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Potential role of protein stabilizers in amelioration of Parkinson's disease and associated effects in transgenic *Caenorhabditis elegans* model expressing alpha-synuclein

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Abstract

Protein stabilizers/chemical chaperones/osmolytes find significant industrial application in maintaining native state of protein and averting their misfolding or aggregation during repetitive freeze-thawing and various other stresses. Existing literature presents evidence towards possible therapeutic benefits of these protein stabilizers in evading or delaying disorders arising from protein misfolding and aggregation, usually termed as proteo-pathies. Parkinson's disease (PD) is one such disorder marked with alpha synuclein aggregation forming inclusions resulting in dopaminergic neurodegeneration and impaired motor function.

We carried out this study in order to delineate the potential of protein stabilizers in attenuating manifestations associated with Parkinsonism using transgenic *Caenorhabditis elegans* model expressing human alpha synuclein. We studied protein stabilizers sorbitol, trehalose and xylitol and evaluated various endpoints associated with alpha synuclein aggregation. Lower concentrations of protein stabilizers were inefficient in exhibiting any effect, however a higher concentrations (10mM) did exhibit significant effect on studied phenotypes. Trehalose, at 10mM concentration, showed reduction in alpha synuclein levels and reactive oxygen species, while showing significant increase in motility, dopamine levels and up-regulation of autophagic and chaperonic genes *bec-1*, *lgg-1*, *epg-8*, *hsp-60* and *hsp-4* in alpha synuclein expressing model, implicating its beneficial effects in Parkinsonism.

Our studies established the potential role of trehalose in alleviating manifestations associated with Parkinsonism via its inherent activity and through induction of autophagic machinery *in vivo*. We conclude that these chemical chaperones have potential in being used as supplementary therapeutic intervention addressing the important factor of protein aggregation in neurodegenerative Parkinson's disease. Further research on the subject will be required towards establishing their mechanism of action and suitability of such intervention, possibly along with existing therapies that address other important factors of the disease.

Keywords: Chemical chaperones, osmolytes, *C. elegans*, Parkinson's disease

Introduction

Protein stabilizers or osmolytes are a class of simple, biocompatible compounds bearing potential to alleviate protein aggregation and correct misfolded proteins. With the mechanism of action still unknown¹²³ there are studies implicating action of these molecules on different parts of the protein quality control system, inducing expression of various molecular chaperones⁴⁵. Molecular chaperones, amino acids and osmolytes can potentiate rescue of aggregated proteins, simultaneously averting protein misfolding. However the potential activity is often limited to a certain extent depending on the type of interacting protein⁶. This ability to alleviate protein misfolding has stimulated researchers to proceed for de-novo design of these chemical chaperones. It is hypothesized that chemical chaperones being lower solvents for protein backbone render protein in its native state⁷. Industrially these protein stabilizers find use in thwarting aggregation of recombinant protein⁸ and in preserving enzyme activity both in solution and freeze dried state⁹. Protein stabilizers are thought to build up in cytoplasm raising osmotic pressure against environmental water stresses, enhancing protein stability¹⁰. Osmolytes such as trehalose have the property to envelope proteins increasing hydrogen bonding between proteins and water molecules¹¹. Research in last few decades has witnessed the potential of these chaperones in countering the protein associated pathologies¹². Parkinson's disease is a slow progressive disorder affecting the nervous system afflicting manifestations in the motor function of patients, initiating from the accumulation of alpha-synuclein aggregates in the form of Lewy bodies in the substantia nigra of patients, resulting in degeneration of dopaminergic neurons culminating detrimental effects on motor functions. Selective degeneration of the dopaminergic neurons resulting in impaired levels of dopamine is the characteristic feature of Parkinson's disease. The major constituent of pathological hallmark, Lewy bodies is aggregated alpha-synuclein protein. Parkinson's being a slow progressive disorder augments majority of symptoms when 80% of the dopaminergic neurons are degenerated due to toxic effects of accumulated alpha synuclein. With the current strategies limited to symptomatic relief, quest for newer pharmacological interventions is underway. Protein aggregation is considered one of the hallmarks of this disease resulting in malfunctioning or damage to nerve cells. Molecular chaperones with the ability to decrease the aggregated proteins can be used for the treatment of protein aggregated diseases. The current scientific research is majorly focused on exploring naturally occurring and synthetic protein stabilizers as a complementary or complete therapy to counter the diseases associated with protein misfolding or aggregation, especially when beneficial effects of trehalose have already been reported in context to Huntington's disease¹³ and Alzheimer's disease¹⁴. Parkinsonism with the known culprit, alpha synuclein holds an intriguing model in investigating osmolytes as potential therapeutic agents. Research on Protein stabilizers in context to Parkinsonism is speculated to not only present better treatment approaches but as a model for management of other proteopathic neurodegenerative disorders.

Investigation into mechanisms underlying chaperone activity of these osmolytes and clearance of aggregation prone biological entities hold significant potential for understanding and amelioration of proteo-pathies. We employed three types of osmolytes i.e. Trehalose, Sorbitol and Xylitol to elucidate their effect on alpha-synuclein aggregation and pathologies associated with Parkinsonism using *Caenorhabditis elegans* as a model.

Materials and Methods

C. elegans strains and culture conditions

C. elegans strains used in the study were maintained on a nematode growth medium (NGM) at 22⁰C. The food source for these nematodes was *Escherichia coli* OP50 as described by Brenner 1974 previously¹⁵. The wild type strain used was N2 Bristol and the transgenic strains used in the study were NL5901(Punc-54::alpha-synuclein::YFP+unc-119), TJ356(daf-16p::daf-16a/b::GFP + rol-6), BZ555(Pdat-1::GFP) and DA2123(GFP::LGG-1). These strains were procured from *Caenorhabditis* Genetics Center, (University of Minnesota, MN, USA).

Treatment with compounds and dose optimization

Three protein stabilizers including D-(+)-Trehalose (Sigma-Aldrich), D-Sorbitol (Sigma-Aldrich) and Xylitol (Sigma-Aldrich) were used in the study. These were dissolved in distilled water to obtain two concentrations 5mM and 10mM. These molecules were mixed with *E. coli* OP50 and then seeded onto the NGM plates. With respect to that of Control group worms treated with 5mM dose of all protein stabilizers showed no reduction in alpha-synuclein levels but the effect of 10mM concentration was significant on the protein levels. So, the further studies to check the multi-factorial aspects of Parkinson's were carried out using 10mM concentration.

