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Toxic potential of botulinum toxin type A on senescence in a *Drosophila melanogaster* model

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ABSTRACT

Botulinum toxin type-A (BoNT/A) application, especially neurological disorders, has been spread nowadays while it may cause side effects. The current study aimed to assess the BoNT/A dose-dependent effect on induction of aging in the *Drosophila melanogaster* model. The third instar larvae of *Drosophila melanogaster* were exposed to ¼ LC_{50}, ½ LC_{50}, and LC_{50} of BoNT/A in the *Drosophila* diet for 48 h while H_2O_2 1% was used as a positive control. After the exposure time, some larvae were collected for molecular study, including gene expression analysis, comet assay, oxidative stress markers, and the phenotype changes. BoNT/A induced dose-dependent cytotoxicity, elevated reactive oxygen species (ROS) levels, and superoxide dismutase (SOD) enzyme activity. In addition, it caused DNA damage and activated caspase-3 and -9, and reduced the body size of the fly, especially in high doses. In line with the purpose of the study, aging markers, including β-galactosidase (β-gal), p16, p21, p38, and p53, were up-regulated by BoNT/A low dose. BoNT/A activates the aging pathway in the low dose, and increasing the dose induces toxicity, including oxidative stress, DNA damage, and apoptosis.

1. Introduction

Botulinum toxin is produced by a gram-positive bacterium called *Clostridium botulinum,* which can induce botulism poisoning. This toxin is made up of botulinum proteins encoded by two gene clusters near each other [1–3]. It demonstrated that botulinum induced inhibition of viability, growth cells, and apoptosis [4,5].

Seven botulinum immunological subtypes (A–G) were reported; of these, just subtypes A and B were commonly used in clinics [6,7]. Botulinum toxin type-A (BoNT/A) has been applied for medical therapy since the early 1970s [8,9]. It was used since 1994 to reduce facial wrinkles as a cosmetic treatment [10]. Using BoNT/A as a cosmetic for glabellar frown lines was approved in 2002 by the United States Food and Drug Administration (USFDA) [11]. The common BoNT/A medications in the market are including abobotulinumtoxinA (Dysport®, Ipsen Ltd., Berkshire, UK), onabotulinumtoxinA (Botox®, Allergan Inc., Irvine, California, USA), and incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH, Frankfurt, Germany) [12].

Besides the widespread use of BoNT/A in cosmetics, it has also been prepared as a pharmaceutical product for a variable number of disorders resulting from muscle tone elevation, including blepharospasm and hemifacial spasm (spasticity), and cervical dystonia [13]. Moreover, BoNT/A is a helpful treatment for other diseases like axillary and urologic disorders [14]. It is demonstrated, BoNT/A induced temporary inhibition of acetylcholine secretion at the neuromuscular junction, decreased muscle activity, and reduced muscle tone and spasticity [6]. The above activities reach their peak three weeks post-injection [15]. BoNT/A has also been expected to have a potential role in toxicity

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(especially genotoxic effects) and senescence; however, this is not well studied.

This study was designed to investigate BoNT/A 's role in the senescence and toxicity process. In the present study, LC_{50} and lower doses of BoNT/A were evaluated by developing the experimentally and genetically approved biologic model, Drosophila *melanogaster*.

As reported in previous studies, the Drosophila genome has been completely sequenced, with a nearly 75 % correlation between human disease-related genes and sequences in fly genes [16,17]. Furthermore, there is an approximately 40 % homology of the nucleotide level or protein sequence between mammals and flies, which is believed to be 80–90 % higher for the functional domains [18]. It is also demonstrated that the Drosophila model is a fundamental *in-vivo* model for studying the probable effect of drugs, toxicity, aging, and environmental contaminants [19–22]. This study evaluates the impact of BoNT/A on senescence and toxicity on the wild type of *Drosophila* model.

