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Investigation of The Relationship between The Pesticide Fluopyram and Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease defined as a shaky stroke. It is clinically characterized by; resting tremor, cogwheel rigidity, bradykinesia, and postural reflex impairment. It is also pathologically characterized by Lewy bodies (LBs) and formed by the loss of dopaminergic neurons in the Substantia nigra pars compacta (SNpc) region of the brain and the accumulation of Alpha-Synuclein (α-Syn) proteins. Pesticides are the hugest risk factor of PD. They cause the formation of PD by using different mechanisms. They also permanently disrupt the function of the electron transport complex (ETC) located in the mitochondria. Researches show that the pesticide Rotenone (ROT) causes PD in vivo and in vitro conditions by inhibiting mitochondrial complex I and causing oxidative stress. Fluopyram (Flu) is a frequently used pesticide that causes mitochondrial toxicity like ROT. When literature is searched, it makes one think about a connection with PD. it seems that there is no available study on Flu and PD. Flu is one of the fungicides utilized in Isparta city at a high rate for all planting types. The Flu is a mitochondrial complex II inhibitor. The inhibition of mitochondrial complexes is the main pathway in the PD mechanism, therefore complex II inhibitor pesticide may lead to the same result. In this context, we created a Parkinson's model in mice with ROT and compare the result of Flu with this ROT to be sure whether that lead to Parkinson's or not. Swiss albino male and female mice were the testing animals of our study. Positive control (ROT Parkinson model, 1 mg/kg/day), negative control (Solvent only, DMSO), and Flu (0.5,1 and 2 mg/kg/day) were administrated to mice daily doses subcutaneously (SC). The experiment got completed after 21 days. Motor Function Test, Histochemical and Immunohistochemical Studies, Comet assay, and quantitative real-time PCR (qRT-PCR) were utilized in the study as well. In our results, no statistically significant changes were observable in animal weights in all groups. In the motor function test, a significant decrease in the ROT group was observable, On the other hand, a significant decrease was detected specifically in the Flu value which was applied at high doses. When the brain tissues that belong to the ROT and Flu tested groups got examined, all-important structural changes such as LBs got observed in the SN. When brain tissues belonging to ROT and Flu groups were examined in α-Syn immunohistochemical staining, more positive markings were observable. When the brain tissues belonging to the ROT and Flu groups were examined in Tyrosine hydroxylase (TH) immunohistochemical staining, fewer positive markings were observed. ROT has been found to cause DNA damage in blood and brain tissues. It has been determined that groups with Flu applied in the brain do not have DNA damage. ROT was observed to increase SNCA mRNA expression levels while decreasing TH and DJ-1 mRNA expression levels. Comparing the Flu and ROT results, SNCA mRNA expression levels increased but were not significant. With the detection of decreased TH and DJ-1 mRNA expression levels, the difference was shown in our ROT results. In conclusion, The study results have proved that in mice Flu's induce of PD phenotype depends on Flu doses. It is recommended to do more research with Flu especially in high doses and with different methods.

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INTRODUCTION

PD is the most common neurodegenerative disease seen after Alzheimer's disease (1) Affects about 1% of the over-60 population (2,3).80-85% reduction in dopamine (DA) levels as a result of loss of approximately 60-65% of dopaminergic neurons causes symptoms of PD (4).Basic clinical identification of PD could not be made. It is thought that genetics, aging, pesticides have a role in the formation of PD (5,6).As a result of the studies, many genes that are claimed to cause the formation of PD have been identified. Mutations were found in many genes such as SNCA (7), TH (8), LRRK2 (9), Parkin (10), DJ-1 (11) and were found to be associated with PD.The SNCA (PARK1 and 4) gene provides instructions for making a small protein called α - Syn.

KEYWORDS:
Parkinson's disease (PD),
ROT,
Flu

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The pathological role of α-Syn in PD is supported by mounting evidence. For instance, multiplications of the α-Syn gene (SNCA) and various point mutations in this gene result in dominant familial parkinsonism (12,13,14,15). The gene TH provides instructions for making the Tyrosine hydroxylase enzyme. This enzyme uses one of the oxygen atoms hydroxylates the tyrosine molecule to form L-DOPA. L-DOPA is converted to DA by the AADC enzyme and stored in vesicles (16). When the TH enzyme decreases DA production will also decrease it causes motor dysfunction one of the main findings of PD (17). PARK8 (LRRK2) is a Leucine-rich repeat kinase 2. Mutations in LRRK2 are the most common changes associated with familial PD identified to date (18,19). PARK7 (DJ-1) mutations are related to autosomal recessive early-onset Parkinsonism (20). PARK2 (Parkin) is an E3 ubiquitin ligase that plays a critical role in ubiquitination. Mutations in this gene are related to PD (21 , 22). Pesticides are the hugest risk factor of PD. They cause the formation of PD by using different mechanisms (23).Researches show that the pesticide ROT causes PD in vivo and in vitro conditions (3) by inhibiting mitochondrial complex I and causing oxidative stress. (24). Based on this observation, it was borne in the view that some substances similar to the chemical structure ROT can be found in the environment or in certain foods and may be responsible for the disease. Flu is a frequently used pesticide that causes mitochondrial toxicity like ROT. When literature is searched, it makes one think about a connection with PD. it seems that there is no available study on Flu and PD. Flu (396.72 g/mol, C16H11ClF6N2O, CAS Number: 658066-35-4) is one of the fungicides utilized in Isparta city at a high rate for all planting types. The Flu is a mitochondrial complex II inhibitor (25,26). The inhibition of mitochondrial complexes is the main pathway in the PD mechanism, therefore complex II inhibitor pesticide may lead to the same result. In this context, this ROT to be sure whether that lead to Parkinson's or not .