Estimation of alpha-synuclein aggregation using fluorescence microscopy

To assess alpha-synuclein aggregation transgenic strain NL5901 was used. This transgenic strain expresses alpha-synuclein protein tagged with yellow fluorescent protein (YFP) in the body wall muscle. The eggs were isolated using hypochlorite treatment and added onto NGM plates seeded with three compounds like trehalose, sorbitol and xylitol. After 48hrs of treatment adult worms were washed thrice with M9 buffer to remove adhering bacteria and transferred to microscopy glass slides with agar pads (2% agarose). For immobilization worms were mounted using sodium azide (100mM) and then covered with a cover slip. The aggregates were monitored using upright fluorescence microscope (Carl-Zeiss). The exposure time was same for all the worms photographed. The mean fluorescence intensity was measured in both control and treated groups by taking six worms from each group using Image-J analysis.

Measurement of Reactive Oxygen Species (ROS)

The quantitative measurement of ROS was done to study the extent of oxidative stress in control and treated worms. The method was followed as described previously with slight modifications¹⁶. After 48 hrs, worms were washed with M9 buffer and then with phosphate buffer saline (PBS). Then, approximately 100 worms from each treatment group were transferred to each well of black plates containing 100µl PBS. To this, 100µM stock of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen) was added to get the final concentration of 50µM. Fluorescence was taken immediately after adding H2DCFDA and after one hour of addition using fluorimeter. Initial readings were subtracted from the final readings and fluorescence per 100 worms was calculated. The experiment was conducted in triplicate sets.

Effect on lipid content

Nile red (Invitrogen) is a lipid specific dye dissolved in acetone (0.5 mg/mL) to prepare stock solution. The stock is then further diluted (1:250) and mixed with *E.coli OP50*¹⁷. For the treatment with these compounds, Nile red was added to *E.coli OP50* mixed with trehalose, sorbitol and xylitol. Embryos were added to these treatment plates and after 48 hrs adult worms were washed thrice with M9 buffer and transferred to microscopy glass slides with agar pads (2% agarose). For mounting 100mM sodium azide (Sigma, cat no. 71289) for immobilization was used and then overlaid with the cover slip. These immobilized worms were imaged using Zeiss Axioplan fluorescence microscopy (Rhodamine channel). All worms were photographed at the same exposure time.

Motility assay

Motility assay was done to check the thrashing pattern of nematodes as they exhibit a typical thrashing behavior when placed in any liquid. The thrashing pattern depends upon adequate excitatory neurotransmitter content and if any alteration in the excitatory neurotransmitter content, the thrashing behavior of the worm changes¹⁸. Worms from control and treated groups (ten from each group) were transferred to a clean glass slide containing a drop of M9 buffer. A single worm was placed in the drop of buffer and allowed to stabilize followed by the counting of the number of thrashes per 30 seconds using stereo-zoom microscope (Leica, EZ4D). One thrash was defined as complete bending of the body one way to the outermost angle and back to the initial posture. The numbers of thrashes (Mean + SE) for each group were evaluated.

Estimation of dopamine content using Repulsion assay

An established behavioral assay using 1-nonanol is employed to study the dopamine associated effects in nematodes. The assay described earlier was employed with minor modifications to study the response of control and treated groups against 1-nonanol¹⁹. The neurotransmitter dopamine (DA) has an important role in the motor functions of *C. elegans* in response to food and repellents. Any alteration in the levels of DA leads the nematodes to respond differently to volatile attractants or repellents. In this assay, after 48 hrs worms were washed thrice with M9 buffer and subjected to 1-nonanol treatment by placing a drop of 1-nonanol near the head of a worm placed in NGM plate. The quick repulsion response is observed in worms with normal dopamine content and in case of altered dopamine, worms show delayed repulsion behavior. We recorded the response time of worms in a batch of ten subjects. The mean response time \pm SE for each group was evaluated.

Expression of LGG-1 to check autophagy

LGG-1 encodes the ortholog of *Saccharomyces cerevisiae* Atg8p and mammalian MAP-LC3 in *C. elegans*. LGG-1 is predicted to be involved in the degradation of cellular components by autophagy²⁰. The effect of various treatments on LGG-1 expression was checked by using DA2123 strain that expresses GFP::LGG-1. After 48 hrs of treatment, GFP expression was monitored in early adult worms after washing the worms with M9, immobilizing the worms using 100mM sodium azide and mounting on agar padded glass slides. GFP-positive punctate regions were visualized in lateral hypodermal seam cells using the fluorescence microscope (Carl Zeiss).

Lifespan assay

Embryos isolated with hypochlorite treatment were added to control and treatment plates and 48 hours, the worms were picked and transferred to new treatment plates using the platinum loop. The worms were transferred to fresh plates daily and the numbers of surviving, dead and missing worms were counted until the death of last worm. Worms were considered dead when they did not respond to a mechanical stimulus. Results of survival assays were analyzed using the Kaplan-Meier analysis. The experiment was done in duplicate sets.

RNA isolation, cDNA synthesis, and quantitative Real-Time PCR

For total RNA isolation, after 48hrs of treatment adult age-synchronized animals were washed using 0.1% diethylpyrocarbonate (DEPC) water. Total RNA was extracted using Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. After washing worms were crushed in trizol reagent followed by an addition of 200µl of Chloroform per 1ml of Trizol and centrifuged. 500µl of chilled Isopropanol was used to precipitate RNA, collecting gently the aqueous phase. RNA pellet was washed twice with 75% ethanol (chilled) and finally dissolved in 15µl of 0.1% DEPC water and RNA was quantified using Nanodrop. For cDNA synthesis, 1 µg of total *C. elegans* RNA in a 96 well thermal cycler using High capacity cDNA synthesis Kit (Applied Biosystems) according to manufacturer's protocol.