2. Material and method

2.1. Chemicals

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and SYBR green were obtained from Sigma-Aldrich (GmbH, Munich, Germany). Superoxide dismutase (SOD) activity kit was obtained from Teb Pazhouhan Razi (Iran). BoNT/A (Dysport®, 500 units/vial) was purchased from IPSEN (UK). Agarose powder was gained from Invitrogen (Carlsbad, CA, USA). Other chemicals were purchased from Merck (Germany).

2.2. Methodology

The wild type of *Drosophila melanogaster* stocks was obtained from the cell and molecular biology department, School of Biology, University of Tehran. All molecular experiments were performed on the third instar larvae cultured at 25 ± 2 °C and humidity of 60 % on the standard *Drosophila* culture medium containing corn flour (80 g/L), jaggery (70 g/L), agar (9 g/L), yeast powder (15 g/L), propionic acid (4.4 mL/L) and water up to one liter.

2.3. Determination of LC_{50} of BoNT/A on Drosophila melanogaster

The third instar larvae were treated with different doses of BoNT/A in the medium for determination of the median lethal concentration (LC_{50}). The control group did not have BoNT/A. In each group, there were 40 larvae. All deaths during the first 24 h were recorded, and LC_{50} of BoNT/A was estimated approximately 10^{-5} U/mL using the probit test.

2.4. Treatment protocol

The larvae were first randomly and equally divided into five groups, with 60 larvae in each. The groups were as follows: Group 1 were grown on Drosophila diet for 48 h (Control group); Group 2 received H_2O_2 1% on Drosophila diet as a positive control for 48 h (H_2O_2 group); Group 3 received $^1/_4$ LC_{50} of BoNT/A on Drosophila diet for 48 h (BoNT/A-1 group); Group 4 received ½ LC_{50} of BoNT/A on Drosophila diet for 48 h (BoNT/A-2 group); Group 5 received LC_{50} of BoNT/A on Drosophila diet for 48 h (BoNT/A-3 group). The experiments were done in triplicate, and finally, the 30 live larvae were chosen for molecular analysis and the rest for phenotypic analysis.

In treated groups, the dosage of BoNT/A and H_2O_2 and treatment strategy was obtained based on our pilot studies. All experimental protocols were conducted following the Institute Ethics Committee's guidelines and approved with the code of IR.TUMS.VCR.REC.1397.482.

2.5. Sampling of larvae for molecular analysis

After treatment, for molecular analysis, the larvae were gathered and washed out by phosphate-buffered saline (PBS) buffer, and the guts of larvae in each group were separated and homogenized. All samples were instantly frozen at −80 °C.

2.6. Measurements of protein levels

The larvae protein detection was performed spectrophotometrically at 595 nm, according to the previous reports by Bradford [23].

2.7. Measurement of oxidative stress markers

The SOD activity was measured according to the kit instruction and reported as units (U) per mg of protein. Reactive oxygen species (ROS) evaluation was done according to the previous method for the oxidation of DCFH-DA to DCF reported by Rahimifard et al. [23]. The ROS levels in each group were determined based on the standard curve and reported as U per mg of protein. The malondialdehyde (MDA) levels were determined spectrophotometrically at 532 nm based on the previous report by (Baeeri et al. [24]). MDA concentration was calculated based on the standard curve and reported as μmol per mg of protein.

2.8. Comet assay

We used the alkaline single-cell Comet assay to evaluate genotoxicity, previously reported by (Hodjat et al. [25]). Briefly, after exposure to each group, the gut tissue was turned into single cells using the collagenase enzyme. After that, 10,000 cells were mixed with 0.7 % low melting agarose in PBS at 37 °C. Then, the cells were lysed with lysis buffer, and the slides were electrophoresed in a horizontal electrophoresis chamber of alkaline running buffer. Subsequently, the slides were neutralized and washed with pure ethanol and dried overnight at 4 °C. The slides were stained with SYBR green and assayed with the fluorescence microscope. The analysis was calculated by CASP software (CaspLab, Poland).

2.9. Caspase-3 and -9 activities

As reported previously, the caspase-3 and -9 activities were measured using spectrophotometrically at 405 nm [26]. The caspases activities were assumed 100 % in the treatment groups as compared to the control.