MATERIAL AND METHOD

Animal Model, Parkinson's Model and Study Design

58 number (30-37 g), 12 - 20 weeks old, male and female Swiss albino mice were the testing animals of our pilot and main study. Mice had free access to food and water. Daily dose applications were performed between 14:00 and 15:00. During the experiment, the general condition of the animals was observed and recorded. Positive control (ROT 2 mg/kg/day), negative control (DMSO), and Flu (0.1,1 and 2 mg/kg/day) were administrated to mice daily doses SC. The experiment got completed after 21 days. According to the pilot study results obtained, the doses of the main study were determined. Positive control (ROT 1 mg/kg/day), negative control (DMSO), and Flu (0.5,1 and 2 mg/kg/day)

Motor Function Test Spontaneous Activity in the Cylinder Procedure

The method of work was used as mentioned by Fleming et al (27). Groups comparisons were conducted using ANOVA with post hoc test. avalue of $p<0.05$ was considered to be statistically significant.

Histochemical and Immunohistochemical Studies Studies

Histochemical studies were performed according to the manufacturer's protocol. Tissue sections (4-µm thick) of formalin-fixed, paraffin embedded specimens were deparaffinized dehydrated in graded alcohol. The slides were then rinsed in distilled water, counterstained with hematoxylin (H) for 2 minutes. the slides were washed three times with water. The slides were placed in the alcoholic acid for 3-4 seconds. the slides were washed three times with water, counterstained with eosin (E) for 2 minutes. the slides were washed three times with water, dehydrated in graded alcohol then kept in xylol for 1 night until the sections became transparent was covered. Immunohistochemical was performed according to the manufacturer's protocol with small modification. The method of work was used as mentioned by Refaiy et al. HC and IHC Painted samples were examined and evaluated under a binocular microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan, and photos were obtained with imaging hardware.The staining score was evaluated as follows: - negative, +/- weak, + few ,++ moderate and +++ strong positivity (28.(

Comet assay

It is important that the cellular material to be used in the Comet assay is alive. The procedure should be performed no later than 2 hours after tissue or blood is taken. Blood and brain tissue were removed from mice by euthanasia under anesthesia. Blood was collected in a 1.5- ml Eppendrof tube and stabilized with 50 U heparin. Histopaque- 1083 (Sigma, St.Louise, MO) 1/1 ratio with blood are added to the prepared eppendrof tube. The samples were centrifuged at 20 min at 2000 rpm. Leukocytes were collected then Phosphate-buffered saline (PBS) is prepared by placing 1/1 on eppendrof tube. Centrifuged 10 min at 2500 rpm. Supernatant part removed, 20 µl cells and 100 µl Lower melting Agarose (LMA) , (type VII, sigma.St, Louis, MO) are mixed in a separate eppendorf. Brain tissue was washed in PBS and then cut into small pieces. the remaining solution is taken to eppendorf and centrifuged for 15 min at 2500 rpm. Depending on the cell density at the bottom Watering is carried out with 25-50 ml of PBS. Mixes 20 µl cells and 100 µl LMA in a separate eppendorf .At this stage, In order not to hurt LMA cells, they must be at body temperature. Cells from pretreated and control animals were run at the same time to allow comparison. The technique by Ostling, et al. and Singh, et al. was followed with minor modification (29,30). The Slide was filmed in the darkroom with the Nikon DSRİ2 camera under a fluorescent microscope from Nikon Eclipse. DNA image automatically evaluated in Open Comet open-source visual evaluation program. Tail DNA percentage (TDNAP) and tail length (TL) parameters are selected to reflect the amount of DNA damage. Results SPSS V20 package program (IBM,2012) via " One-way Anova (Tukey)" statistical test evaluated and considered significant when p <

0.05 .(31)