Quantitative Real-Time (qRT-PCR) studies were done using Light Cycler 480 machine (Roche Diagnostics, Germany) using equal amount of cDNA template (125nano molar per reaction). Differential expression was calculated by the $2^{-\Delta\Delta CT}$ method. Gpd-1 was used as internal control and used to normalize ratios between samples²¹. Oligonucleotide sequences used for qRT-PCR Orientation (5'→3')

Gene name	Forward	Reverse
Bec-1	AGG AGC TGG AGC AAC AGT TGA AGA	ATA TTG ACG TTC GGC TTC CAG CGA
Lgg-1	AAC AAC TTT GAG AAG CGT CGT GCC	ATC TTC TGG ACG AAG TTG GAT GCG
Vps-34	TGG ATC CCT TTG CAT CAC GAC GTA	CGA AAC AAT CCC AAC ACC ACC GTT
Epg-8	ACG AAA CTC GAA GAG GAA GTC GCT	AGC TCT GAA TCG TTC ACA ACT GCC
Hsp-60	AAG GAT ATG GGA ATT GCG ACG GGA	TGT GCT CGA TTC GCT TCT CGA TCT
Hsp-4	AAC TTC GAT GTC ACC GGA ATC CCA	ATC ATG CGC TCG ATG TCT TCT GGA

Gpd-1	AAA GTC ATT CCG GAG CTG AAC GGA	AGC GGC CTT GAC TAC CTT CTT GAT
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Statistical analysis

Statistical analysis was done using SPSS-15. To calculate significance independent t-test and ANOVA were used, wherever applicable. Kaplan-Meier test was used to calculate mean life span and plot lifespan curves.

Results

Treatment with 5mM concentration of sorbitol increased the alpha-synuclein aggregation whereas 10mM trehalose significantly reduced the alpha-synuclein aggregation in NL5901 worms.

Alpha synuclein aggregation is the major manifestation in Parkinson's disease, leading to demise of dopaminergic neurons in substantia nigra of PD patients. In order to elucidate the effect of protein stabilizers on synucleinopathies we subjected the worms expressing alpha-synuclein to 5mM concentration of sorbitol, trehalose and xylitol. In comparison to that of control (Fig. 1A), no alteration in alpha-synuclein levels was observed upon treatment with trehalose (Fig. 1C) and xylitol (Fig. 1D), whereas a significant increase in alpha-synuclein levels was observed upon treatment with sorbitol (Fig. 1B). The results so obtained from fluorescence microscopy were quantified using Image J (Fig. 1E) and in comparison to the fluorescence observed in Control, *OP50* ($2.79 \pm 0.30 \times 10^6$) there was increase in alpha-synuclein levels upon treatment with 5mM sorbitol ($4.51 \pm 0.47 \times 10^6$), whereas no alteration was observed upon treatment with 5mM trehalose ($3.15 \pm 0.55 \times 10^6$) and 5mM xylitol ($3.14 \pm 0.64 \times 10^6$).

After assessing effect of 5mM concentration of protein stabilizers on alpha-synuclein levels, we determined the effect of 10mM concentration of protein stabilizers on alpha-synuclein levels. In comparison to Control worms treated with *E. coli OP50* (Fig. 1A), we observed a significant decrease upon treatment with 10mM Trehalose (Fig. 1C), whereas no alteration in alpha-synuclein levels was observed upon treatment with 10mM sorbitol (Fig. 1B) and 10mM xylitol (Fig. 1D). The fluorescent images were further assessed semi-quantitatively using Image J. In comparison to the levels of alpha-synuclein levels in *OP50* ($4.53 \pm 0.24 \times 10^6$), we observed a significant decrease upon treatment with 10mM trehalose ($2.53 \pm 0.06 \times 10^6$), whereas a significant increase in alpha-synuclein levels was observed upon treatment with 10mM xylitol ($6.93 \pm 0.12 \times 10^6$). However no alteration in alpha-synuclein levels was observed upon treatment with 10mM sorbitol ($4.48 \pm 0.69 \times 10^6$) (Fig: 2E).

Treatment with 5mM and 10mM trehalose and xylitol decreased the ROS levels in wild-type worms.

Toxic alpha-synuclein aggregates tend to cause neuronal damage in substantia-nigra of PD patients. Increased ROS has been considered as a reason and consequence of neuronal damage in Parkinsonism. In order to investigate the effect on ROS levels we employed H₂DCFDA, a cell permeable dye which reacts with reactive oxygen species to generate fluorescence. In comparison to the ROS levels observed in *E.coli OP50* (340.35 ± 8.15), we observed a significant increase in ROS levels upon treatment with 5mM sorbitol (388.88 ± 5.34), whereas a significant decrease was observed upon treatment with 5mM trehalose (238.34 ± 5.98) and 5mM xylitol (267.77 ± 1.46),

implicating beneficial effects of trehalose and xylitol on ROS levels (Fig. 1F).

In line with the effects observed in 5mM treatment groups we also determined the effect of higher concentration (10mM) of protein stabilizers on ROS levels in wild type worms. In comparison to the ROS levels in *OP50* (478.66 ± 3.76), we observed a significant decrease in ROS levels in worms treated with 10mM trehalose (343.93 ± 3.49) and 10mM xylitol (390.53 ± 2.74), whereas a significant increase in ROS levels was exhibited by the worms treated with 10mM sorbitol (504.76 ± 1.81) (Fig. 2F).

Treatment with 10mM trehalose reduced lipid-levels in wild type strain N2 whereas there was no change in lipid-content in transgenic NL5901 worms upon treatment with trehalose, sorbitol and xylitol.

We studied the effect of 10mM concentration of protein stabilizers on lipid content using Nile red staining in both N2 and NL5901 worms. In comparison to the optimum lipid-levels in *E.coli OP50* (Fig. 3A) in wild type worms, we observed a significant decrease in lipid-content in worms treated with trehalose (Fig. 3C), whereas sorbitol (Fig. 3B) and xylitol (Fig. 3D) did not alter lipid-levels. The worms subjected to 10mM xylitol were relatively thin in comparison to Control.

In comparison to the lipid-content in N2 worms we observed a significant increase in lipid-content in NL5901 worms expressing alpha-synuclein. However, in comparison to the levels of lipid-content in Control (Fig. 3E) in NL5901 worms we did not observe any alteration in lipid content upon treatment with 10mM concentration of trehalose (Fig. 3F), sorbitol (Fig. 3G) and xylitol (Fig. 3H).