2.10. Real-time PCR

A real-time PCR method was done to examine and analyze catalase, β-gal, p16, p21, p38, and p53 gene expression, using LightCycler ® 96 (Roche, Mannheim, Germany) [27].

The specific primers used in the present study are as follows:

GAPDH Forward: GTTGTGGATCTTACCGTCCG
GAPDH Reverse: GAACACAGACGAATGGGTGT
Catalase Forward: TTCCCCAGCTTCATTCACAC
Catalase Reverse: TTCATGTGGCAGTAACCGTC
β gal Forward: ATACTCTACACTGGCAGCCT
β gal Reverse: CTCGCCAGTTGCTGTCTTAT
P16 Forward: CCTTGGACCAGAACGACCTA
P16 Reverse: GGGAACAGTTGGGGTAGTGA
P21 Forward: TATTAAGGGCGCCAAAACGA
P21 Reverse: AAGAAATGAGAGCGCGACAA
P38 Forward: CATGCAAAGAGGACGTACCG
P38 Reverse: GCGTCCATCAAGTGGGTAAC
P53 Forward: AATGATAGCCGAGACTGCGA
P53 Reverse: CCATTCCTTATTGGGGCACG

2.11. Phenotype study

For the study of fly's phenotype features after the treatment, the larvae (n = 20) were transferred to the medium without H_2O_2 or BoNT/A. Then, when they turned into flies, the flies were anesthetized with diethyl ether. Some morphological parameters, including eye, body-color changes, body size, and wings, were assessed under the stereo-microscope. Data of size were analyzed using ImageJ software.

2.12. Statistical analysis

The data were demonstrated as Mean ± SEM. Statistically, the analysis was performed with a one-way ANOVA test followed by Tukey's multi-comparison. The StatsDirect 3.3.5 software was used.

3. Results

3.1. Oxidative stress markers assay

3.1.1. SOD activity

BoNT/A at all doses, especially in ½ LC_{50} and LC_{50}, significantly elevated the SOD activity compared to the control group ($p < 0.001$). The SOD activity of all groups is demonstrated in Table 1.

3.1.2. Evaluation of ROS level

ROS production in H_2O_2 and different concentrations of BoNT/A were increased in the dosage of ½ LC_{50} and LC_{50} of BoNT/A compared to the control group ($p < 0.05$, Table 1).

3.1.3. MDA assay

The level of MDA as lipid peroxidation (LPO) activity index was elevated in H_2O_2 and BoNT/A groups compared to the control group ($p < 0.001$). Furthermore, MDA generation in BoNT/A groups at the concentration of ½ LC_{50} and LC_{50} U/mL was significantly elevated compared to the H_2O_2 group ($p < 0.01$ and $p < 0.001$, respectively) (Table 1).

3.2. The mRNA gene expression level of catalase

As shown in Fig. 1, catalase gene expression was up-regulated in H_2O_2 and BoNT/A groups. Data analysis showed that the expression of catalase at the concentration of ½ LC_{50} and LC_{50} of BoNT/A was the highest compared with the control (untreated) group (with $p < 0.01$; 1.93-fold and $p < 0.001$; 2.35-fold, respectively) (Fig. 1).

3.3. The genotoxic effect of BoNT/A

The comet assay was perfumed to indicate BoNT/A induced DNA damage response (DDR) to display the DNA breaks. DNA in damaged cells appeared as the Comet with a head and tail, while intact cells had a round intact DNA with no tail. It was found that Comet Olive tail was significantly elevated in H_2O_2 and BoNT/A groups, especially at the concentration of ½ LC_{50} and LC_{50} compared with the non-treated control group, as shown in Fig. 2 ($p < 0.001$).

Table 1
Effects of BoNT/A on oxidative stress parameters.

