in DJ-1 KO rats

*Log transformed total number of TH(+) DA neurons was non-statistically significant among the three groups. ** Lipocurc DJ-1(Park7) KO group II statistically different from DJ-1 (Park7) PBS treated group with respect to DA neuron maturity ratio ($P < 0.05$)

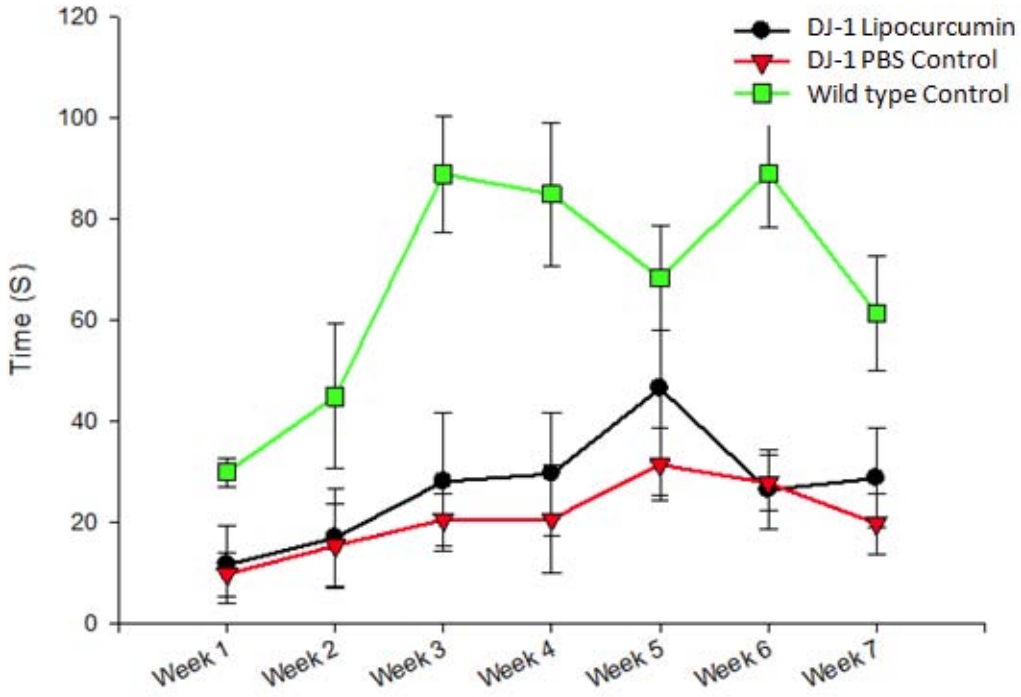

Figure 1. Behavioral results of Rotarod session by treatment groups. Mean ratio of time spent on the rotarod by session for each treatment type. DJ-1 Lipocurc™ (DJ-1 KO Lipocurc treated) n = 4 ; DJ-1 PBS control (DJ-1 KO PBS treated) : n = 4 ; Wild type control: DJ-1 WT PBS Longevans controls : n= 4. The results are expressed as mean ± SEM.