NL5901 worms showed improved motility upon treatment with trehalose and xylitol, which was limited to trehalose only in case of wild type worms

Parkinsonism is associated with impaired motor function. In order to study the effect of protein stabilizers on motility we performed thrashing assay for worms treated with 10mM concentration of protein stabilizers against *E.coli OP50* treated worms (68.50 ± 0.05). We observed a significant increase in number of thrashes in worms subjected to 10mM trehalose (74.66 ± 0.23) whereas worms treated with 10mM sorbitol exhibited decrease in number of thrashes (59.16 ± 0.08). The alteration in number of thrashes shown by 10mM xylitol (68.00 ± 0.05) however was non-significant with respect to control, *E.coli OP50* (Fig. 3I). In NL5901, the number of thrashes in *OP50* (63.33 ± 0.29), we observed a significant increase in number of thrashes in NL5901 worms treated with 10mM trehalose (67.70 ± 0.15) and 10mM xylitol (66.60 ± 0.17). Treatment with 10mM sorbitol (59.16 ± 0.08) exhibited decrease in number of thrashes as was the case in wild-type worms (Fig. 3J).

Trehalose treatment increased the dopamine levels as indicated by Repulsion assay in wild-type and NL5901 worms.

We studied the effect of higher doses of protein stabilizers on dopamine levels, a major contributor to the neuro-motor function of body in both wild-type and transgenic NL5901 worms by employing an indirect assay called Repulsion assay. The nonanol assay is a chemo-taxis based evaluation; relying on the fact that 1-nonanol is a chemo-repellant which causes worms to exhibit a strong repulsive behavior upon olfaction of nonanol. As a rule, greater repulsion time indicates lower dopamine levels and vice versa. We observed a significant decrease in repulsion time

in worms treated with 10mM trehalose (1.61 ± 0.00) as compared to that of control, *E. coli OP50* (1.82 ± 0.01) implicating an increase in dopamine levels upon treatment with 10mM trehalose. Conversely, a significant increase in repulsion time indicative of reduced dopamine levels was observed in wild type worms subjected to 10mM sorbitol (2.02 ± 0.00) and 10mM xylitol (1.91 ± 0.00) (Fig. 4A)

In NL5901 worms, compared to the repulsion time in control (*E. coli OP50*) (2.02 ± 0.00), we observed a significant decrease in repulsion time in worms treated with 10mM trehalose (1.84 ± 0.00), implicating an increase in dopamine levels. The treatment groups 10mM sorbitol (2.11 ± 0.01) and 10mM xylitol (1.90 ± 0.00) however exhibited opposite trend indicating reduced dopamine levels (Fig. 4B).

Trehalose treatment induced autophagy via increased expression of LGG-1

LGG-1 is an autophagy marker in *C. elegans* that shows diffused or punctate expression in cells. A large number of punctate structures are representative of increased autophagy. We observed a significant increase in punctate LGG-1::GFP structures, especially in seam cells upon treatment with trehalose (Fig. 4D) as compared to control (Fig. 4C), implicating an increase in autophagy.

Trehalose treatment increased the lifespan of the transgenic NL5901 worms

After ascertaining the effect of 10mM trehalose on life span in wild-type worms we checked the effect of trehalose treatment on the modulation of lifespan in NL5901 worms. Contrary to the results observed in wild-type worms, we observed an increase in life span in worms treated with trehalose (10.60 ± 0.49) as compared to that of control, OP50 (9.96 ± 0.33) in NL5901 strain (Fig. 4E). These results implicated that higher concentration of trehalose was beneficial for longevity in disease condition.

Treatment with trehalose up-regulated the mRNA expression of genes associated with autophagy and heat-shock in transgenic NL5901 worms but not in wild type N2 strain.

Protein stabilizers are said to possess chaperonic activity and are hence considered as potential agents with property to alleviate mis-folded proteins. In order to detect any possible role of the inherent autophagic and chaperonic system by trehalose we performed real time PCR to check the levels of mRNA expression in both wild-type worms and NL5901 worms treated with 10mM trehalose.

- i. **Effect in wild type worms:** In wild type worms we observed a significant decrease in mRNA expression of genes *bec-1* (0.18 ± 0.04), *lgg-1* (0.23 ± 0.04), *epg-8* (0.26 ± 0.02) and *hsp-60* (0.23 ± 0.02). Genes *vps-34* (0.23 ± 0.01) and *hsp-4* (0.51 ± 0.09) also exhibited reduction in mRNA levels however the decrease was statistically non-significant (Fig. 5A)
- ii. **Effect in NL5901 worms:** Contrary to the down-regulation of autophagic and chaperonic genes in wild-type worms we observed a highly significant increase in expression of genes, *bec-1* (42.66 ± 7.03), *lgg-1* (52.04 ± 8.92), *epg-8* (4.49 ± 0.34) and *hsp-4* (134.11 ± 13.43). The increase in mRNA levels of *vps-34* (1.11 ± 0.06) and *hsp-60* (55.91 ± 15.83) was however statistically non-significant (Fig. 5B)

Discussion

Protein misfolding and aggregation are a cause of majority of neurodegenerative disorders like Alzheimer's and