Group	Control	H_2O_2	¼ LC_{50} BoNT/A	½ LC_{50} BoNT/A	LC_{50} BoNT/A
ROS (U/mg protein)	0.86 ± 0.09	1.38 ± 0.09*	1.02 ± 0.042	1.4 ± 0.16*	1.4 ± 0.095*
LPO (μM/mg protein)	93.13 ± 3.36	196.34 ± 16.56***	180.56 ± 13.01***	277.57 ± 6.54***, ##	308.23 ± 5.13***, ###
SOD activity (U/mg protein)	0.74 ± 0.03	1.11 ± 0.04*	1.15 ± 0.12*	1.32 ± 0.07***	1.23 ± 0.08***

The values are expressed as mean ± SEM for each experimental group. The significance of changes was reported as * $P < 0.05$, and *** $P < 0.001$ versus the control group, ## $P < 0.01$, and ### $P < 0.001$ versus H_2O_2 group. Reactive oxygen species (ROS), lipid peroxidation (LPO), superoxide dismutase (SOD).

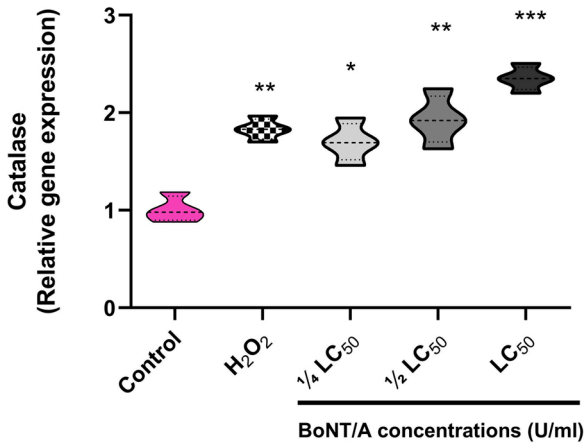


Fig. 1. The effects of BoNT/A toxin on catalase activity in *Drosophila melanogaster* larva. The values are expressed as mean ± SEM for each experimental group. The significance of changes was reported as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus the control group.

3.4. Caspase-3 and -9 activities

The activities of caspase-3 and -9 were elevated in the H_2O_2 group, not significantly. On the other hand, BoNT/A significantly elevated caspase-3 and -9 activities at the concentration of LC_{50} versus the control group ($p < 0.001$; with 1.48-fold, and $p < 0.01$; with 1.26-fold, respectively). BoNT/A, at the concentration of ½ LC_{50} increased caspase-3 activity compared with the control group ($p < 0.001$; with 1.30-fold). Furthermore, a substantial elevation in caspase-3 activation was found in ½ LC_{50} and LC_{50} U/mL of BoNT/A compared to the H_2O_2 group ($p < 0.001$) (Table 2).

3.5. Senescence markers evaluation

3.5.1. β-gal gene expression

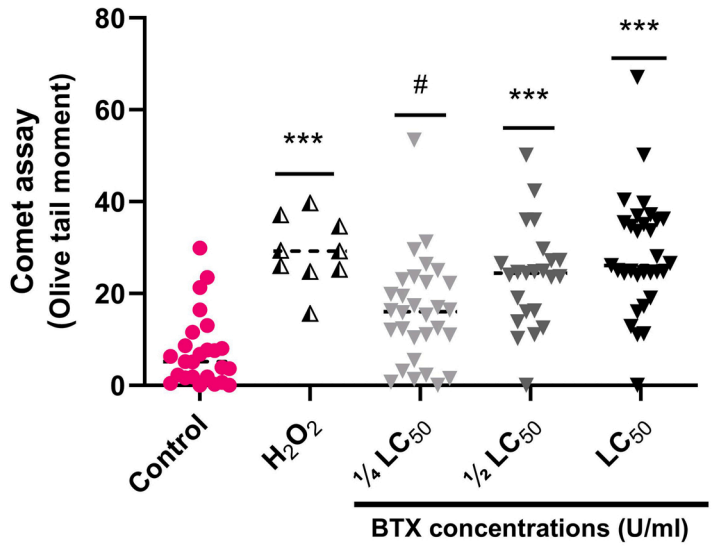
The mRNA expression of β-gal, as an indicator of cellular senescence, was considerably augmented in H_2O_2 and BoNT/A groups. The β-gal gene expression in the H_2O_2 group increased significantly compared to the control group ($p < 0.01$). Also, the concentration of ¼ LC_{50} and ½ LC_{50} of BoNT/A was overexpressed compared with the control group ($p < 0.001$; with 1.76-fold, $p < 0.05$ and 1.33-fold, respectively) (Fig. 3).