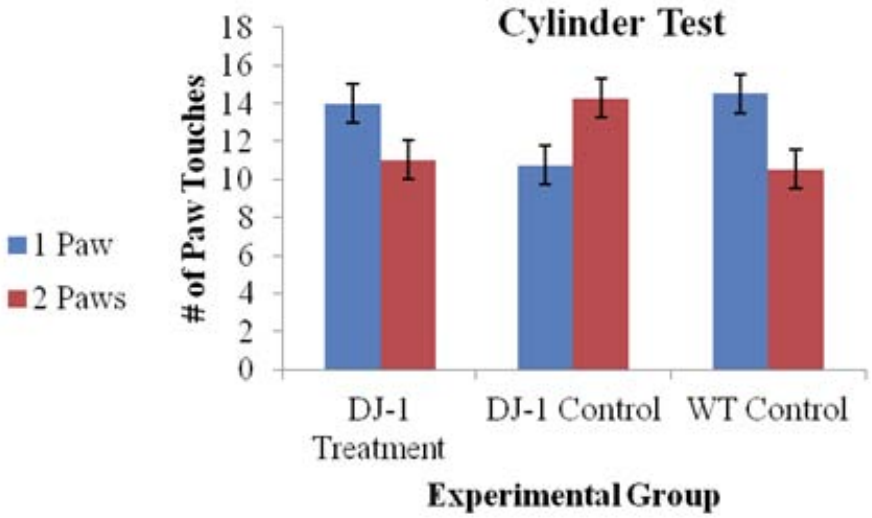

Figure 2. Behavioral results for Cylinder test. Mean ratio of 1 paw versus 2 paw extension avererged over a total of 25 touches in the Cylinder test for each treatment type. DJ-1 KO Lipocurcumin (DJ-1 KO Lipocurc treated): n = 4 ; DJ-1 KO Control(DJ_1 KO PBS treated): n = 4 ; Long-evans controls. WT (DJ-1 WT PBS

treated) n = 4 .Data are presented as mean ± SEM.

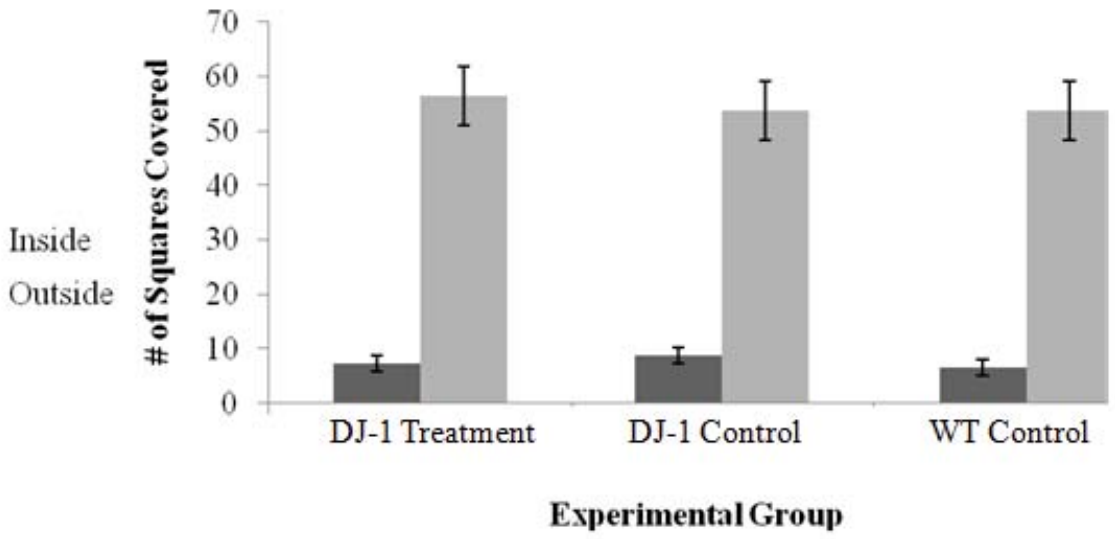

Figure 3. **Behavioral results for Open Field test.** Mean ratio of exploratory behaviour for inside versus outside squares in the open field test for each treatment type. n = 4/DJ-1 KO Lipocurcumin (DJ-1 KO Lipocurc treated) , n = 4; DJ-1 KO Control (DJ-1 KO PBS treated n = 4; Longevans controls (DJ-1 WT PBS treated) Data are presented as mean ± SEM.