Parkinson's. Protein stabilizers possess the potential to inhibit protein aggregation or orchestrate the correct folding of proteins. Trehalose is a simple naturally occurring disaccharide of glucose found in diverse organisms including plants, fungi, bacteria and yeasts and is known to avert denaturation, misfolding and aggregation of proteins. Trehalose inhibits aggregation of proteins involved in polyglutamine and polyalanine disorders²². Sorbitol has been reported to stabilize protein aggregation through direct interaction. Xylitol is reported to protect proteins from heat-induced denaturation in aqueous solutions²³. Similar to trehalose, Xylitol protects various micro-organisms and biological macromolecules including proteins from inactivation or viability loss during freeze-drying and freeze-thawing²⁴ dependent on various physical properties like molecular mobility and crystallinity. In this study, we have tried to unleash the potential of rationally selected osmolytes like trehalose, sorbitol and xylitol in amelioration of synucleinopathies in *C. elegans* model of Parkinson's disease. The exact mechanism of these osmolytes and whether they have any effect on the inherent chaperonic machinery, made this study quite intriguing. We carried out our initial studies using 5mM concentration of selected osmolytes i.e. trehalose, sorbitol and xylitol. We observed marginal increase in alpha-synuclein aggregation upon treatment with sorbitol with some reduction in ROS levels upon treatment with trehalose and xylitol in wild-type worms. Since the results observed upon treatment with 5mM osmolytes were not significantly compelling to proceed for further studies, we studied the effect of higher concentration of osmolytes i.e. 10mM, where we observed significant reduction in alpha-synuclein levels upon treatment with 10mM trehalose. Paradoxically a significant increase in protein aggregation was observed upon treatment with 10mM xylitol. The results implicated effect of trehalose in countering the toxic protein load specific to Parkinson's disease. Altered lipid levels are considered as one of the characteristics in PD patients, our next aim was to elucidate the effect of osmolytes on lipid-content. Significant reduction in lipid-levels upon treatment with 10mM trehalose specific to wild-type worms only. These results substantiated the reports about altered lipid-levels in PD patients along with lipid lowering properties of osmolytes. Dopaminergic neuronal damage being caused due to increased ROS has been a major manifestation in Parkinsonism²⁵. In order to ascertain the effect of these osmolytes on ROS levels we elucidated the ROS content and observed a significant reduction in ROS levels upon treatment with 10mM trehalose, whereas an increase in ROS levels was observed in worms treated with 10mM sorbitol. These results not only indicated decreased ROS as a possible protective mechanism in case of trehalose but also posed a probable clue for deleterious effects of higher concentration of sorbitol, which was very much in concurrence with the results observed in the previous studies employing higher concentration of sorbitol. Damage to motor neurons has been linked to impaired motor function in Parkinsonism²⁶. We tried to access the effect of 10mM concentration on motility in wild-type and NL5901 worms. We observed a significant increase in number of thrashes upon treatment with 10mM trehalose in both wild type and NL5901 worms, whereas treatment with 10mM sorbitol exhibited reduction in motility in both NL5901 and N2 worms. The beneficial effects of 10mM xylitol on motility were however limited to NL5901 worms only. In continuation to the effect on motor function we also studied the effect of osmolytes on dopamine levels employing an indirect Repulsion assay. In this assay, repulsion time in response to 1-nonanol is inversely proportional to the dopamine levels. Higher repulsion time is representative of lower dopamine levels and *vice-versa*. We observed a significant increase in dopamine levels as indicated by lower repulsion time in case of wild type and NL5901 worms treated with 10mM trehalose, whereas a

significant decrease in dopamine levels was observed in case of N2 and NL5901 worms subjected to 10mM sorbitol and xylitol. After assessing the effects of osmolytes on the above parameters related to Parkinsonism we observed that trehalose at higher concentrations was most potent of all osmolytes in countering PD related manifestations represented in *C. elegans*. We selected trehalose for its effect on life span, where we observed that 10mM trehalose led to a significant increase in lifespan in NL5901 worms expressing alpha-synuclein. As there are studies which suggest that trehalose induces autophagy so we also checked the effect of trehalose on autophagy and observed relatively increased punctate LGG-1::GFP structures indicating amassed autophagy. We next aimed to determine the protective effect of trehalose on mRNA expression of genes related to autophagy and heat-shock system in both wild-type and NL5901 worms. We observed a significant down-regulation of genes, *bec-1*, *lgg-1*, *vps-34*, *hsp-60* and *hsp-4* in wild type worms, which might be attributed to the autophagic or protein stabilizing potential of trehalose. Since trehalose treatment is expected to reduce the proteotoxic load within the biochemical milieu causing down-regulation of autophagic and chaperonic machinery in a kind of feedback inhibitory manner. As speculated in the NL5901 worms, with significant amount of proteotoxic load a significant up-regulation in expression of *bec-1*, *lgg-1*, *epg-8*, *hsp-60* and *hsp-4* was observed implicating that trehalose, besides possessing inherent protein stabilizing potential works in collaboration with the cellular machinery, resulting in up-regulation of autophagy and chaperonic genes to counter the toxic build up of misfolded and aggregated proteins. The results from this study implicate the protective role of trehalose treatment in reducing mutant protein via inherent and cellular mechanisms along with exerting beneficial effects towards other manifestations such as dopaminergic and motility functions.

Our findings are in line with the reported beneficial effects of trehalose in various protein aggregation related neurological disorders, with existence of sufficient research literature corroborating our results. Ameliorative potential of trehalose on MPTP model of Parkinsonism in mice has recently been reported, where trehalose treatment was found to mitigate microglial activation and astrocytic hypertrophy, besides sufficing with protection to micro vessels and endothelial cells against the toxic effects of MPTP²⁷. Trehalose has also been known for its inhibitory effects on alpha synuclein fibril formation²⁸. Besides possessing inherent protein stabilization properties trehalose has also been reported to enhance autophagy, mitigating aggregation of mutant huntingtin and alpha synuclein protein in an mTOR independent manner²⁹.

Conclusion:

Our findings present evidence regarding the potential role of trehalose in ameliorating synucleinopathies and associated manifestations in Parkinsonism, which are in concurrence with the reported chaperonic activity of protein stabilizers. We conclude that further research towards comprehending the underlying mechanisms arising from direct or indirect Protein stabilizer-protein association and synthesis of novel Protein stabilizers will not only buildup understanding about the mode of action of osmolytes but will also open up avenues for therapeutic attenuation of proteopathic disorders through molecular chaperones. Research on the subject will be required towards establishing their mechanism of action and suitability of such intervention, possibly along with existing therapies that address other important factors of the disease

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Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

Conceived and designed the experiments: AN and SK, performed the experiments: SK Analyzed the data: SK; Wrote and edited the paper: SK, AN.