3.5.2. Gene expression of p16, p21, p38, and p53

Data of p16, p21, p38, and p53 mRNA expressions illustrated in Fig. 4. The p16 gene in the H_2O_2 group, and ¼ LC_{50} of BoNT/A, was significantly over-expressed compared with the control group ($p < 0.001$; with 1.66-fold and 1.56-fold, respectively). However, this gene was down-regulated at ½ LC_{50} and LC_{50} of BoNT/A compared with the H_2O_2 group ($p < 0.001$; with 1.04-fold and 1.11-fold, respectively).

Analyzing p21 gene expression among the groups showed the highest expression with BoNT/A at the concentration of ¼ LC_{50}, whereas concentrations of ½ LC_{50} and LC_{50} of BoNT/A showed down-regulation compared with the H_2O_2 group ($p < 0.01$; with 0.69-fold).

The mRNA expression p38 was significantly increased in H_2O_2 and BoNT/A groups at the concentrations of ¼ LC_{50} and ½ LC_{50} compared with the control group ($p < 0.05$, with 1.95-fold, $p < 0.001$, 3.37-fold, and 3.48-fold, respectively).



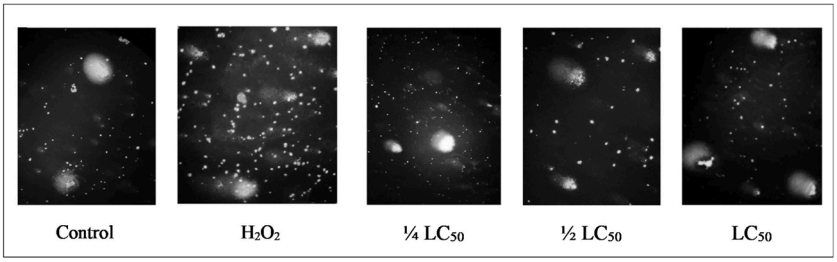


Fig. 2. Evaluation of BoNT/A-induced DNA damage in *Drosophila melanogaster* larvae by comet assay. The graph depicts the Olive tail moment post-exposure to BoNT/A. Comet parameters were quantified by CASP software. The values are expressed as mean ± SEM for each experimental group. Values are shown (horizontal lines) are the mean of 50 to 100 randomly selected images of each sample. The significance of changes was reported as * $P < 0.05$, and *** $P < 0.001$ versus the control group, $^{\#}$ $P < 0.05$ versus to H_2O_2 group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Effects of BoNT/A on caspase-3 & -9 activities.

Caspase activity (% of control)	Control	H_2O_2	¼ LC_{50} BoNT/A	½ LC_{50} BoNT/A	LC_{50} BoNT/A
Caspase-3	100 ± 0.76	110 ± 4.24	109.19 ± 2.07	130.25 ± 1.70***, ###	148.45 ± 1.33***, ###
Caspase-9	100 ± 1.55	115.26 ± 6.27	104.54 ± 1.23	110.56 ± 2.23	126.41 ± 2.60**

The values are expressed as mean ± SEM for each experimental group. The significance of changes was reported as ** $P < 0.01$, and *** $P < 0.001$ versus the control group, $^{\#\#\#}$ $P < 0.001$ versus H_2O_2 group.

Furthermore, H_2O_2 (with $p < 0.05$) and ¼ LC_{50}, ½ LC_{50} and LC_{50} U/mL of BoNT/A groups markedly up-regulated p53 compared with the control group ($p < 0.001$; with 3.13-fold and 3.39-fold, $p < 0.05$; with 2.25-fold, respectively).