8 -QRT-PCR

Levels of mRNA were analyzed in real-time. Total RNA was extracted from brain tissues using PureZOL(Trizol reagent) Bio-Rad (USA). Total RNA concentration was determined by Nanodrop™ Spectrophotometer 2000 (ThermoFisher Scientific™). and reversed transcribed into cDNA using iScript™ RT supermix for RT-qPCR- Bio-Rad (USA). The primers sequences for genes SNCA, Parkin, DJ-1, LRRK2, TH and housekeeping genes ß-glucuronidase (GUSB) and Transferrin receptor (TFRC) were designed by us using a primer design tool. First, specific mRNA sequences of the above mentioned genes were determined from http://blast.ncbi.nlm.nih.gov/Blast.cgi. Then, possible primers were selected considering the general primer design rules. The site http://www.premierbiosoft.com/netprimer/index.html was used to check whether it formed any dimers or conflicted with the sequences of other genes. primers synthesized by (oligomer biotechnology Ankara) company.The following primer pairs were used: PARK 1 ve 4 (SNCA), forward 5′ - GCAAGGGTGAGGAGGGGTA- 3′, reverse 5′ - AGGCTTCAGGCTCATAGTCTTG- 3′; PARK 2 (Parkin), forward 5′ - TCTTCCAGTGTAACCACCGTC- 3′, reverse 5′- ATCAGGGAGTTGGGACAGC- 3′; PARK 7 (DJ1), forward 5′- AGGTCCTACGGCTCTGTTGG-3′, reverse 5′- GTCCTTAGCCAGTGGGTGTG- 3′; PARK 8 (LRRK2), forward 5′- CAATGCCTGCCTTACCTTCT- 3′, reverse 5- ′AGTTCAGAGACTTCCAATGTGC- 3′; TH (Tirozin hidroksilaz), forward 5′-GTCTCAGAGCAGGATACCAAGC- 3′, reverse 5′- CGAATACCACAGCCTCCAAT- 3′; GUSB (HouseKeeping), forward 5′-GGCTGGTGACCTACTGGATTT-3′, reverse 5′- AAGTTGACCCTGGTTCCCTG- 3′; TFRC (HouseKeeping), forward 5′-CCGCTCGTGGAGACTACTTC-3′, reverse 5′- GAGATACATAGGGCGACAGGA-3′.All qRT-PCR reactions were performed using the QIAGEN- Rotor-Gene Q Fast Real-Time PCR System and iTaqTM Universal SYBR® Green Supermix kit.The relative quantification of gene expression was calculated as the 2−ΔΔCt method.

RESULTS

Body weight results and Motor Function Test Results

No statistically significant changes were observable in animal weights in all groups before and after experiment. ROT group saw at 10.56 value it is mean the motor function decreased significantly in the ROT group Compared to control (*p < 0.05) and DMSO groups (**p<0.05). In the Flu group, the motor function decreased depending on the dose, on the other hand, a significant decrease was detected specifically in the Flu value which was applied at high doses (Fig.1).

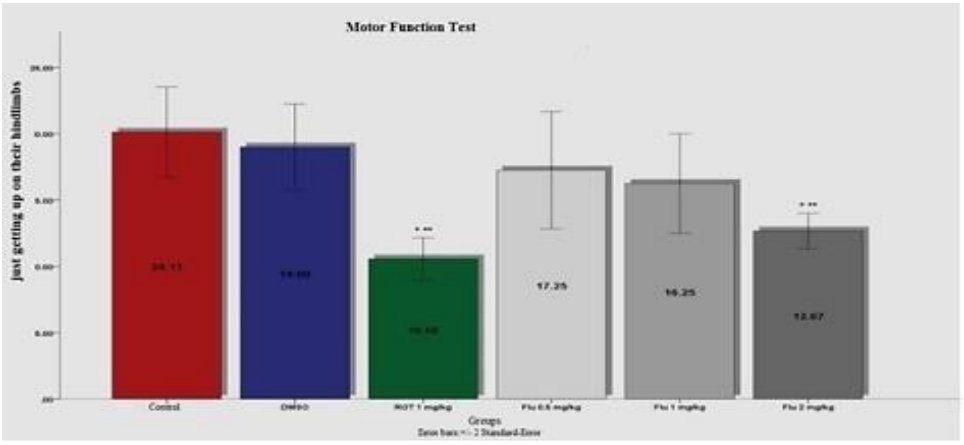


Fig. 1. Motor Function compared to Control and DMSO groups statistically significant (*p < 0.05) (**p<0.05)**.**

The Histochemical Studies results

Table 1: Grading of histological structural changes according to groups

	LBs	*neurodegeneration*	*Decrease in the Number of Neurons*	*hemorrhagic areas*	*mononuclear Cell Infiltration*	*neuropil vacuolization*	*pycnotic nuclei*
Control Group	-	-	-	-/+	-	-/+	-
DMSO Group	-	-	-	-/+	-	-/+	-
ROT Group	++/+++	++/+++	+++	+++	+++	+++	++/+++
0.5 Flu Group	+	+	+	+	+	+	+
1 Flu Group	+/++	+/++	++	++	++	+/++	+/++
2 Flu Group	++/+++	+++	+++	+++	+++	++/+++	++/+++