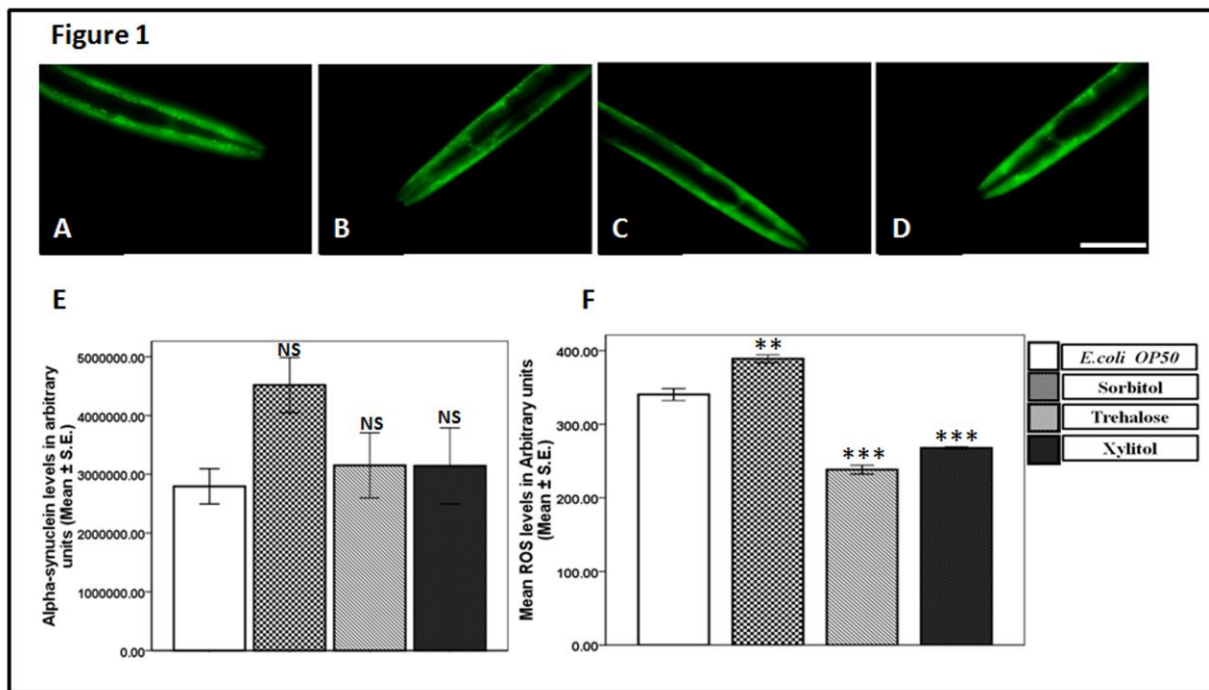
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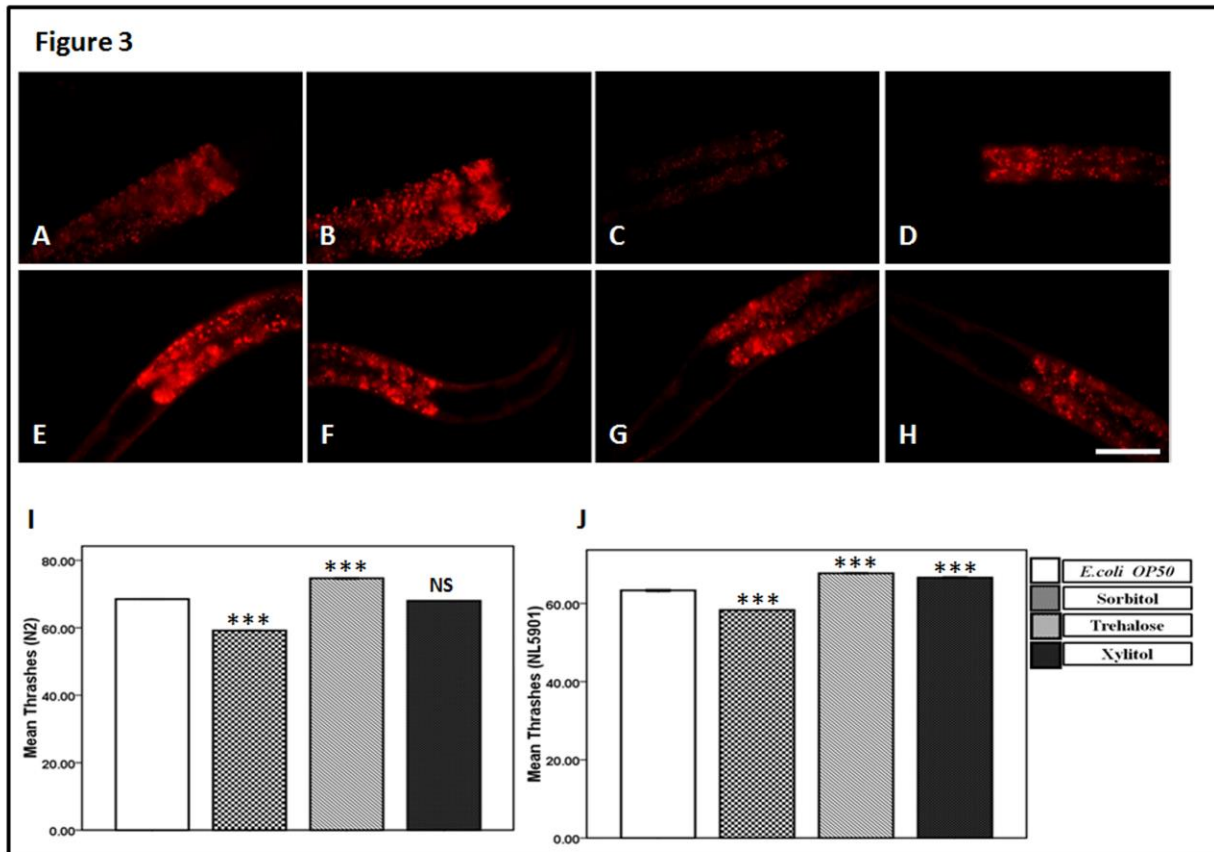
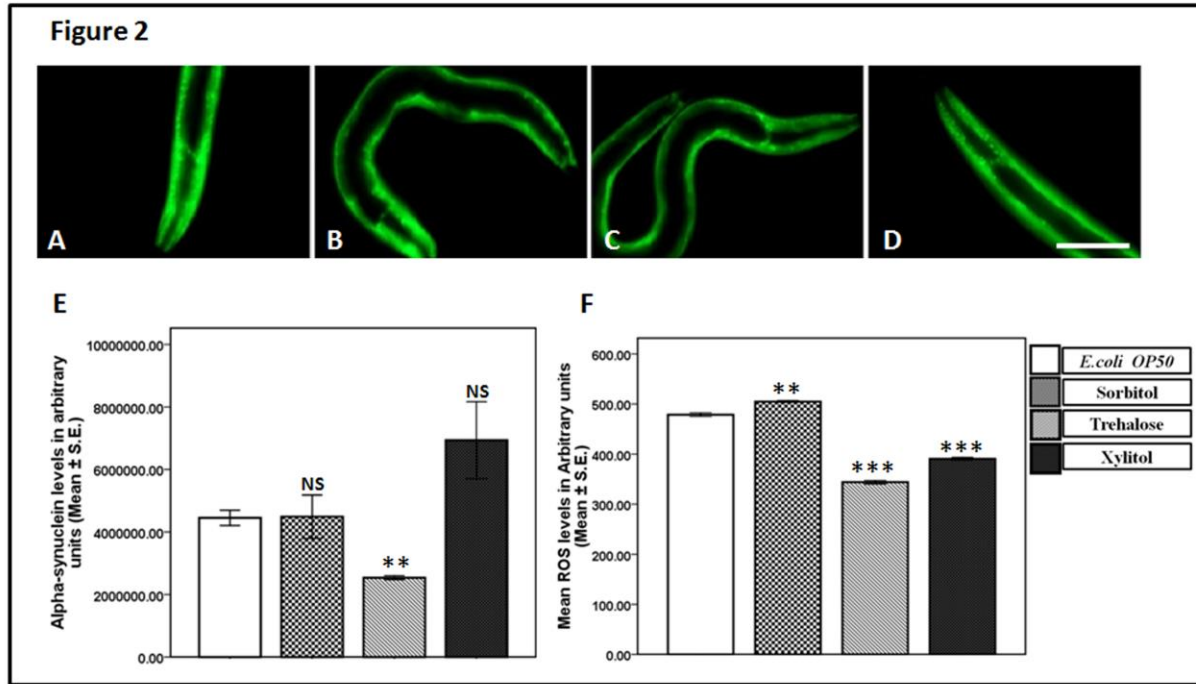
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Figures