3.6. Morphological analysis of drosophila flies

Investigation of possible Drosophila phenotypic changes of ten pairs of males and females in different groups of BoNT/A are shown in Fig. 5. Results demonstrated a remarkable reduction in the female group's body size at the concentration of LC_{50} of BoNT/A ($p < 0.001$) compared to the control group; however, other features as eye color, wing length, and body-color remained unchanged. Treating with BoNT/A (½ LC_{50}) dramatically changed the body size of both female and male groups ($p < 0.05$) without affecting eye color, wing length, and body-color. According to the analyzed pictures from BoNT/A at the concentration of ¼ LC_{50}, there was no meaningful difference between body size and morphological features than the control group in both male and female flies.

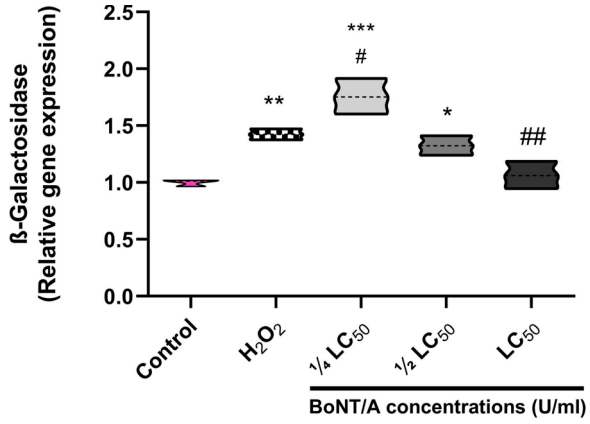


Fig. 3. Effects of BoNT/A on expression patterns of β-gal gene in *Drosophila melanogaster* larvae. The values are expressed as mean ± SEM for each experimental group. The significance of changes was reported as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus the control group, $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ versus H_2O_2 group.

4. Discussion

BoNT/A is a non-specific neurotoxin that can induce impairment in neurons with surface SV2 protein and need SNAP25 protein to fuse synaptic vesicle [28]. Moreover, it is reported BoNT/A can cause oxidative stress and inflammation [29]. It was considering the extensive clinical use of BoNT/A during managing facial wrinkles or plastic surgery [30,31]. This study was performed to understand BoNT/A's safety profile better, mainly focusing on the senescence pathway, toxicity, and