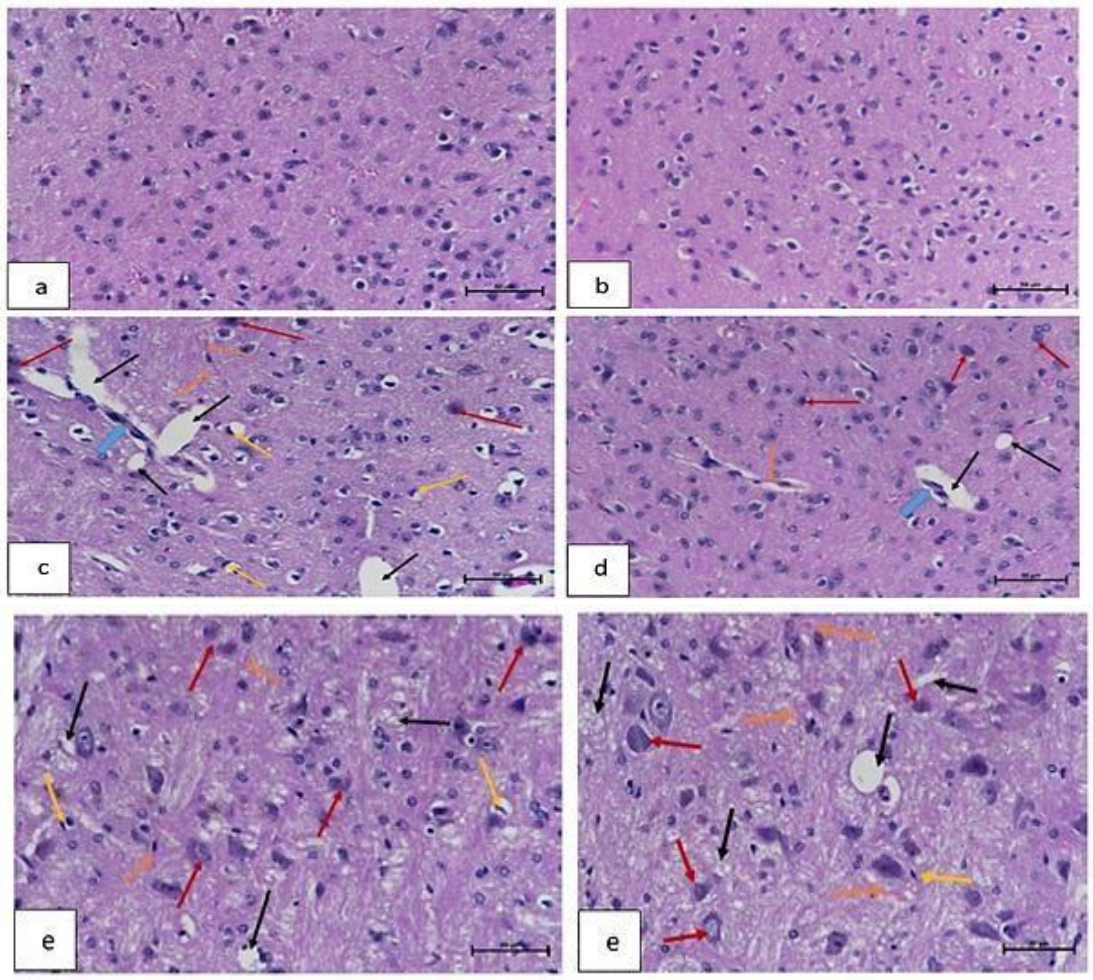


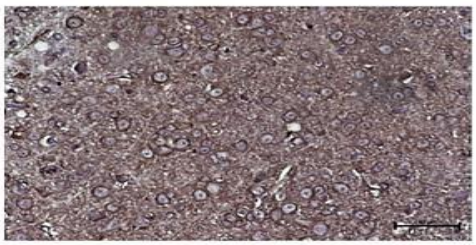
Fig.2.a. Brain tissue section of the control group and b.brain tissue section of the DMSO group hemorrhagic areas and neuropil vacuolization are present in some areas.c.Brain tissue section of the ROT group. more histopathological findings were observed: black arrows; neuropil vacuolization, yellow arrows; pycnotic nuclei in neurons, red arrows; LBs, orange arrows; hemorrhagic areas and blue arrow; shows mononuclear cell infiltration. d. Brain tissue section of the Flu group (0.5) histopathological findings were observed but its Less than ROT group e. Brain tissue section of the Flu group (1). Results decreased compared to the ROT group, increased compared to the Flu 0.5 group. f. Brain tissue section of the Flu group (2) Results equally with ROT group, increased compared to the Flu group 0.5 and 1 mg/kg. (H-E, x400)**.**

Immunohistochemical Studies results

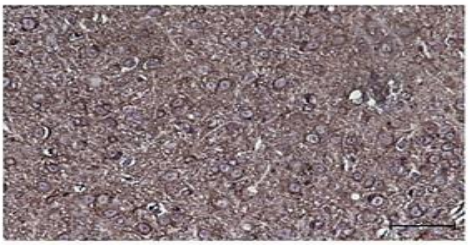
A significant difference was found between the groups in α-Syn and TH immunohistochemical staining of brain tissue sections. There was no significant difference between Group I and Group II in labeling with α-Syn and TH (Table.2 - Fig.3a –b- g - h). In labeling with α Syn, more positive markings were observed in the sections belonging to group III (ROT) and group IV (0.5 - 1 - 2 Flu) compared to the sections belonging to group I (Control) and group II (DMSO) (Table.2 – c – d - e – f. When group III (ROT) and group IV are compared, the most positive staining is respectively; group IV Flu 2 in equal ratio and group III (Table.2..c- f).then in group IV Flu 1 (Table.2 – e) and the least staining was observed in group IV Flu 0.5 (Table.2 – d).In labeling with TH, more positive markings were observed in the sections belonging to group I and group II, more than other groups (Table.2. – g- h) Group III (ROT) and group IV the least positive staining groups, respectively; Equal ratio of group IV Flu 2 and group III (Table.2 – i- l). then, group IV Flu 1 (Table.2. – k) and the most positive staining were observed in group IV Flu 0.5 (Table.2. – j x400). In addition, no significant difference was found between male and female brains in any of the groups in the histochemical and Immunohistochemical results.