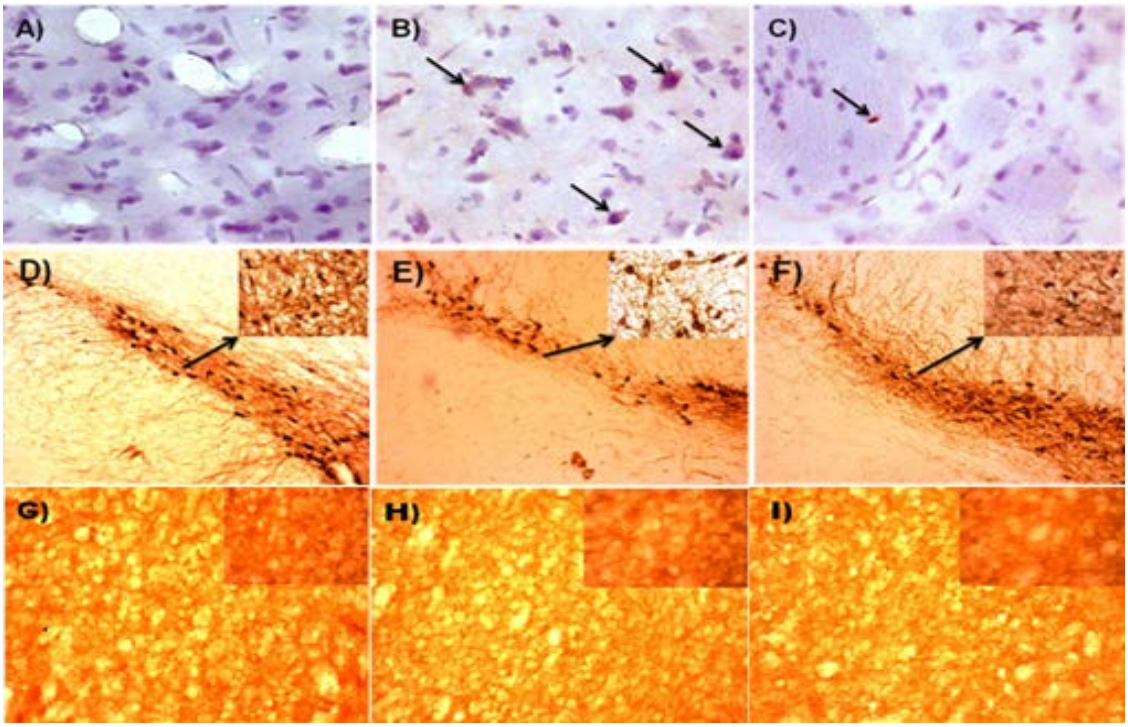

Figure 4 (A-C). Striatal TUNEL staining immunohistochemistry results for 60X magnification. Dark brown indicates apoptotic cells. A) Wild type controls (DJ-1 WT PBS treated) , B) DJ-1 KO PBS treated , C) DJ-1 KO Lipocurc treated , $p < .001$. *Figure 4 (D-F).* Substantia nigra tyrosine hydroxylase staining immunohistochemistry results for 10X Lipocurc™ and 40X magnification. Dark brown indicates tyrosine hydroxylase cells. D) Wild type controls (DJ-1 WT PBS treated), E) DJ-1 KO PBS treated , F) DJ-1 KO Lipocurc™ treated . TH (+) cells from treated DJ-1 KO rats, showed higher ratio of immature (TH+) cells to mature (TH+) cells,DJ-1 KO Lipocurc™ treated : 0.13 vs. DJ-1 KO PBS treated : 0.58, t-test, $p, < 0.05$). *Figure 4 (G-I).* Striatal tyrosine hydroxylase staining immunohistochemistry results for 10X and 40X magnification. Dark brown indicates tyrosine hydroxylase cells. D) Wild type controls (DJ-1 WT PBS treated), E) DJ-1 KO PBS treated , F) DJ-1 KO Lipocurc™ treated . TH (+) cells from Lipocurc™ - treated DJ-1 KO rats. No statistically significant difference in TH (+) cells among the three groups at $p < 0.05$ level

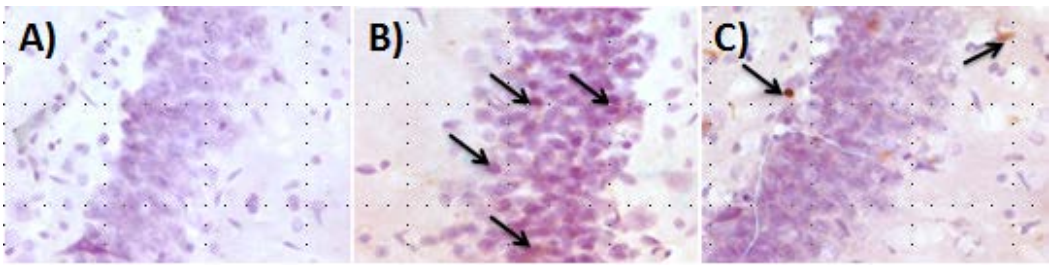

***Figure 5.* Hippocampal TUNEL staining immunohistochemistry.** Data for 60X magnification. Dark brown indicates apoptotic cells. A) Wild type controls (DJ-1 WT PBS treated) , B) DJ-1 KO PBS treated , C) DJ-1 KO Lipocurc™ treated.