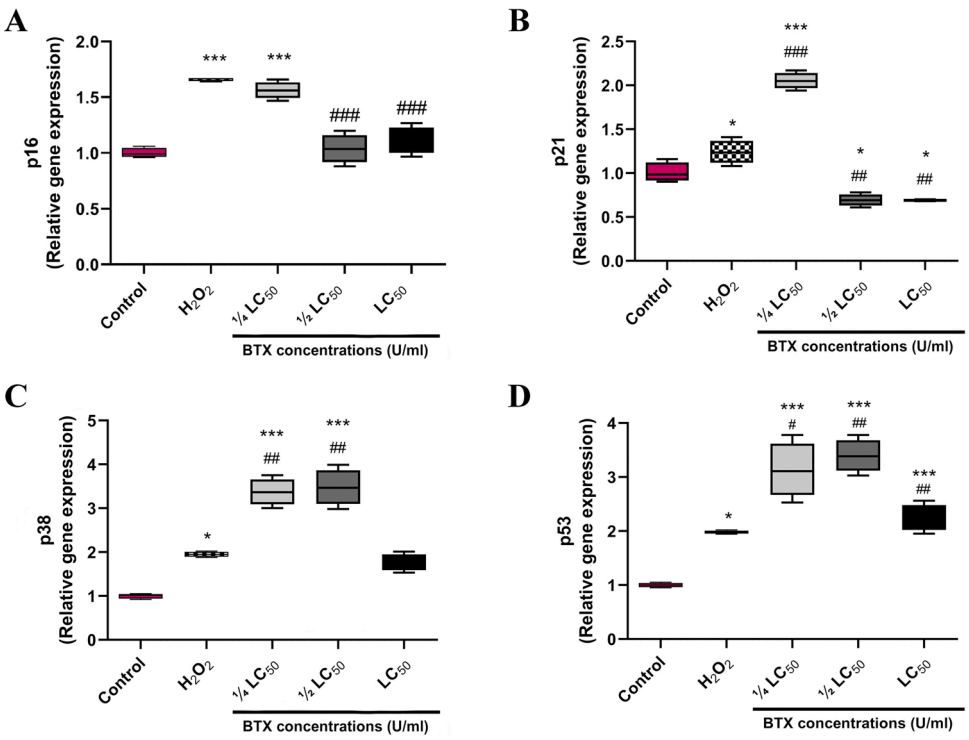


Fig. 4. Effects of BoNT/A on expression patterns of (A) p16, (B) p21, (C) p38, and (D) p53 genes in *Drosophila melanogaster* larvae. The values are expressed as mean ± SEM for each experimental group. The significance of changes was reported as * $P < 0.05$ and *** $P < 0.001$ versus the control group, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, and $^{\#\#\#}$ $P < 0.001$ versus H_2O_2 group.

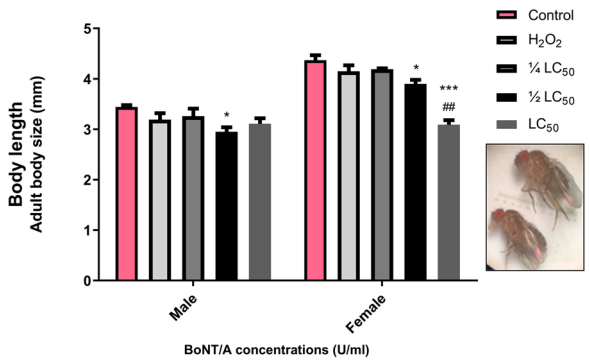


Fig. 5. Effects of BoNT/A on body size of *Drosophila melanogaster* fly. The values are expressed as mean ± SEM per each experimental group. The significance of changes was reported as * $P < 0.05$, and *** $P < 0.001$ versus the control group, $^{\#\#\#}$ $P < 0.001$ versus the H_2O_2 group.

measuring related parameters in a dose-response manner using the *Drosophila melanogaster* model. Overall, this study demonstrated that BoNT/A in the low dose activates the aging path and increases the dose, triggering oxidative stress and apoptosis.

Oxidative stress is an imbalance between anti-oxidant defenses and free radicals production [32,33]. As demonstrated previously, the formation of H_2O_2 (non-radical ROS) induced further production of hydroxyl radicals (OH^-) through the Fenton and Haber-Weiss Net pathway. H_2O_2 and the resulted radicals are then neutralized by catalase enzyme [34] and glutathione peroxidase (GPx), respectively [35]. According to previous studies, oxidative stress mainly involves senescence, apoptosis, inflammation, malfunction of macromolecules [36–38]. The results of the present study indicated that BoNT/A induced oxidative stress. BoNT/A increased the level of ROS and SOD activity. The current study also confirmed the dose-dependently elevation of catalase expression and MDA activity in *Drosophila melanogaster* larvae exposed to BoNT/A. These results (BoNT/A-induced oxidative stress) in agree with those previous results revealing the injection of BoNT/A induced oxidative stress [39,40]. Furthermore, in another study, it is demonstrated that BoNT/A reduces the induction of oxidative stress during cutaneous ischemia-reperfusion injury [41].

The most apparent finding that emerged from the previous studies demonstrated a diversity of free radicals inducing DNA damage. The Comet assay data in the current study indicated that BoNT/A caused DNA damage dose-dependently, which seems to be mediated through oxidative stress, as explained [42]. This finding aligns with the earlier research suggesting DNA damage with cell senescence/cell malfunction and cell death [43,44]. Moreover, it is demonstrated ROS through activation of p53 and then translocation of p53 to nucleus and binding to DNA induced up-regulation of several genes which are involved in inflammation and apoptosis [42,45].