Table.2 α-Syn and TH Marking Degrees

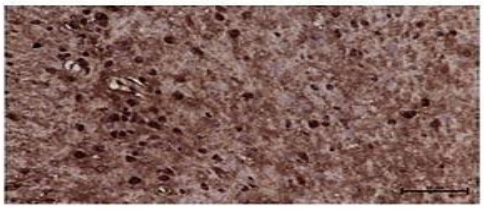
	Control Group	*DMSO Group*	*ROT Group*	*0.5 Flu Group*	*1 Flu Group*	*2 Flu Group*
α-Syn	-/+	-/+	++	+	++/+	++
TH	+++	+++	+	++	++/+	+



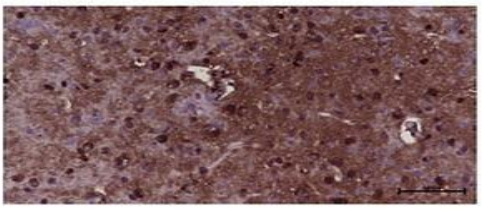
a. Brain tissue section of the control group with α-Syn immunostaining, x400



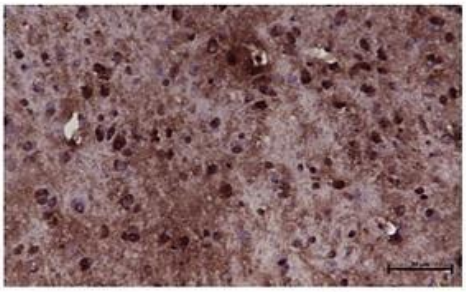
b. Brain tissue section of the DMSO group with α-Syn immunostaining,x400



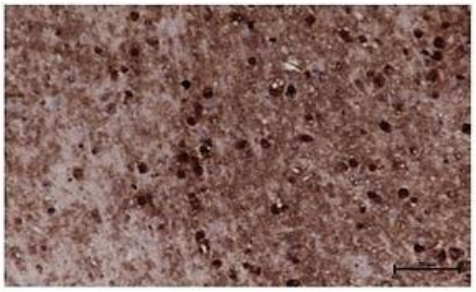
c. Brain tissue section of the ROT group with α-Syn immunostaining, x400



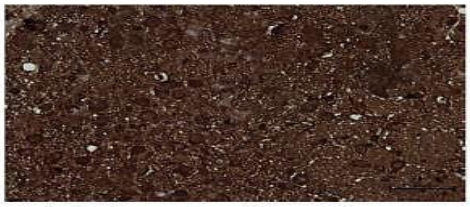
d. Brain tissue section of the Flu group (0.5) with α-Syn immunostaining,x400



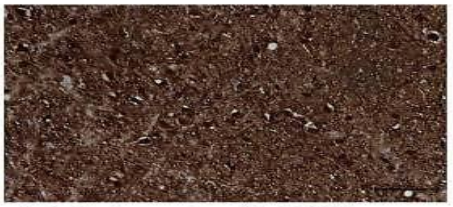
e. Brain tissue section of the Flu group (1) with α-Syn immunostaining,x400



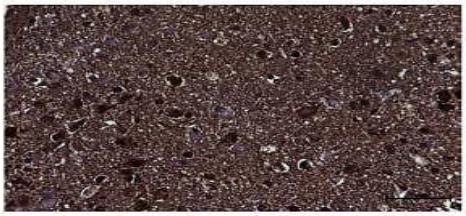
f. Brain tissue section of the Flu group (2) with α-Syn immunostaining,x400



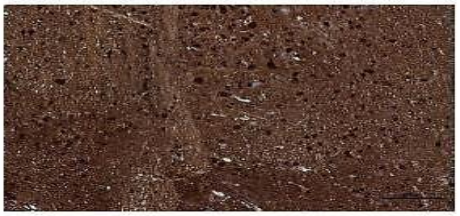
g. Brain tissue section of the control group with TH immunostaining,x400



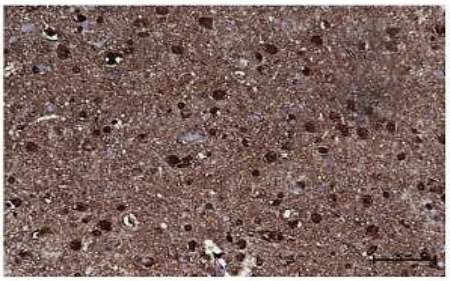
h. Brain tissue section of the DMSO group with TH immunostaining,x400



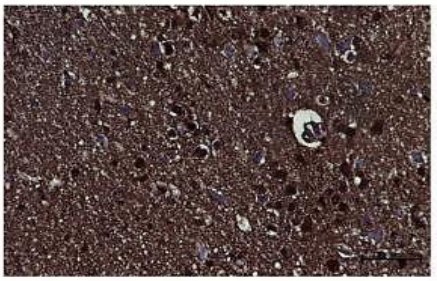
i. Brain tissue section of the ROT group with TH immunostaining,x400



j. Brain tissue section of the Flu group (0.5) with TH immunostaining,x400



k. Brain tissue section of the Flu group (1) with TH immunostaining,x400



l. Brain tissue section of the Flu group (2) with TH immunostaining, x400

Fig.3.a,b,c,d,e,f,i,j,k,l α-Syn and TH immunohistochemical staining of brain tissue sections.