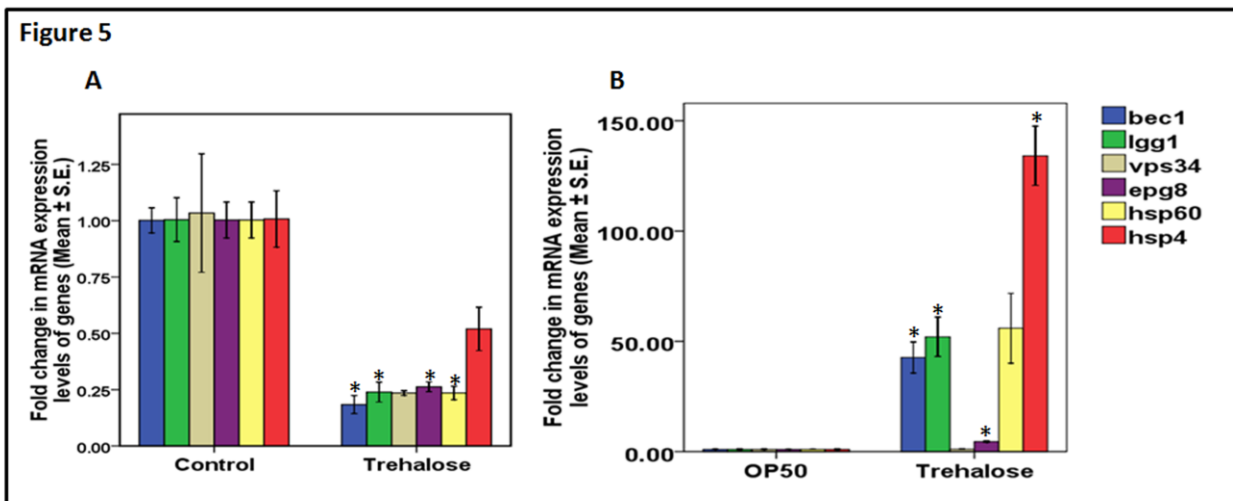
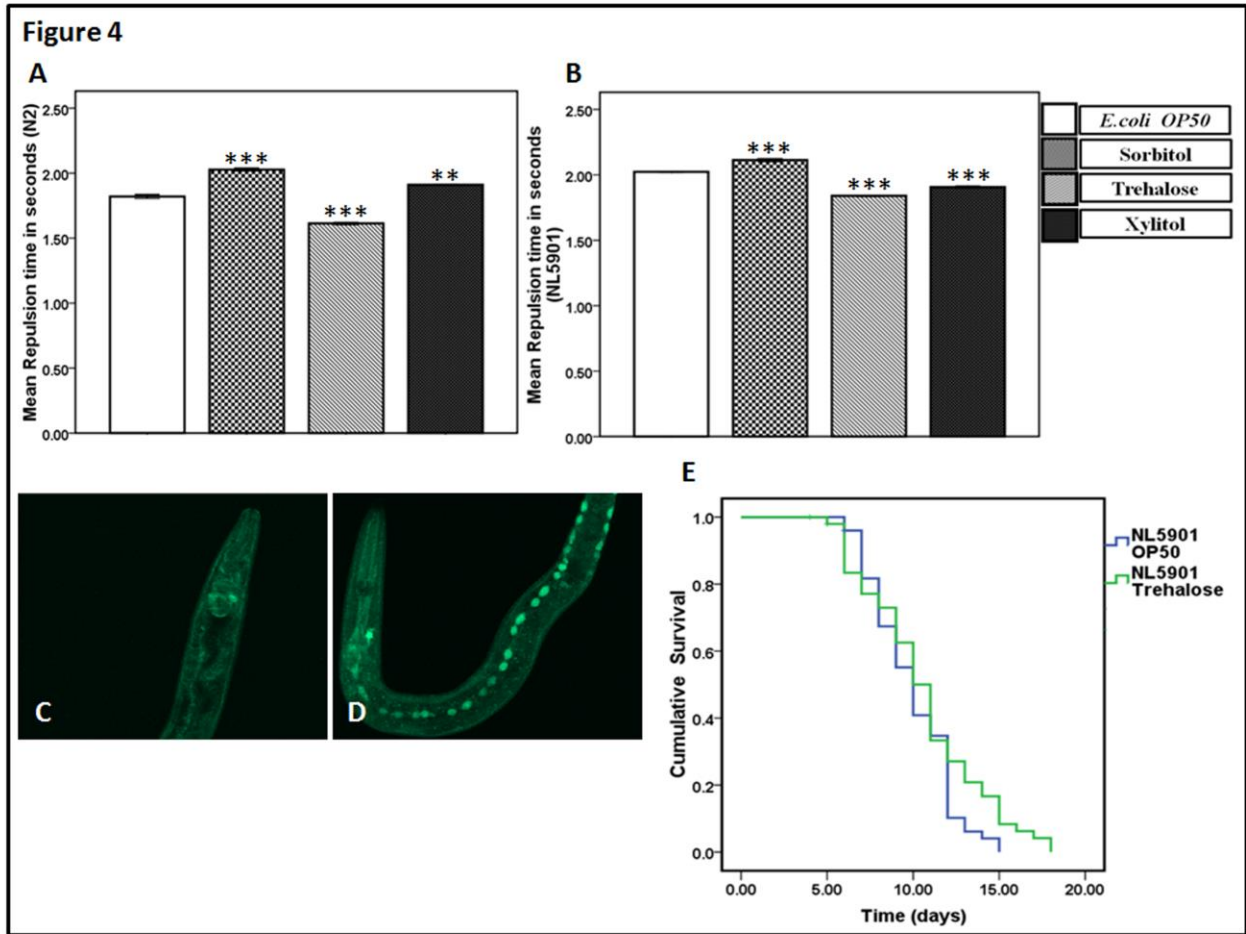