Apoptosis is an essential regulatory pathway for regulating cell death during the homeostasis of multicellular organisms during many physiological or pathological situations [46,47]. This cellular pathway is required to destroy harmed tissue (pathological or physiological conditions), tissue's shaping, transformed cells, and control cell numbers [48,49]. The possible mechanisms for activation of caspase-3 and-9 possibly are through the free radical effect on mitochondrial malfunction and opening of mitochondrial permeability transition pore (mPTP) [50,51]. Moreover, oxidative stress-induced DNA damage leads to p53 activation and the opening of mPTP. The opening of mPTP induced releases of some factors, including cytochrome c, ATP, etc., from mitochondria to cytoplasm, which reacts with apoptotic protease activating factor-1 (apaf-1) and ATP to produce apoptosome [52]. Then, apoptosome turns procaspase-9 to caspase-9, activation of caspase-3, and finally, cell death/apoptosis occurs in a high dose of BoNT/A [53–55]. In the present study, the induction of the apoptosis pathway by BoNT/A treatment was confirmed by dose-dependently activation of caspase-3 and-9. In a low dosage of BoNT/A, there is no significant elevation of caspase-3 and -9. However, in a high dosage of BoNT/A, caspase-3 and -9 were significantly up-regulated, which means by increasing the dose

of BoNT/A, the senescence (aging) pathway turned to apoptosis and toxicity. However, studies demonstrated conflicting results in the role of BoNT/A in activating apoptosis. The result by Gorgal et al. [28] and Bandala et al. [56] indicated BoNT/A elevated apoptosis in the rat prostate and T47D breast cancer cell line, respectively. Moreover, another study by (Shi et al. [57]) showed BoNT/A reduced apoptosis in human dermal microvascular endothelial cells exposed in the ischemia/reperfusion injury model. The possible mechanism for this conflict can be through the dose usage of BoNT/A or the models.

Cellular senescence, or cell growth arrest, is a permanent cell cycle arrest induced by oxidative stress, DNA damage, age-linked telomere shortening, and oncogene activation [58–60]. β-gal is an important senescence marker, detection of cellular senescence (aging). The present study's data demonstrated β-gal elevated in a low dose of BoNT/A and reduced dose-dependently by increasing BoNT/A concentrations. As mentioned in the literature review, two critical signaling pathways involved in cell senescence are p53/p21 and p38/p16 pathways [42,61]. The current study found that β-gal, p53/p21, and p38/p16 increased in a low dose of BX and then dose-dependently down-regulated in larvae exposed to higher amounts BoNT/A, which these results of markers of senescence are in line with previous results.

Due to the frequent administration of BoNT/A in humans, further research should be undertaken to investigate the exact role of BoNT/A in several models. Moreover, this is an essential issue for future research to understand the role of BoNT/A for facial wrinkles, which might cause aging in long-term use.

5. Conclusion

In this study, BoNT/A showed dual effects in different concentrations. The cell senescence pathway was activated in low dose treatment of BoNT/A, which the expression of β-gal, p53, p21, p38, and p16 gene elevated. This is while the increment in the dose of BoNT/A was accompanied by the induction of toxicity markers, including elevation of oxidative stress, apoptosis, and reduction of flies' body size.

Author statement

Farnoosh Fooladvand: Conceptualization; Methodology; Investigation; Writing - original draft; **Vida Tahouri:** Conceptualization; Methodology; Investigation; Writing - original draft; **Maryam Baeeri:** Methodology; Data curation; Writing - original draft; **Tayebeh Minaei:** Methodology; Data curation; Writing - original draft; **Mahban Rahimifard:** Methodology; Data curation; Original draft; Writing - original draft; **Mahshid Hodjat:** Methodology; Software; Writing - original draft; **Reza Khorasani:** Conceptualization; Methodology; Writing - original draft; **Hamed Haghi-Aminjan:** Conceptualization; Writing - review & editing; **Mohammad Abdollahi:** Supervision; Conceptualization; Original draft; Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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