DNA Damage Results

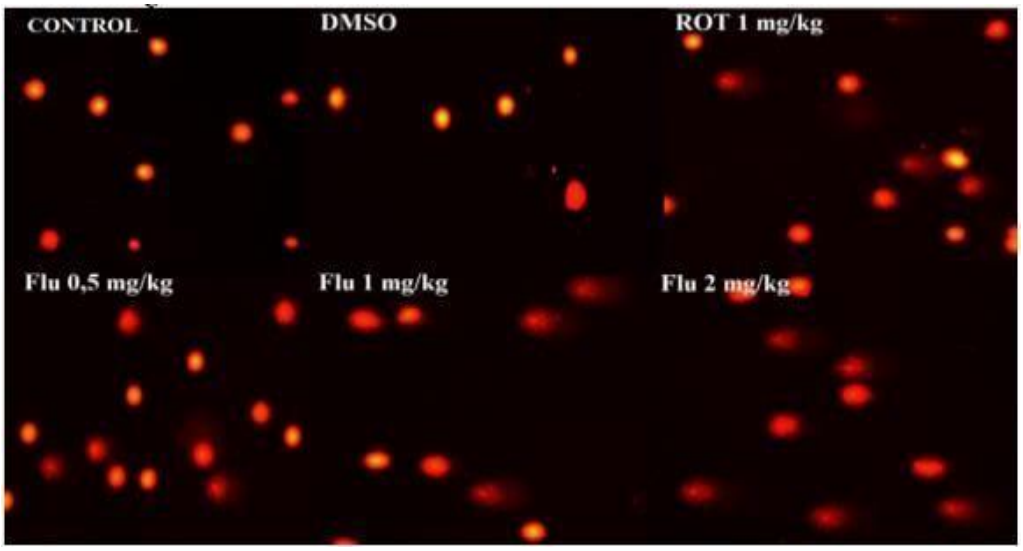


Fig.4. Sample comet photos from blood groups.

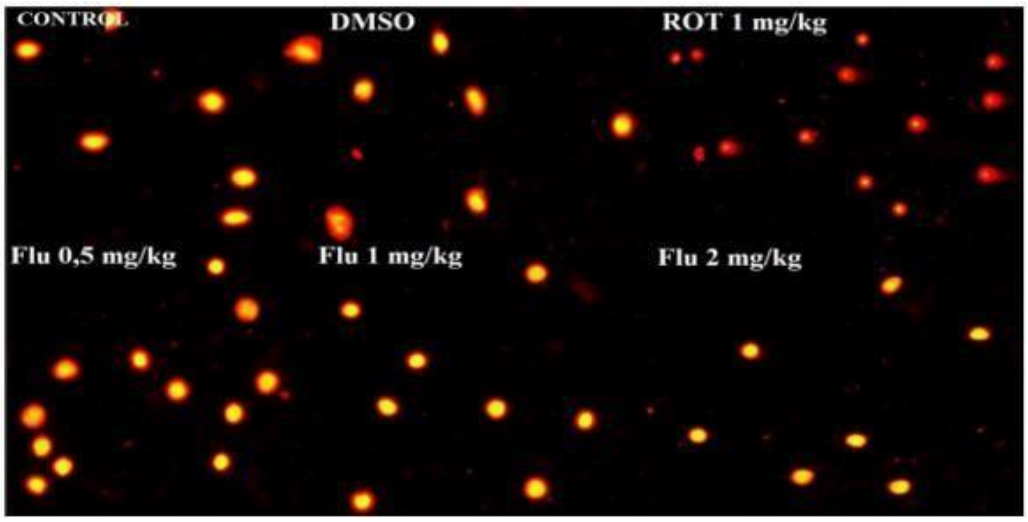


Fig.5. Sample comet photos from brain tissue groups

Table 3. Blood tissue mean DNA damage

Groups	Means of Tail DNA Percentage Values
Control	4,36±0,24
DMSO	4,43±0,21
ROT	8,40±0,38 * **
Flu 0.5 mg/kg	8,09±0,29 * **
Flu 1 mg/kg	8,31±0,32 * **
Flu 2 mg/kg	8,58±0,35 * **

(Mean± Std.Err) *: Statistically significant when compared with the control group (p<0.001), **: statistically significant when compared with the DMSO group (p<0.001).

Table 4. Brain tissue mean DNA damage

Groups	Means of Tail DNA Percentage Values
Control	5,76±0,27
DMSO	5,80±0,29
ROT	6,94±0,30 * **
Flu 0.5 mg/kg	5,58±0,25
Flu 1 mg/kg	5,71±0,26
Flu 2 mg/kg	5,91±0,31

(Mean± Std.Err) *: Statistically significant when the ROT group was compared with the Control group (p<0.05), **: statistically significant when compared with the DMSO group (p<0.05).

ROT have been found to cause DNA damage in blood and brain tissues. ROT significantly increased Tail DNA percentage compared with the control group (p<0.001) (p<0.05).DNA damage in blood tissues increased depending on the dose of Flu.Flu significantly increased Tail DNA percentage compared with the control group (p<0.001) (p<0.05).It has been determined that groups with Flu applied in the brain not have DNA damage.

QRT-PCR Results

Table.5 In the DMSO group, SNCA, Parkin, DJ-1, LRRK2 and TH genes mRNA expression levels

genes	*mRNA Expression Level*	*Std.Err*	*P value*
SNCA	1.41	0.26-6.47	0.34
Parkin	1.32	0.69-2.53	0.10
DJ-1	0.94	0.53-1.69	0.71
LRRK2	1.33	0.35-5.52	0.43
TH	0.78	0.41-1.46	0.11

($p<0.05$) significant, ($p>0.05$) not significant

SNCA, Parkin, DJ-1, LRRK2 and TH group were not different from the controlgroup.