Figure Legends

Figure 1

Effect of 5mM concentration of sorbitol, trehalose and xylitol on the alpha-synuclein and ROS levels:

We studied the effect of 5mM protein stabilizers on alpha-synuclein aggregation in NL5901 worms. In comparison to the alpha-synuclein levels in Control (A) there was an increase in alpha-synuclein levels in worms treated with 5mM sorbitol (C). Treatment with 5mM trehalose (B) and xylitol (D) however did not alter alpha-synuclein levels.

Upon semi-quantitative analysis, we observed a significant increase in alpha-synuclein levels in NL5901 worms treated with 5mM sorbitol as compared to that of control (E). Increase in alpha synuclein levels exhibited by 5mM trehalose and xylitol was non- significant.

Treatment with 5mM Sorbitol exhibited a slight increase in ROS levels, whereas 5mM trehalose and xylitol exhibited a significant reduction ion ROS levels as compared to that of control (F). ** indicates p Value ≤ 0.005 , *** indicates p Value ≤ 0.001 .

Figure 2

Effect of 10mM concentration of sorbitol, trehalose and xylitol on the alpha-synuclein and ROS levels:

We studied the effect of 10mM concentration of protein stabilizers on alpha- synuclein levels. In comparison to the alpha-synuclein levels in *E. coli OP50* (Control A), we observed a significant reduction in alpha-synuclein levels in worms treated with 10mM trehalose (C). Treatment with 10mM concentration of sorbitol (B) and xylitol (D) however did not alter alpha- synuclein levels. In order to further ascertain the amount of reduction in alpha-synuclein levels, we performed semi-quantitative analysis using Image J. As compared levels of alpha-synuclein in *E. coli OP50*, we observed a significant decrease in alpha-synuclein levels in NL5901 worms treated with trehalose. A non-significant decrease in alpha-synuclein levels was also observed in case of sorbitol and xylitol (E).

In comparison to the ROS levels in *E. coli OP50* (Control), we observed a significant decrease in ROS levels in worms treated with 10mM trehalose followed by 10mM xylitol. 10mM sorbitol however, did not exhibit any significant alteration in ROS levels (F). ** indicates p Value ≤ 0.005 , *** indicates p Value ≤ 0.001 .

Figure 3

Effect of 10mM concentration of sorbitol, trehalose and xylitol on the lipid-levels and motility in wild-type N2 and NL5901 worms:

In comparison to the lipid-levels in OP50 (A), we observed a significant decrease in lipid content upon treatment with trehalose (C), whereas sorbitol (B) and xylitol (D) did not alter lipid-levels in wild type worms. In comparison to lipid-content in N2 worms we observed a significant increase in lipid- content in NL5901 worms expressing alpha-synuclein. However, in comparison to the levels of lipid-content in Control (E) in NL5901 worms we did not observe any alteration in lipid-content upon treatment with 10mM concentration of trehalose (F), sorbitol (G) and xylitol (H).

In comparison to the number of thrashes in *E. coli OP50* (Control), we observed a significant decrease in number of thrashes in worms treated with 10mM sorbitol whereas a significant effect on motility was observed in worms treated with 10mM trehalose. Treatment with 10mM xylitol

was devoid of any significant alteration (I). In NL5901 worms, we observed a significant decrease in number of thrashes in worms treated with 10mM sorbitol, whereas a significant increase in number of thrashes was observed in worms subjected to 10mM trehalose followed by 10mM xylitol (J) as compared to that of control.; ** indicates p Value \leq 0.005, *** indicates p Value \leq 0.001.

Figure 4

Effect of 10mM concentration of sorbitol, trehalose and xylitol on the dopamine-content, autophagy and lifespan:

We checked the effect of 10mM concentration of protein stabilizers on dopamine levels. In comparison to the repulsion time in *E. coli OP50* (Control) N2 worms we observed a significant decrease in repulsion time in worms treated with 10mM sorbitol and xylitol. Treatment of worms with trehalose resulted in a significant decrease in repulsion time indicating increased dopamine levels. However, the worms treated with sorbitol and xylitol increased the repulsion time indicating the decreased dopamine levels (A).

In NL5901 worms, in comparison to the repulsion time in *E. coli OP50* (Control), we observed increased repulsion time in worms treated with 10mM sorbitol whereas a significant reduction in repulsion time was observed in worms treated with 10mM trehalose and 10mM xylitol (B).

We checked the effect of the trehalose treatment on autophagy using DA2123 strain and we observed that as compared to control (C) there was increase in punctate structures in trehalose treatment (D) pointing towards induction of autophagy by trehalose.

We checked the effect of the 10mM concentration of trehalose in NL5901 worms for modulation of lifespan. We observed an increase in lifespan in worms treated with trehalose (10.60 ± 0.49) as compared to that of control, OP50 (9.96 ± 0.33) (E). ** indicates p Value \leq 0.005, *** indicates p Value \leq 0.001.

Figure 5

Effect of trehalose treatment on the mRNA expression of genes *bec-1*, *lgg-1*, *vps-34*, *hsp-60* and *hsp-4*:

We checked the effect of 10mM trehalose on mRNA expression of autophagy and heat-shock related genes i.e. *bec-1*, *lgg-1*, *vps-34*, *epg-8*, *hsp-60* and *hsp-4*. We observed a significant decrease in mRNA expression of all the studied genes where *bec-1* exhibited extreme down-regulation in its expression (A) in wild-type strain N2.

We checked the effect of 10mM trehalose on the mRNA expression of autophagy and heat-shock related genes i.e. *bec-1*, *lgg-1*, *vps-34*, *epg-8*, *hsp-60* and *hsp-4*. We observed a significant increase in mRNA expression of all the studied genes except *vps-34* (B) in transgenic NL5901 worms.