Table.6 In the ROT 1mg/kg group, SNCA, Parkin, DJ-1, LRRK2 and TH genes mRNA expression levels

genes	*mRNA Expression Level*	*Std.Err*	*P value*	*Results*
SNCA	*2.20*	*0.40 - 11.14*	*0.02*	*increased*
Parkin	*1.06*	*0.48 - 2.19*	*0.77*	
DJ-1	*0.35*	*0.15 - 0.80*	*0.00*	*decrease*
LRRK2	*1.81*	*0.36 - 8.97*	*0.10*	
TH	*0.35*	*0.05 - 1.66*	*0.00*	*decrease*

($p<0.05$) significant, ($p>0.05$) not significant

Table.7 In the Flu 0.5 mg/kg group, SNCA, Parkin, DJ-1, LRRK2 and TH genes mRNA expression levels

genes	*mRNA Expression Level*	*Std.Err*	*P value*	*Results*
SNCA	*1.25*	*0.20 - 8.05*	*0.61*	
Parkin	*0.38*	*0.10 -1.54*	*0.00*	*decrease*
DJ-1	*0.50*	*0.15 - 1.63*	*0.02*	*decrease*
LRRK2	*0.59*	*0.11 - 3.19*	*0.22*	
TH	*0.27*	*0.09 - 0.62*	*0.00*	*decrease*

($p<0.05$) significant, ($p>0.05$) not significant

Table.8 In the Flu 1 mg/kg group, SNCA, Parkin, DJ-1, LRRK2 and TH genes mRNA expression levels

genes	*mRNA Expression Level*	*Std.Err*	*P value*	*Results*
SNCA	*1.28*	*0.11- 13.48*	*0.66*	
Parkin	*0.18*	*0.05 -0.57*	*0.00*	*decrease*
DJ-1	*0.32*	*0.02 -1.66*	*0.04*	*decrease*
LRRK2	*0.33*	*0.01 -3.69*	*0.10*	
TH	*0.41*	*0.06 -1.82*	*0.02*	*decrease*

($p<0.05$) significant, ($p>0.05$) not significant

Table.9 In the Flu 2 mg/kg group, SNCA, Parkin, DJ-1, LRRK2 and TH genes mRNA expression levels

genes	*mRNA Expression Level*	*Std.Err*	*P value*	*Results*
SNCA	*1.81*	*0.32 - 11.06*	*0.15*	
Parkin	*0.17*	*0.03 - 0.59*	*0.00*	*decrease*
DJ-1	*0.18*	*0.02 -1.00*	*0.00*	*decrease*
LRRK2	*0.25*	*0.03 -2.04*	*0.01*	*decrease*
TH	*0.29*	*0.07 -1.02*	*0.00*	*decrease*

($p<0.05$) significant, ($p>0.05$) not significant

DISCUSSION

In the present study, we created a Parkinson model in mice with ROT and compare the result of Flu with this ROT to be

sure whether that lead to Parkinson's or not. Changes in body weight have been observed in several studies of ROT.11 months old Lewis rats had osmotic minipumps implanted SC on their back containing (ROT which was delivered at the rate of 1mg/kg/day) for 6 weeks. Body mass decreased about 10% at the end of the experimental protocol as compared with the initial situation for rats (32). İn another study no change was observed in the bodyweight of the male Wistar rats injected SC with ROT (2 mg/kg) daily for 5 weeks (33). In our results, no statistically significant changes were observable in animal weights in all groups.A clear parkinsonian phenotype was produced by ROT that was highlighted by decreased motor functions (34).In the Parkinsonism model, oxidative stress caused by ROT decreases the TH enzyme (17). It causes motor dysfunction (35). When the TH enzyme decreases DA production will also decrease it causes motor dysfunction one of the main findings of PD (17).Rats injected with ROT (1.5 mg/ kg /day, SC. for 28 days). It caused an increase in ROS and NO levels, decreased TH + cell numbers, and caused a significant decrease in the TH enzyme, and in rats prolonged immobility periods were seen (6,33, 36).Zhang, et al. performed a study in Rats who were injected with ROT 2.5 mg/ kg /day, ip. for 14 days to assess the effect of ROT on TH+ cells in the SN. It caused motor dysfunctions by decreasing the number of TH+ cells in SN. After 42 days of ROT treatment, neurons in the SN presented with obvious apoptotic features and more severe motor dysfunctions. This result of ROT-induced apoptosis suggests that it may be associated with motor dysfunction (37).In our results, In the Flu group, it was determined that the motor function decreased depending on the dose. a significant decrease was detected specifically in the Flu value which was applied at high doses.The formation of the PD phenotypic pattern can be detected by histochemical and immunohistochemical methods (38). decreased TH expression in SNpc (33) and LBs developed by increased expression of α-Syn indicates the formation of the PD model (39).More positive markings were observed when α-Syn cell number and expression increased while less positive markings were observed when TH cell number and expression decreased. In a study conducted by Alzahranı et al, male Swiss albino mice were injected with ROT 1 mg/ kg 48 hours, SC) for 9 times to formation ROT-parkinsonian mice. the motor dysfunctions induced by ROT as well as markers DNA damage 8-hydroxydeoxyguanosine (8-OHdG) expression. Routine examination of histologic sections from the SNpc indicated that mice treated with ROT group showing mixed normal neurons and degenerated neurons the degenerated neurons exhibit, cytoplasmic vacuoles with irregular faint nuclei, or irregular pyknotic nuclei. Immunohistochemical staining for nigral TH demonstrated a lower percentage of TH-positive neurons in the ROT group (40, 33,41). Zeng, et al, performed a study in Rats were treated with ROT (1.0 mg/kg/day.SC) for 5 weeks. Immunohistochemical analysis showed a significant loss in DA neurons in the SN of rats treated with ROT accompanied by an increase in the accumulation of α-Syn (42). The α-Syn is a component of LBs which is a hallmark of PD (43,44). In our results, When the brain tissues that belong to the ROT and Flu tested groups got examined, all-important structural changes such as LBs, hemorrhagic areas, mononuclear cell infiltration, neuropil vacuolization, pycnotic nuclei in neurons got observed in the SN. In our results, When brain tissues belonging to ROT and Flu groups were examined in α-Syn immunohistochemical staining, more positive markings were observable. When the brain tissues belonging to the ROT and Flu groups were examined in TH immunohistochemical staining, less positive markings were observed.ROT causes the formation of ROS It can cause cell death by interacting with DNA, RNA and proteins. Verma, et al. reported that ROT caused significantly decreased level of glutathione along with increased level of nitrite and lipid peroxidation in rat. Significant oxidative and nitrosative stress was also observed after ROT administration. ROT induced DNA fragmentation was also assessed in all studied rat brain regions by utilizing comet assay (45).The pathogenesis of PD is associated with numerous factors including oxidative stress, mitochondrial dysfunction and apoptosis. In a study conducted by Nataraj, et al. Following the exposure of SH-SY5Y cells to ROT there was a marked overproduction of ROS, mitochondrial dysfunction (as indexed by the decrease in mitochondrial membrane potential), and apoptosis (Hoechst and dual staining, comet assay; expressions of pro-apoptotic and anti-apoptotic indices (46,47,48,49).Luna Experience SC-400 is a new generation fungicide that combinated with Flu and Tebuconazole. İn a study conducted by Aktaş, et al. showed that exposing adult rats to Luna Experience SC 400 fungicides (5, 10, and 20 mg/kg) led to an increase in Micronucleus (MN) frequency and damage levels in Comet assay in both liver and blood tissues and a decrease in catalase activity. This could be explained by oxidative stress induced by Luna Experience SC 400 in both blood and liver (50).In our results ROT have been found to cause DNA damage in blood and brain tissues. It was determined that DNA damage in blood tissue increased depending on the dose of Flu. It has been determined that groups with Flu applied in the brain not have DNA damage.No similar studies were found on this subject. However, It is thought that the barrier tissue in the brain contributes to the protection of the brain from toxic substances and is the reason for less DNA damage. As a result of the studies carried out to date, many genes that are suggested to cause the formation of PD have been determined. C/EBPβ acts as an age-dependent transcription factor for both α-Syn and MAOB and initiates the PD pathologies by upregulating these two pivotal players, in addition to escalating δ-secretase activity to cleave α-Syn and promotes its neurotoxicity. Human neuroblastoma SH-SY5Y cells were treated with 200 nM ROT for 24 h. It was observed that SNCA mRNA and protein expression levels increased when ROT mediated the upregulation of C/EBPβ (51, 52). ATP-sensitive potassium (KATP) channels found in plasma membrane. It has been determined that the opening of the KATP channel with the increase of ROS causes a decrease in TH expression levels.With patch clamp technology, results showed that treatment of PC12 cells with ROT (0.2-1 μg/ml) for 15 min can cause KATP channel opening with significantly increased intracellular ROS production. Treatment with ROT (2-16 ng/ml) for 24 h also caused the channels to open with gently increased ROS and reduced TH expression (53).İn our results, ROT was observed to increase SNCA mRNA expression levels while decreasing TH and DJ-1 mRNA expression levels Comparing the

Flu and ROT results, It was determined that TH mRNA expression levels were significantly decreased while SNCA mRNA expression levels increased but were not significant. If more doses were given than the administered doses SNCA expression could be detected significantly. In dopaminergic neurons,PD proteins including LRRk2, DJ-1 and Parkin have been shown to play important roles in maintaining normal mitochondrial function, protecting against oxidative stress and preventing cell death (54). LRRK2 mutations predispose to familial and sporadic PD (55). DJ-1 is seen in only 1-2% of early-onset PD cases. It is involved in mitochondrial protection against oxidative stress. Therefore A decrease in D-J1 expression,, may make dopaminergic neurons more vulnerable to ROT-induced oxidative stress (11).Parkin gene mutations are the major cause of autosomal recessive parkinsonism. Parkin is the gene known to cause early-onset PD (56). It prevents α-Syn accumulation by regulating its degradation of it.Berger, et al. they determined that the Parkin mRNA expression levels was decreased after microglial BV2 cells were treated with 20 MmROT for 12 hours (57).In a study conducted by Wanga, et al. ROT 2 mg/L/ for 4 weeks. It was dropped in water to 5-7 months old male zebrafish.ROT has caused impaird motor function, anxiety, and behavioral disorders such as depression.Significantly increases LRRK2 and Parkin mRNA expression levels in the zebrafish brain (58).With the detection of decreased TH and DJ-1 mRNA expression levels,the difference were shown in our ROT results.

In conclusion, The study results have proved that in mice Flu's induce of PD phenotype depends on Flu doses. It is recommended to do more research with Flu especially in high doses and with different methods.

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